

**Evaluation of amino acid transport and protein metabolism in the mammary gland  
of dairy cattle**

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## **ABSTRACT**

Improving our understanding of milk protein production regulation and AA transport is important for successfully formulating diets for AA and improving N efficiency. The objectives were to study protein synthesis regulation and AA transport using in vitro and in vivo models. In the first experiment, the objective was to evaluate the ability of five distinct AA profiles and balancing Lys to Met ratio to 3:1 to stimulate protein translation. No single AA profile uniquely stimulated phosphorylation of translational machinery related proteins suggesting identification of a single optimal AA profile as unlikely. In the second experiment, an in vitro method using three different AA isotopes was developed to trace AA movement. The method assesses bi-directional transport of multiple AA simultaneously enabling evaluation of unidirectional uptake kinetics. This method was used to evaluate AA concentrations representing 16, 100, 186, and 271% of cow plasma AA concentrations. Amino acid uptake was not saturable within the in vivo range for eleven AA. Arginine, Val, and Pro exhibited saturation with the Michaelis-Menten  $k_m$  being 95, 49, and 65% of in vivo concentrations. Results suggest that AA transport is generally non-saturable and that high bi-directional transport exists which enables a mechanism for mitigating AA shortages. In experiment 3, the objective was to evaluate milk protein production and regulation from infusing Met, Lys, and His (MKH) or Ile and Leu (IL). The two EAA groups independently and additively increased milk protein yield. This finding contradicts the single limiting AA theory that a single nutrient will limit milk protein yield. Changes in udder AA extraction and blood flow from supplemental EAA reveal flexible delivery mechanisms. The phosphorylation state of proteins associated with the mTOR pathway was

impacted by both EAA treatments. Changes in the udder proteome suggest negative feedback on mTOR pathway activation when milk protein yield was increased by the EAA groups separately but when supplemented together, negative feedback was lessened. Results indicate that multiple EAA can stimulate milk protein production, the ability of AA transport to match intracellular needs, and that the single limiting AA theory or existence of a unique optimal AA profile is likely irrelevant in dairy cows.

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## **GENERAL AUDIENCE ABSTRACT**

Post absorptive metabolism of dietary protein and conversion to milk protein is not well described in dairy nutrition models resulting in poor predictions of response to changing protein supply. This partially constrains diet formulation with respect to successfully balancing diets for protein and amino acids or for improving N efficiency. The efficiency of absorbed AA into milk protein varies and udder AA uptake may contribute to this varying efficiency through transport regulation in an attempt to maintain intracellular AA homeostasis. Amino acid transport was assessed when AA supplies were varied below and above in vivo supplies. High bi-directional AA transport was saturating uptake for Arg, Pro, and Val within the normal in vivo range for lactating dairy cows. The high AA exchange suggest strong ability to manage changes in AA supply to meet needs for milk protein translation. When intracellular AA supply declines, efflux of the limiting AA out of the cell declines which results in greater uptake of the limiting AA by the cell. The theory that milk protein yield is limited by a single most limiting amino acid (e.g., the barrel and stave analogy) and that a single optimal EAA profile exists predominates in the field of animal nutrition, but implementation of this theory has not greatly improved N efficiency or been adopted widely. We observed that various AA profiles can equally stimulate milk protein translational machinery in mammary epithelial cells and that balancing Lys to Met ratio to 3 to 1 only had a minor effect. Multiple EAA can regulate milk protein production through signaling to synthesis machinery and delivery of AA to the udder. Supplementation of two

groups of EAA, 1) methionine, lysine, and histidine and 2) isoleucine and leucine, independently and additively increased milk protein yield in dairy cows. These increases were achieved by changes in blood flow in the udder, AA uptake, and nutrient signaling related to protein translation regulation. Hence, results of this dissertation tend to not support the idea of a single limiting amino acid or a unique optimal profile of AA for milk protein production in dairy cattle.

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**Figure 7. 1.** Phosphorylation abundance, total abundance, and the ratio of these abundances determined by western immunoblotting for the following proteins in mammary tissue from dairy cows supplemented with AA; (A) mTORC1, (B) rpS6 = ribosomal protein S6, (C) S6K1 = p70 S6 kinase, (D) eIF2a=eukaryotic initiation factor 2 a subunit, (E) 4EBP1 = eukaryotic initiation factor 4E binding protein 1. Treatments were the following; CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu. .... 327

**Figure 7. 2.** Phosphorylation abundance, total abundance, and the ratio of these abundances determined by western immunoblotting for the following proteins in muscle tissue from dairy cows supplemented with AA; (A) mTOR, (B) rpS6 = ribosomal protein S6, (C) S6K1 = p70 S6 kinase, and (D) eIF2a=eukaryotic initiation factor 2 a subunit. Treatments were the following; CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu. .... 329

## **CHAPTER 1: Introduction**

Milk protein is an important revenue source for dairy farms. Maximizing milk protein income over nutrient costs is critical for financial sustainability of individual dairy farms. At the macro level, producing milk protein in larger quantities with a lower cost per unit while reducing the environmental footprint per unit of milk protein is needed to sustain a growing world population.

The marginal efficiency of conversion of absorbed protein to milk protein assumed in common nutrition models is 67% (NRC, 2001, VanAmburgh et al., 2015). However, studies have shown the marginal efficiency to be 15-30% (Hanigan et al., 1998a, Doepel et al., 2004, Martineau et al., 2016, Moraes et al., 2018). The inaccurate marginal efficiency represented in current nutrition models reduces their utility for decision-making related to maximizing milk protein income over feed costs, optimal protein supplies for heterogeneous groups of cattle, and strategies to reduce environmental N output. Varying the metabolizable protein AA profile and effects on milk protein or predicting milk protein yield from known biological mechanism (energy, hormones) are not encoded in common nutrition models. Use of the single-limiting AA theory within nutrition models also misrepresents biological observations. When nutritionist implement this theory, variable results are often observed (Hanigan et al., 2000, Sinclair et al., 2014).

Significant progress has been made in gaining a better understanding of post absorptive AA metabolism and milk protein regulation in dairy cows. Post absorptive N loss is the largest contributor to dairy cattle inefficiency and also the most variable (Arriola Apelo et al., 2014b). Splanchnic tissues represent the largest loss of post absorptive metabolism. This splanchnic tissue loss primarily consists of first pass catabolism during absorption and recirculating AA

from the venous blood. Recirculating AA catabolized by the splanchnic tissues represent approximately 66% of the post-absorptive N loss (Hanigan et al., 2004) whereas first pass ranges from 1-8% loss for absorbed EAA (Estes et al., 2018b). Decreasing dietary protein supplies is the most effective strategy for reducing splanchnic tissue N catabolism and improving N efficiency (Kebreab et al., 2010). However, decreasing dietary protein on average reduces milk and milk protein production (Zanton, 2016).

To prevent decreased production when reducing N supplies, enhanced AA uptake needs to occur when blood AA supplies are reduced. The mean AA uptake by the udder as a proportion of arterial supplies was observed to only be 22% (Hanigan et al., 2004) which suggests opportunity for increasing net extraction. Milk protein demand for AA is considered the largest factor dictating net uptake of AA by the udder (Cant et al., 2018). Approximately 90% of the net AA extracted from arterial blood ends up in milk protein (Cant et al., 1993). Therefore, the milk protein use is the outsized demand sink for AA in udder tissue with little opportunity existing for decreasing udder catabolism of AA in general. Hence, understanding how to maintain milk protein synthesis with declining AA supply or increase milk protein synthesis by provision of metabolites that are either limiting synthesis rates or that can stimulate the regulatory mechanism controlling the rate is the most logical strategy for increasing udder net extraction of AA. If the latter can be achieved without changing dietary AA supplies or when supplies are reduced, N efficiency will be enhanced by reduced AA supplies exiting the udder via venous drainage and being catabolized to urea by the gut tissues.

Experimental evidence however suggests AA supply is not the sole regulator of milk protein production. Starch and glucose supplementation or euglycemic insulin infusion have increased milk protein production independent of AA supplies (Mackle et al., 2000, Rius et al.,

2010b, Curtis et al., 2018). The increase in milk protein yield from these non-amino nitrogen sources has been traced to changes in regulation of protein translation, specifically the rate of initiation of mRNA translation (Rius et al., 2010a, Appuhamy et al., 2011a, Appuhamy et al., 2014, Cant et al., 2018). In vitro cell culture and mammary explant studies as well as short term animal trials (< 2 d) have demonstrated the effects of EAA, and in particular Met, Ile, Leu, and Thr, on increasing the phosphorylation state of direct substrates of mTORC1 (i.e., S6K1, rpS6, 4EBP1) which increases casein synthesis rate (Toerien et al., 2010, Appuhamy et al., 2011a, Appuhamy et al., 2012, Arriola Apelo et al., 2014c, Arriola Apelo et al., 2014d, Luo et al., 2018). This has resulted in the hypothesis that specific EAA and energy could be manipulated with diet formulation to maintain or increase milk protein yield with constant, or even reduced, dietary protein supply. If this hypothesis is correct, increases in gross N efficiency from 25 to 35% or even greater should be feasible which would represent a 40% gain in N efficiency.

Validation of the hypothesis that the mTORC1 pathway and increased protein translation initiation is a regulator of milk protein translation initiation has proved fleeting in longer term dairy cow trials (>4 d). Supplementation of EAA that have been shown to activate the mTORC1 pathway have not elicited increased milk or milk protein yield increases (Korhonen et al., 2002, Appuhamy et al., 2011b, Arriola Apelo et al., 2014a, Kassube et al., 2017, Curtis et al., 2018). Conversely, sizeable increases in milk protein yield of 29% and 16% in dairy cows in response to EAA infusion or increased dietary concentrate have not been accompanied by changes in the phosphorylation state of mTORC1 pathway proteins (Bajramaj et al., 2017, Nichols et al., 2017). Hence, it is unclear how large of a role mTORC1 plays in regulating rates of protein synthesis or how important regulation of rates of translational initiation in response to AA and energy

supplies is in controlling milk protein synthesis in dairy cattle. Clearly, these mechanisms can exert control, but it is unclear how much control they may exert within the in vivo range.

Activation of the mTORC1 pathway by AA generally requires uptake of AA by the udder and increased intracellular concentration. Within in vivo concentration ranges, AA uptake is thought to be governed by mass action kinetics, and thus a fixed proportion of extracellular supplies are extracted (Hanigan et al., 1992, Hurley et al., 2000, Jackson et al., 2000). This means that reducing dietary supplies of protein will reduce intracellular AA supplies which will potentially downregulate mTORC1 pathway. Empirical evidence shows that the efficiency of conversion of absorbed AA to milk protein increases in a curvilinear manner as dietary protein supplies are reduced (Doepel et al., 2004, Moraes et al., 2018). This curvilinear increase might occur because of changes in AA transport extraction. The ability of AA transporters to change activity to mitigate single AA deficiencies has been demonstrated (Bequette et al., 2000, Hanigan et al., 2000). Most AA transport experiments have evaluated a single AA or only accounted for net uptake and not bi-directional fluxes. A better understanding of AA transport and how it changes given varying supplies of AA should enable a better understanding of the efficiency of extraction of AA by mammary tissue. This would lead to a better representation of post absorptive AA efficiency and better predictions of milk protein yield in nutrition models.

Understanding how AA transport changes in response to varying extracellular supplies and impacts intracellular AA supplies is important for predicting milk protein production. The lack of definitive evidence of mTORC1 regulation on milk protein yield also suggests more research is needed to characterize the mechanism controlling milk protein synthesis. The multitude of factors regulating mTORC1 including feedback loops and challenges in the analysis

of signaling protein phosphorylation states should be considered before ruling out mTORC1 signaling as a mediator of rates of milk protein synthesis.

The overarching objectives of this dissertation are as follows:

- 1) Conduct a review of the current literature relative to AA transport and regulation of protein synthesis in mammary tissue.
- 2) Develop an improved in vitro method for assessment of AA transport in mammary epithelial cells.
- 3) Evaluate AA transport responses to varying supplies of AA.
- 4) Perform an in vitro trial evaluating mTORC1 and ISR pathway signaling with various distinct AA profiles and effects of an unaltered or balanced 3 to 1 ratio of Lys to Met.
- 5) Evaluate udder AA uptake, blood flow, and protein expression and activation in udder and muscle tissues from increasing the supply of EAA known to stimulate milk protein yield as well as EAA thought to stimulate mTORC1 pathway.

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## **CHAPTER 2: Literature Review**

### **2.1. Introduction**

Milk sales represent 80-90% of the revenue on a dairy farm (Karszes and Windecker, 2018). Of the components in milk, 99% of the economic value correspond to milk fat and milk protein on average (St-Pierre, 2017). Maximization of milk protein income over feed costs is therefore critical for dairy farm profitability. Milk protein income over feed costs is defined as milk protein revenue minus feed costs and can be calculated on individual cow or herd basis. Of the protein fed to dairy cows, typically only 20-25% is converted to milk protein with the remainder being excreted into the environment (Hristov et al., 2011). If milk protein efficiency could be improved while maintaining or even increasing yield, farm profitability will be enhanced.

The software commonly used for decision-making with protein feeding in dairy cows assume a fixed 67% conversion efficiency of absorbed protein into milk protein below calculated requirements and zero conversion above requirements (NRC, 2001, CNCPS v6.55). The marginal efficiency when supply is close to requirements has been observed to be 15-30% (Hanigan et al., 1998a, Doepel et al., 2004, Martineau et al., 2016, Moraes et al., 2018). The entrenched 67% marginal efficiency in current models means these models are somewhat inept for decision-making relating to maximizing milk protein income over feed costs given varying prices of inputs and outputs (Yoder et al., 2016). Additionally, the high inferred marginal metabolizable protein (MP) efficiency informs nutritionists and farmers alike that reducing protein supplementation has a high milk protein yield penalty cost. If a more correct marginal MP efficiency was encoded into common nutrition models, then formulating for lower CP diets and consideration of environmental objectives become much more feasible. For example,

feeding an extra 100 g of MP which costs on average \$0.609 / lb (2013-2017) (St-Pierre, 2017), is predicted by nutrition models to return 67 g of milk protein valued at \$2.00 / lb (September 2018) yielding an income over feed cost of \$0.16 per cow/d. However, if the marginal MP efficiency is 20%, feeding an extra 100 g of MP yields a negative income over feed costs of -\$0.046 for every 100 g increase in MP.

The post-absorptive AA framework of common field nutrition models is quite empirical and insensitive to AA supply relative to demand, AA profile, energy, and other factors (NRC, 2001, CNCPS v6.55). The current state of field nutrition models is well described with this quote “Post absorptive metabolism is essentially considered an inflexible black box”, page 348 (Bequette et al., 2003). This inflexibility renders nutritionist and farmers without decision-making software to optimize milk protein yield. This literature review will discuss industry nitrogen efficiency, nutrition models, AA balancing, N losses in the cow, AA uptake by the udder, and regulation of milk protein synthesis. Translating biological understanding to nutrition models and field application is necessary for providing a better solution for decision-making regarding protein supplementation.

## **2.2. Nitrogen Emissions by the Dairy Industry**

Animal production in the US is implicated for 70-85% of US ammonia emissions with dairy cows being blamed for 12.3% of the animal contribution (EPA, 2014). Ammonia ( $\text{NH}_3$ ) arises from N excreted in urine and feces by dairy cows. Urinary excreted urea is rapidly hydrolyzed to ammonia. Feces N is converted to ammonia by urease. Approximately half of nitrogen fed to dairy cows is lost as atmospheric N (Todd et al., 2015). Ammonia is a precursor for  $\text{PM}_{2.5}$  particulates (particulates with an aerodynamic diameter of 2.5  $\mu\text{m}$ ) and nitrous oxide. Those two compounds contribute to eutrophication and hypoxia of waterways, increased nitrate

concentration in precipitation, soil, and surface water, and air quality and visibility issues (Wolfe and Patz, 2002, Leytem, 2014).

Rapid consolidation of the US dairy industry into larger farms has occurred to sustain and maximize profits by producing milk at the lowest economic cost. The US is now a major exporter of dairy products, 18% in 2018 (Council, 2018), which is predicated on having competitive cost of production on a global basis. This rapid consolidation of dairy farms has resulted in approximately 59% or 91,241 herds exiting the dairy business between 1992 and 2012. Nowadays, 50% of cows are located on herds larger than 900 cows (USDA, 2015). Just 13 counties now produce 25% of US milk production, which is 0.80% of US counties that contain dairy farms (USDA, 2015). This concentration of cows on large farms is expected to continue and will greatly exacerbate ammonia emissions in the geographic areas that contain high densities of dairy cattle.

High concentrations of dairy cows in certain geographic areas arising from this industry consolidation have resulted in unsafe drinking water in some areas. Idaho counties with a substantial number of dairy cows now have an increased numbers of water wells with nitrates exceeding 10 mg/L (maximum level considered safe for human consumption) (Leytem, 2014). Nitrates in drinking water can cause nitrate poisoning, particularly in babies, by reducing the concentration of oxygen in the baby's blood (Giammarino and Quatto, 2015). A field study of a 3500-cow dairy in New Mexico showed an average N intake of 2139 kg d<sup>-1</sup> with 19% partitioned to milk, 2% to cow body retained N, and 79% to urine and feces (Todd et al., 2015). This resulted in daily farm ammonia emission of 1120 kg d<sup>-1</sup>. This amount is far in excess of the Emergency Planning and Community Right to Know Act regulation of ammonia emissions of 45 kg d<sup>-1</sup> (EPA, 2009) and may result in local ground water contamination. Another example is the

Chesapeake Bay located in eastern USA. This watershed has approximately 300 million tons of polluting N entering the Chesapeake Bay on an annual basis. The annual polluting N amount reaching the Bay is about six times of what occurred in the 1600s (Foundation, 2012). Of this polluting N, 40% is associated with agriculture and dairy is a significant contributor of this agriculture portion.

The rapid consolidation into large dairy farms in certain geographic locations heightens the need to reduce the amount of N expelled per unit of milk protein produced. The swine industry, through advances such as AA balancing and phase feeding, can achieve overall N efficiency in lactating sows or pigs for meat production of 43 and 57% N efficiency respectively (Millet et al., 2018). If dairy N emissions are not addressed soon, future environmental regulations may be imposed and risk derailing the ongoing strategy of using economy of scale to reduce the cost of production. Improvement of protein efficiency by 10% units (i.e. 35%) for dairy production would reduce output by 475 million tons annually (~40% reduction) (assumed 9.3 million cows consuming 17.8% CP diet). The latter achievement is important for the US maintaining global cost competitiveness for producing milk.

### **2.3. Protein Supplementation and Nutrition Models**

The amount of nitrogen supplemented to cows on most farms is determined using computer software nutrition models. Decreasing N supplementation is the most effective strategy for increasing N efficiency (Kebreab et al., 2010). A recent meta-analysis of 253 treatment means demonstrated that protein efficiency decreased linearly as dietary CP concentrations increased with an intercept of 67.7 g milk N / g intake N (SE=2.96,  $P < 0.001$ ) and a slope of -3.2 g milk N / g intake N (SE=0.33,  $P < 0.001$ ) (Zanton, 2016). This infers a 6.4 percentage unit gain in N efficiency if dietary CP were simply reduced from typical industry concentrations of 17.8% (Caraviello et al., 2006) to 15.8% CP, which would represent a sizeable

25% gain in dairy production N efficiency. However, regression from this study also predicted a 1.36 kg/d and 31.7 g/d losses of milk and milk protein yield, respectively, from a reduction of 17.8% CP to 15.8% CP.

While these findings might infer disappointment in the ability to increase efficiency and maintain production, variation exists in the responses to dietary CP, as the observed R-squares were 0.73, 0.79, and 0.94 for milk yield, milk protein yield, and efficiency, respectively (Zanton, 2016). The establishment of the metabolizable protein system improved that ability to lower CP by accounting for microbial protein and feed protein supplies that escape rumen degradation (NRC, 2001; CNCPS v6.55). The prediction of absorbed AA from dietary intake and microbial protein supplies by software (NRC, 2001; CNCPS v6.0) is considered to be robust by some (Pacheco et al., 2012). However, a meta-analysis recently demonstrated a 46% root mean square prediction error with 19% and 22% of that error being mean and slope bias for predicting dietary RUP flow (NANMN) (White et al., 2017). This review will focus on post-absorptive N metabolism, but it is worth mentioning that supply side estimates may need refinement which is necessary to accurately parameterize post-absorptive N metabolism models.

#### **2.4. Nutrition Model Shortcomings**

The current metabolism protein system has not moved the industry beyond 20-25% N efficiency (Hristov et al., 2011). Some of the shortcomings of the current metabolizable protein approach for improving N efficiency have been reviewed previously (Arriola Apelo et al., 2014b). Briefly, the following are proposed issues with current nutrition models; 1) fixed efficiency of maintenance and lactation MP and AA use, 2) aggregating AA into MP, and 3) regulation of milk protein yield by the single-limiting AA theory.

The encoding of a fixed efficiency of AA use manifests into a larger issue of nutrition models being unable to predict responses to varying AA supply as the marginal efficiencies are

quite incorrect. This renders nutrition programs as only being able to predict requirements which are derived from the observed outputs (milk components, growth, etc.) (St-Pierre, 2012). If current nutrition models are considered for their response predictions, the results will be erroneous in most cases. For example, undersupplying 100 g/d of MP will predict a milk yield loss of 2.2 kg/d whereas experimental evidence would suggest a loss of only 0.6 kg/d (assumed 3.1% true milk protein) (Hanigan et al., 1998a, Doepel et al., 2004). In contrast, oversupplying MP to requirements results in no predicted increase in milk yield where experimental evidence would suggest an increase, though with diminishing returns (Moraes et al., 2018).

Nutrition formulation generally consists of consideration for milk component prices, nutrient costs, milk production, animal heterogeneity within a group, animal health, farm specific factors, and possibly environment criteria (Yoder, 2015). For example, the high heterogeneity of animals in a one-group lactation diet scenario infers that the optimal CP concentration of the diet should be 19% when considering lead factors and using the NRC 2001 nutrition model (Kalantari et al., 2016). This was based on simulation of the observed characteristics of 5 commercial dairies located in Wisconsin ranging from 313 to 1460 lactating cows. The high CP concentration optimal solution is driven by the wrong marginal cost associated with undersupplying MP to lactating cows and fixed maintenance efficiency that overestimates needs for cows. Multiple grouping of cows according to dietary needs will enable more precisely meeting the protein needs of individual cows and improve efficiency as observed with the swine industry. That industry has proposed that maximal efficiency could be achieved with targeting protein supply to daily requirements, hence formulating a unique diet on a daily basis (Millet et al., 2018). To achieve higher N efficiency in the dairy industry as observed in the swine industry, marginal response surfaces that reflect biological observations must be incorporated. If

the latter improvement is not conducted, unrealistic lead factors for feeding groups of cows will continue to be recommended.

## **2.5. Proposed Improvements for Incorporation into Nutrition Models**

Progress in better representation of post-absorptive efficiency of N use is occurring (Lapierre et al., 2016, Hanigan et al., 2018, Moraes et al., 2018) and will result in more accurate marginal response surface predictions. Research by Lapierre's group demonstrated that the concept of meeting AA needs of maintenance prior to milk production is not valid as cows will enter into negative protein balance to maintain production. In addition to being able to mobilize N, cows will change the amount of AA extracted for splanchnic tissue use (discussed in a later section) depending on the level of supply relative to anabolic demand (Raggio et al., 2004). Mammals in general possess strong regulation mechanisms to maintain systemic homeostatic balance of plasma AA (Broer and Broer, 2017). Variable catabolism by the gut tissues is one physiological mechanism employed to maintain homeostasis of blood AA supplies. As plasma supplies of AA decrease, a smaller amount of AA will be catabolized by the gut. Another finding by Lapierre's group is that catabolism varies by tissue and individual AA (discussed in a later section).

A combined efficiency encompassing maintenance and production has been incorporated into the CNCPS model, thereby no longer restraining absorbed AA supply to first meeting maintenance needs. While an improvement, the efficiency value is still fixed, regardless of supply, relative to tissue demand, and derivation of these values is constrained to available experimental data. For example, the data used to derive fixed EAA efficiencies are predominately Met and Lys infusion studies (n=33) versus only four studies involving BCAA supplementation (Doepel et al., 2004). Additionally, the average milk production was 25.6 kg/d in the dataset of Doepel et al. (2004) and the maximum milk protein yield was 1105 g/d. In

comparison, the completed trial in Chapter 6 reports milk protein yields of 1500-1600 g/d. As maintenance catabolism of gut tissues is a function of tissue demand, higher production may change maintenance demand for AA. From a statistical standpoint, extrapolation of a model outside the range of the data used to derive the model is incorrect (Quinn, 2002).

Recently, the proposed logistic model by Doepel et al. (2004) was derived using a Bayesian approach to account for between study variability to derive MP requirement for a given observed milk protein yield (Moraes et al., 2018). This function specified variable MP and a combined lactation and maintenance efficiency, which ranged from 18 to 75% depending on level of MP supply versus milk protein yield. While the model provided a reasonable fit to the literature data (18-20% RMSE), individual EAA were not considered and again, the dataset used to derive the equations limits the ability for extrapolation to modern dairy cows. The average milk and milk protein yields for the two datasets within this study were 23.6 and 28.9 kg/d and 729 and 891 g/d milk and milk protein yield respectively. Maximum milk protein yields were 1140 and 1490 g/d, respectively. The one dataset consisted entirely of infused casein studies, which represents a distinct AA profile versus feed RUP. Hence, while the work provides progress with variable efficiency, extrapolation to dairy cows today issue and lack of consideration for individual AA limit applicability.

Lastly, recent work by Hanigan et al. (2018) addresses the three previously outlined issues with post-absorptive metabolism. To reiterate, these issues are; 1) variable efficiency, 2) accounting for individual EAA, not the aggregated MP, and 3) incorporation of multiple AA and other factors (e.g. energy stimulating milk protein yield). This work involved a large database (n=724 treatment means), incorporating varying efficiency with quadratic functions, and accounts for several EAA in multiple regression to allow for independent effects. The model

indicates that increasing supplies of AA will decrease post-absorptive N efficiency resulting in more appropriate marginal response surface. Additionally, the model predicts that various profiles of AA may increase milk protein yield. In summary, progression in experimental data generation and model development should yield a representation of post-absorptive metabolism that aligns with observed biological findings and enables users to address the objectives of ration balancing much more robustly. If the latter can be achieved, increasing N efficiency from 25% to 35% might be possible.

## **2.6. Amino Acid Balancing in Lactating Dairy Cows**

Meeting the AA needs of lactating dairy cows while reducing dietary protein represents the most recommended strategy for increasing N efficiency. The dogma is that only providing the AA at amounts equal to requirement will result in maintaining milk protein yields while improving N efficiency (Schwab, 2014). This strategy is predicated on the theory that the most limiting AA relative to demand will limit protein synthesis and, thereby, milk production (Mitchell and Block, 1946). The strategy is usually represented with a water barrel with varying lengths of staves and the shortest stave represents the maximum water or milk protein production that is allowed. Staves with a length in excess of production are thought to contribute to inefficiency via AA catabolism. Methionine and lysine are implicated as being the lowest staves in typical US corn-based diets for dairy cattle (NRC, 2001). The only other EAA implicated as being possibly limiting in US diets is His (Lee et al., 2012, Giallongo et al., 2016). Met and Lys are often inferred as limiting as the concentration in feed protein is low compared to microbial and milk protein. Positive milk and milk protein responses to Met are routinely observed in individual studies (Noftsker and St-Pierre, 2003, St-Pierre and Sylvester, 2005, Appuhamy et al., 2011b, Chen et al., 2011, Lee et al., 2012) and by several meta-analyses (Patton, 2010, Zanton et al., 2014). Positive Lys responses are less frequent. Common nutrition models also identify

most diets as being limiting in Met and sometimes Lys, indicating need for increased feed or crystalline sources.

Industry adoption of balancing for Met and Lys while reducing dietary CP has occurred but does not dominate the industry like swine and poultry. Industry estimates indicate 20-30% adoption by the US dairy industry on a per cow basis (November 2018, Brian Sloan, Personal Communication, Addiseseo, Alpharreta, Georgia, US). This means the majority of the industry has not embraced feeding lower CP diets and improving N efficiency by balancing for limiting EAA. The latter observation is likely why the industry efficiency has not progressed beyond 25% gross N efficiency. Besides cost of synthetic AA, several distinct challenges exist; 1) rumen protection of synthetic AA and uncertainty with bioavailability, 2) AA supply estimation from diet and rumen microbial supplies, and 3) uncertainty with predicting animal requirements and responses from varying AA supplies. A meta-analysis of 14 studies involving Met and Lys supplementation to cows fed diets less than 15% CP resulted in no benefit in milk yield and only a small increase in milk protein yield (Sinclair et al., 2014). Hanigan et al. (2000) demonstrated that multiple EAA and energy better predicted animal performance than the single AA theory.

A poor representation of post-absorptive AA metabolism may be the largest factor contributing to the uncertainty with AA balancing and inability to achieve successful implementation at a desirable frequency. Comparing the absorbed AA profile to milk profile for inference in limiting AA is incorrect as the splanchnic tissue bed, non-mammary tissue demand, and AA transport alter the AA profile that mammary epithelial cells will experience. The static efficiency of use for Met and Lys is also incorrect as this efficiency will vary depending on supply and demand (Table 1). Intracellularly, efflux of these AA will decrease when their concentration is reduced, and splanchnic tissue catabolism will also decrease, thereby changing

the efficiency for incorporation into milk protein. The overall efficiency at the optimal concentrations of Met and Lys within CNCPS v6.55 are 57 and 67%, respectively. In comparison, Arg and Leu efficiencies are 58 and 61%. This seems unlikely since Arg and Leu are both taken up well in excess of milk protein, 2.45 and 1.35 respectively, compared to Met which exhibits an uptake to output ratio of 0.94 (Lapierre et al., 2012b). The primary site of catabolism for Met is the liver and this catabolism is driven by mass action, hence as demand changes for Met, the efficiency will vary, changing the requirement for a given level of milk production (Table 1). One may assume there is a 12-g/d deficit of Met supply, thereby limiting milk protein production when modeling the Met requirements of a cow producing 45 kg/d of milk at 3.1% milk protein within CNCPS v6.55 model. However, if the efficiency for use is 90% which is possible (Doepel et al., 2004), then there is a dietary surplus of Met (Table 1). A more dynamic representation of AA efficiency of use should increase accuracy of predicting when AA are deficient and milk protein yield. Enhanced prediction accuracy will increase the frequency of success with amino acid balancing and ability to reduce dietary CP and improve N efficiency.

## **2.7. Post Absorptive Nitrogen Losses**

Nitrogen intake on a dairy farm can be partitioned into body retained N, milk N, manure N, and ammonia N arising from both feces and urine (Todd et al., 2015). Between 79 to 94% of urine N will volatilize as ammonia (Todd et al., 2015). Urinary N loss is greater than fecal N loss and arises primarily from liver synthesis with the N originating from rumen ammonia or catabolized AA (Kebreab et al., 2010). Urinary N loss is easier to manipulate than fecal N loss and correlates well with milk urea nitrogen concentrations (Kauffman and St-Pierre, 2001). This is because fecal N consists of endogenous N (sloughed cells and enzymes, recycled urea) which represent 17-31% of fecal N (Ouellet et al., 2002, Lapierre et al., 2008a, Ouellet et al., 2010) and

microbial undigested N, both which largely cannot be influenced by diet manipulation. Fecal N also contains undigested feed N which is considered constant by the NRC (2001) except for some unique feeds (i.e. blood meal). Protein digestibility variation within and across feed ingredients does exist, particularly for animal protein ingredients (Noftsker and St-Pierre, 2003, Gutierrez-Botero et al., 2014a). However, the opportunity for reducing fecal N via selection for highly digestible protein feed ingredients is considered minor compared to urinary N.

Upon absorption by the gut tissues, ammonia and AA are carried via the hepatic portal vein to the liver. Loss of AA occurs during digestion and absorption in the gut tissues, though it varies by AA with Gln and Glu exhibiting near complete catabolism in sheep (El-Kadi et al., 2006). High catabolism of Gln and EAA supplies at 100 and 67%, respectively, infer large losses of AA during digestion and absorption (Berthiaume et al., 2001). These studies might be accounting for multiple passes of blood delivering AA to the splanchnic tissues versus first pass absorption. For example, abomasal infusion of casein in dairy heifers and measurement of arterial appearance suggested on average only 1-8% loss of EAA during absorption with the exception of Ile being 24% (Estes et al., 2018b). The latter estimates for EAA losses also agree with a previous study in lactating cows (Hanigan et al., 2004). Hence, first pass losses are likely minor in dairy cows.

## **2.8. Rumen Degradable N**

Excess rumen degradable protein (RDP) results in increased ruminal ammonia which the liver then converts to urea. Insufficient RDP will decrease DMI, NDF digestibility, microbial protein synthesis and will promote energy spilling (Allen, 2000, NRC, 2001, Lee et al., 2012, Giallongo et al., 2015, Hackmann and Firkins, 2015). Reducing dietary RDP from 11.3 to 7.6% of DM decreased milk yield linearly by 4.6 kg/d (Cyriac et al., 2008), however only a numerical 0.90-kg/d loss was observed between 11.3 and 8.8% RDP as a percent of DM.

Allocation of RDP into ammonia, peptides, and AA fractions may improve ability to optimally supply rumen microbes and minimize N losses. Amino nitrogen stimulates more efficient growth of bacteria compared to ammonia (Van Kessel and Russell, 1996). In vitro evidence has also suggested stimulatory effects of peptides versus amino acids (Argyle and Baldwin, 1989). The cost of non-protein N versus amino-nitrogen is different, \$0.06 versus \$0.29 to provide 0.70 lbs of CP from urea (\$500/ton) or soybean meal (\$400/ton). Ration optimizers cannot distinguish between the RDP value of non-protein N versus amino N, which is problematic and needs to be addressed.

The average RDP concentration as percent of DM reported in a survey of 103 US farms was 9.2% (Caraviello et al., 2006), which is surprisingly less than recommended by the NRC (2001) given typical energy concentrations. While this does represent survey data, this indicates that feeding less RDP perhaps represents only a minor opportunity for reducing urine N losses. Instead, supplementing less RUP or a more balanced RUP AA profile appears to be a more viable strategy for reducing urinary N losses.

## **2.9. Gut N Losses**

Oversupplying metabolizable protein relative to the tissue AA needs of the cow will increase urinary N excretion. Increasing dietary CP from 14 to 19% in high producing dairy cows (>40 kg/d milk) across 6 studies in which protein was model inferred to not be first limiting resulted in no change in milk production or fecal N loss (VanAmburgh et al., 2015). The increased dietary CP resulted in a linear increase in urinary N loss as a proportion of dietary intake N from approximately 22 to 41%. Nearly all of the increased N intake was lost as urinary N.

Post absorptive efficiency of N in pigs has been shown to be as high as 85% when AA supply precisely matches AA needs (Baker, 1996). Dairy production is lagging far behind and

post-absorptive metabolism matching of AA supplies to AA needs appears to be the primary culprit. The major demands of N in a dairy cow are the mammary and splanchnic tissues with the latter resulting in recycled urea to the rumen and large intestine and urinary excreted urea. The splanchnic tissue bed includes the portal-drained viscera (PDV) and the liver. The PDV contains the gut, pancreas, spleen, and visceral fat tissues. The mesenteric-drained viscera contain only blood being drained from the small intestine. These tissues are thought to catabolize around 33% of absorbed AA with 69% of that catabolism being recirculated arterial blood (Hanigan et al., 2004). The remaining N loss is considered hair and scurf which is minimal, i.e. 1-2% of dietary N intake (Arriola Apelo et al., 2014b).

Liver catabolism varies by AA and usages are primarily the following; liver and export protein needs, oxidation to urea, and gluconeogenesis (Bequette et al., 2003). Appreciable amounts of His, Met, Phe, Tyr, and Trp are catabolized by the liver (Lapierre et al., 2012b) and increasing absorbed supplies will increase the amount catabolized of these AA (Raggio et al., 2004). Increased AA concentration or blood flow are the primary determinants of liver uptake and catabolism of these AA. Liver AA uptake is well represented by mass action kinetics (Hanigan et al., 2004).

Lysine, Ile, Leu, and Val exhibit minimal catabolism loss in the liver, with BCAA losses considered to be <1% (Hanigan et al., 2004, Lapierre et al., 2012b). Hence, arterial AA concentrations of these AA will be more indicative of protein balance than His, Met, Phe, Tyr, and Trp. Perhaps, this is evolutionary as Leu in particular is considered the most potent AA for stimulating mammalian tissue protein synthesis (Kimball et al., 2016, Sabatini, 2017). Increased circulating plasma BCAA with Leu as the most effective also increases insulin resistance, particularly in skeletal muscle (Saha et al., 2011, Smith et al., 2015).

The AA supply and profile are reduced and altered during absorption by the intestines (MacRae et al., 1997, El-Kadi et al., 2006). The gut uses AA for synthesis of constitutive and secreted proteins and digestive enzymes. While the gut is only 4-8% of body weight, Lobley et al. (1980) implicated this tissue as contributing to 20-35% of whole body protein synthesis in dairy cattle. Only approximately 55-75% of AA supply entering the MDV and non-MDV tissue beds exit the PDV (MacRae et al., 1997, Berthiaume et al., 2001) which indicate excessive catabolism by digestive tissues other than small intestine and endogenous protein secretion. Stage of lactation also plays a role in demand of AA by the gut tissues. Rumen, small intestine, and liver weights, as a % of empty body weight, increase substantially (11-28%) following calving until around 120 d to accommodate increased intake (Bequette et al., 2003, Baldwin et al., 2004) and would demand AA for tissue accretion. Later in lactation, gut tissue mass declines which should provide AA. Additionally, as mentioned for the liver, AA supplies, blood flow, recirculating AA, and tissue demand will also dictate use by the gut tissues.

Maintenance AA requirements are considered as an aggregate in the form of MP and to be a function body weight and DMI. From experimental evidence, the use by the gut tissues suggest this should vary by individual AA and be a function of absorbed supplies, non-splanchnic tissue demand, blood flow, and possibly stage of lactation. Portal blood flow increases linearly by 83.6 L/h per kg of DMI and similar increases are observed for hepatic vein blood flow (Ellis et al., 2016). Concentrate intake, which should be increasing energy content of the diet, increases hepatic vein blood flow as well (Ellis et al., 2016). Greater blood flow in high producing dairy cows (high energy, high intake diets) will reduce portal and interstitial AA concentrations. This should increase net release of AA by the splanchnic tissues (Arriola Apelo et al., 2014b). In contrast, increasing protein supplies and portal and interstitial concentrations

by infusing casein linearly increased catabolism by splanchnic tissues without changing blood flow of these tissues (Hanigan et al., 2004).

In summary, a more dynamic representation of gut and liver removal AA on an individual AA basis with consideration of protein supplies, blood flow, and non-splanchnic tissue demand should greatly enhance our understanding of maintenance needs and what AA supplies are truly available for mammary use. A more dynamic estimation of maintenance needs will address the severe underpredictions of MP milk (>10 kg/d) that is often observed (Doelman et al., 2015a, Doelman et al., 2015b, Doepel et al., 2016). The latter diets reduced AA supplies and diets were high in energy content which likely greatly reduced splanchnic AA uptake of dietary supplied AA. The high marginal efficiency of MP (i.e. 67%) would also likely be corrected with a more dynamic maintenance representation as increasing supplies of MP when already high dietary MP supplies are being fed will result in increased maintenance demand, reflecting higher peripheral AA delivery and portal and interstitial AA concentrations which would increase catabolism. The maintenance requirements calculated in field nutrition models, independent of protein supplies and blood flow and as an aggregate, result in a wrong marginal response surface, thereby rendering economic and environmental decision-making using nutrition models problematic. Amino acid supplies and blood flow (except with AA deficiencies, to be discussed) can be predicted with reasonable accuracy as discussed earlier, and non-mammary and non-splanchnic tissue demand is minor. A mathematical representation of AA-N flows in lactating dairy cows fed a 15% dietary CP diet found only 22% of daily arterial AA influx was captured by the udder (Hanigan et al., 2004). Udder tissue AA uptake is a function of blood flow, arterial AA concentration, and tissue affinity, the latter of which involves

amino acid transporters. Increased efficiency of AA net uptake will reduce venous AA concentrations thereby reducing catabolism by gut tissues and urea production.

Before discussing udder AA transporters, the other variable in AA uptake, i.e. blood flow, is sensitive to AA supplies. Shortages of His, Lys, and Thr or induced AA shortages from glucose or starch have elicited substantial changes in mammary plasma flow (MPF) (Bequette et al., 2000, Rius et al., 2010a, Doepel et al., 2016, Guo et al., 2017, Curtis et al., 2018). However, shortages of all AA in combination do not increase MPF, indicating that only single to several AA imbalances appear to elicit a response. Increased phosphorylation of nitric oxide synthase and increased abundance of nitric oxide have been reported in two of the studies that observed increased MPF (Rius et al., 2010a, Guo et al., 2017). The rate of oxidative metabolism and buildup of ADP and release into interstitium triggers nitric oxide synthesis from arginine (Cieslar et al., 2014). Excessive metabolism results in a need to expel CO<sub>2</sub> and a need for more energy yielding metabolites, hence, ADP is a strong vasodilatory to increase delivery of nutrients and export of CO<sub>2</sub>. Infusion of glucose decreased iliac blood flow within 20 min whereas AA infusion increased blood flow (Cieslar et al., 2014). This work indicates that blood flow is connected to rate of energy metabolism. However, in the AA imbalance studies of Doepel et al., (2016) as an example, no change in udder uptake of acetate, BHB, glucose, or lactate or yield of energy corrected milk was observed. Hence, the 60% rise in blood flow from a Thr deficiency in the latter study cannot be explained by oxidative metabolism regulation alone. Uncharged tRNAs increase phosphorylation of general control nonderepressible 2 (GCN-2) and subsequently eukaryotic translation initiation factor 2 alpha (eIF2 $\alpha$ ) and these changes stemming from AA shortages are associated with increased protein misfolding and ER stress (Baird and Wek, 2012, Han et al., 2013). Increased phosphorylation of eIF2 $\alpha$  increases expression of

activating transcription factor 6 (ATF6) and to some extent activating transcription factor 4 (ATF4), which increase synthesis of chaperones but are also known to have a role in draining ATP supplies and initiating a pro-inflammatory response (Han et al., 2013). Perhaps, severe AA shortages intracellularly lead to a cascade of events that drawdown ATP concentration, thereby triggering a nitric oxide-driven increase in blood flow. If the latter is true, then the regulatory effect of oxidative metabolism rate on blood flow is still the trigger during severe AA shortages.

Excess AA supplies can also decrease MPF. Infusing 0, 8, 16, and 32 g/d of DL-Met decreased MPF linearly and quadratically which resulted in similar uptakes across all treatments but declining extraction efficiency (Guinard and Rulquin, 1995). This decrease in MPF should decrease AA uptakes of the other AA though this was not reported, but if observed, would decrease ability for a limiting AA (Met) to increase milk protein yield. In contrast, infusing 0, 40, 80, and 120 g/d of L-Leu had no effect on MPF and Leu uptake increased linearly and quadratically (Rulquin and Pisulewski, 2006). The same response was observed with Lys in that supplementation had no effect on MPF (Guinard and Rulquin, 1994). Perhaps, for Met, it was limiting and that as supply increased, MPF was reduced as severe shortage was no longer present. In summary, severe AA shortages increase MPF to increase supply. However, whether this is a long-term effect (>5 d) is unknown. Additionally, reductions of all AA do not elicit an increase in MPF, hence, the expectation that feeding lower CP diets and that greater blood flow to the udder will reduce the shortage is not valid. Instead, it appears that increasing the rate of oxidative metabolism (i.e. milk yield) is the best strategy to maintain high blood flow.

## **2.10. Amino Acid Uptake by the Udder**

Intracellular AA supplies determine level of tRNA charging and some AA transmit signals for regulation of translation initiation, therefore, AA uptake to maintain these intracellular supplies for protein translation is critical. How a change in arterial AA supply

translates into a change in intracellular AA supply is necessary to know if one desires to predict milk protein synthesis. The effect of the profile of AA arriving at the mammary gland on individual AA uptake is also a question. Perhaps, use of crystalline AA, which can greatly alter the AA profile, will negatively affect uptake of other certain AA through competitive inhibition.

Amino acid net uptake is assumed to be proportional to extracellularly supplies.

Increasing extracellular supplies of Ile, Thr, Met, and Leu in mammary tissue slices linearly increased intracellular concentrations (Arriola Apelo et al., 2014c). In pig mammary tissue explants, linear increases of intracellular AA were observed within ranges of extracellular AA similar to in-vivo for Val and Lys (Hurley et al., 2000, Jackson et al., 2000). In lactating cows, mammary net uptakes of most AA were linear in response to arterial concentrations (Hanigan et al., 1992).

These findings have resulted in theory that increasing MP supplies will increase net EAA uptake (Cant et al., 2018), the latter which should increase milk protein yield due to rising intracellular EAA concentrations. The other finding is that besides the initiation and stage of lactation and hormones, gene expression of transporters (mRNA) is largely not influenced by dietary supplies (Manjarin et al., 2012, Manjarin et al., 2014, Shennan and Boyd, 2014, Huber et al., 2016, Osorio et al., 2016). These findings suggest that if arterial AA concentrations are reduced during lactation, then intracellular mammary AA concentrations will be reduced, given that mass action kinetics are inferred. This means that feeding a lower CP diet risks decreasing intracellular Leu and Ile concentrations, since these AA have minimal first pass catabolism by the splanchnic tissues. It also infers that dairy cows have minimal ability to cope with changing AA supplies and profile while translating copious amounts of milk protein, thereby, suggesting the importance of balancing AA supplies relative to AA requirements. However, it should be

reiterated, the benefit of balancing for Met and Lys has largely not been demonstrated, i.e. small increases in milk protein yield so this latter statement of dogma might be incorrect.

Marginal efficiency of absorbed AA into milk protein increases quadratically when absorbed supplies are reduced (Whitelaw et al., 1986, Doepel et al., 2004). This may occur by increased extraction of all AA by transporters. Increased net uptake at the same or even reduced extracellular supplies can occur because of a change in protein expression of transporters, movement of transporters to the membrane, transporter conformational change, or reduced AA efflux (Christensen, 1990, Taylor, 2014, Broer and Broer, 2017).

In kinetic terms, transporter activity changes due to an increase or decrease in  $k_m$  or  $V_{max}$  (Souba and Pacitti, 1992). The  $V_{max}$  refers to when all membrane transporters are saturated with AA indicating maximal capacity and rate of AA uptake. The  $k_m$  signifies the extracellular AA concentration when one-half of the maximal rate of AA velocity has been achieved. Affinity is represented by  $k_m$  and result from the transporter protein's ability to bind AA via its structural conformation. The maximal capacity ( $V_{max}$ ) can change by the number of active transporters spanning the membrane which may result from increased gene expression, protein translation, and protein stability or increased translocation of transporters from the cytosol to the membrane (Souba and Pacitti, 1992, Broer and Broer, 2017).

The AA efflux is rarely measured *in vivo* and should be accounted for as cells will decrease efflux of a limiting intracellular AA, which if unidirectional uptake is not affected, will result in higher net uptake. In human muscle, the efflux rate of Phe, Leu, Lys, and Ala exceeded influx rates, inferring net exit of these AA (Miller et al., 2004). In lactating goats, 66% of the Phe taken up by the udder was effluxed (Hanigan et al., 2009). If the intracellular concentration of one AA becomes low, efflux will decrease resulting in a net uptake increase. The homeostatic

intracellular regulation on transport may provide mechanisms that blunt the variation arising from varying arterial supplies (Souba and Pacitti, 1992).

### **2.11. Amino Acid Transporters in the Udder**

Up to 13 different AA transporters are known to be present in mammary tissue (Wu, 2013b, Shennan and Boyd, 2014, Taylor, 2014) (Table 2). The number of identified AA transporters in mammals is approximately 50 with some being specific to tissue types (Broer and Palacin, 2011, Perland and Fredriksson, 2017). Amino acid transporters across species appear to be well conserved (Christensen, 1990), possibly indicating an important role. Amino acids possess distinct residues for creation of unique protein quaternary structures whereas this specificity appears to be lost with membrane transporters. Amino acids can utilize multiple transport systems and transporter proteins to provide efficient uptake. However, this economy renders competition from other AA a possibility and that transport is multivariate (i.e. twenty dimensions) (Christensen, 1990).

Transport of AA into an epithelial cell can be simplified into three mechanisms; passive diffusion, sodium ion driven transport, and sodium independent transport which is driven by AA efflux (often termed exchange transport) (Christensen, 1990, Taylor, 2014, Broer and Broer, 2017) (Table 2.2). The mammary gland exhibits increased AA concentrations relative to blood AA concentrations reflecting a need for energy to drive the concentration gradient (Shennan, 1998). Hence, nearly all AA uptake in dairy cows is considered active transport.

Some transporters can build up steep intracellular concentration gradients to the extracellular concentration (“loaders”) while others act as exchangers (“harmonizer”) to mediate intracellular needs of individual AA (Broer and Broer, 2017). These “loader” transporters are primarily System A, Gly, N, X<sub>ag</sub>, and to some extent, y<sup>+</sup> in dairy cows (Table 2.2). For example, Ala, Gln, and Gly had intracellular to extracellular ratios of 5.5, 5.5, and 9.6 whereas

the Leu, Ile, and Val exhibited ratios of 0.94, 1.34, and 1.08 respectively in mammary tissue of unpublished work illustrated by Shennan et al. (1997). Alanine, Gln, and Gly across mammals, as well as in cows, exhibit high blood plasma concentrations relative to other AA. The high AA supplies and route of uptake (loaders) enable these AAs to function as “exchange currency” for the exchange transporters (Pochini et al., 2014, Bhutia and Ganapathy, 2016).

These NEAA are usually not considered as important in dairy cows, however, several have indicated their recovery in arterial concentrations more slowly than other AA during early lactation and numerical increases in milk yield from Gln supplementation have been observed (Meijer et al., 1995, Doepel et al., 2006). Removal of Gln dramatically decreased Leu uptake and subsequently mTOR signaling and induced autophagy in mammalian HeLa cells (Nicklin et al., 2009). The results highlight the importance of Gln as exchange AA currency. In mammary epithelial cells, positive responses in mTOR signaling and casein synthesis were observed from the addition of NEAA to media containing AA (Luo et al., 2018).

Within this class of “loader” transporters, one transporter, SNAT2, exhibits strong adaptive affinity (Tovar et al., 2000, Gaccioli et al., 2006, Shennan and Boyd, 2014). In mammary tissue explants, amino acid free media increased SNAT2 RNA expression by >25 fold versus an amino acid supplemented media (Lopez et al., 2006). Tovar et al. (2000) found that 60 min AA starvation in mammary tissue explants prior to addition of methylaminoisobutyric acid (MeAIB) (system A analogue) increased uptake by 3-fold over 5 h. Incubation of cells with inhibitors of RNA and protein synthesis (actinomycin D and cycloheximide) during this starvation period negated any increase in MeAIB uptake, thereby providing good evidence of adaptive regulation.

Adaptive regulation of SNAT2 is thought to arise from phosphorylation of eIF2 $\alpha$  kinase, indicative of uncharged tRNAs. This phosphorylation triggers ATF4 transcription factor which binds to the response element of sodium-coupled neutral amino acid transporter 2 (SLC38A2) and increases transcription (Table 1) (Gaccioli et al., 2006). Increased protein stability of SNAT2 is also observed when extracellular AA supplies are depleted (Hoffmann et al., 2018). Lastly, as part of this adaptive regulation, some evidence, though not conclusive, exists that SNAT2 signals to mTOR regarding extracellular AA supplies via a mechanism called “transceptor” (Taylor, 2014, Hoffmann et al., 2018). It appears that a change in extracellular AA supplies can increase mTOR signaling which downstream increases expression of transporters and conversely, if AA become limiting, phosphorylation of eIF2 $\alpha$  kinase will also increase transport. These mechanisms have not been found across all transporters and at this time, have predominantly been identified with sodium dependent transporters.

System y<sup>+</sup> represents the only sodium independent and non-exchange transporter identified in bovine mammary tissue (Baumrucker, 1984) (Table 2.2). This system consists of CAT-1 and CAT-3 and transports namely positively charged cationic AA, His, Arg, and Lys. The mode of uptake is electrical and chemical gradients arising from Na<sup>+</sup>/K<sup>+</sup> ATPase active transport (Deves and Boyd, 1998). This transport system is prone to competitive inhibition as both Arg and Lys have been shown to inhibit uptake of each other in dairy and pig mammary tissue explants (Baumrucker, 1984, Hurley et al., 2000). Uptake of Lys in the presence of extracellular AA supplies similar to in-vivo demonstrated no saturation, with the calculated k<sub>m</sub> being 2-3 times higher than in vivo concentrations (Hurley et al., 2000). Decreasing CP from 17.5 to 13.5 percent increased mammary transport efficiency of Lys and Arg but had no effect on mRNA abundance of the CAT-1 gene (Manjarin et al., 2012). This might infer a functional

change in CAT-1 or movement to the membrane which enabled the increased uptake efficiency. It should be noted this experiment did not account for blood flow in their uptake efficiency calculation, which is incorrect (Hanigan et al., 1998c). A more appropriate calculation would be arterial concentration multiplied by blood flow rate and divided by venous concentration (Hanigan et al., 1998c). Increasing dietary protein concentration from 12 to 24% in lactating sows decreased expression of CAT-2 and numerical decreases were observed for CAT-1 (Laspiur et al., 2009), indicating possible downregulation.

Lysine and Arg net uptake is far in excess of milk protein output, i.e. 1.35 and 2.45 times higher (Lapierre et al., 2012b), and Lys is considered a limiting AA for milk protein production. The nitrogen in Lys can be used for synthesis of Gln, Glu, Asp, Asn, Ser, and Ala whereas N in Arg is utilized primarily for Glu. Abomasum infusion of 23.5 g/d of Arg increased mammary uptake by 23% indicating proportional uptake to an increase in supply (Doepel and Lapierre, 2011). Lysine uptake is considered mass action, hence, increasing or decreasing arterial supplies result in constant proportion extracted by the udder. Supplementing varying amounts of Lys to lactating goats resulted in a corresponding linear change in mammary uptake of Lys relative to Lys supply (Guo et al., 2017). In summary, while these system y<sup>+</sup> transporters are prone to competitive inhibition, demonstration of in-vivo supply changes impacting transporter affinity or saturation is not apparent.

System y<sup>+</sup>L transport system also takes up cationic AA (Arg, His, Lys) but this is achieved by efflux of AA, primarily neutral AA though this can include cationic AA. Several studies have shown that Ala, Met, and Leu inhibit uptake of Lys which is thought to occur via y<sup>+</sup>LAT1 of System y<sup>+</sup>L (Shennan et al., 1994, Hurley et al., 2000). Increased intracellular concentrations of Arg, Lys, and Leu also accelerate efflux of Lys (Shennan et al., 1994).

Decreasing dietary CP from 17.5 to 9.5% had no effect on mRNA expression of a key System y+L transporter, y+LAT2, in lactating sows (Manjarin et al., 2012). Adaptive affinity besides initiation of lactation does not appear evident for this class of transporters.

System ASC transporters take up small neutral AA by exchange driven transport. These exchange transporters are termed “harmonizers” given their ability to synchronize intracellular AA needs. Baumrucker was the first to identify this class of transporters in udder tissue explants and demonstrated that expression is influenced by physiological status and milking frequency (Baumrucker, 1985). These exchangers within System ASC operate much faster than most of the transporters within System A (loaders) based on isotope studies indicating their importance in “harmonizing” the mixture of intracellular AA (Broer et al., 2016). Adaptive regulation given changing AA supplies in mammary tissue explants or in-vivo is sparse at this point (Shennan and Boyd, 2014). However, evidence outside of mammary tissue experiments point towards minimal adaptive regulation of this class of exchangers (Broer and Broer, 2017).

The System L transporters have affinity for the largest number of AA (at least a dozen) and operate by 1:1 AA exchange driven transport. Two heterodimer System L transporter proteins, LAT1 and LAT2, were documented in mammary tissue of cows (Baumrucker, 1985). These transporters do not establish large concentrative gradients versus extracellular supplies (Baumrucker, 1985, Shennan et al., 1997, Broer and Broer, 2017). Activity is rapid like system ASC in order to harmonize supplies of a large number of AA intracellularly. These system L transporters and rapid exchange were evident in lactating goats with a 35  $\mu\text{mol}/\text{min}$  influx and 24  $\mu\text{mol}/\text{min}$  efflux observed for Phe by the udder tissue (Hanigan et al., 2009). The results indicate 69% of Phe taken up by the udder was effluxed.

While these System L transporters are sodium independent, the importance of sodium driven AA (Ala, Gln, Met) for exchange is evident as removal of sodium or Gln from the media will inhibit uptake of System L AA substrates (Jackson et al., 2000, Nicklin et al., 2009). Additionally, excess Ala, Met, Gln, and Leu will competitively inhibit uptake (Jackson et al., 2000). Saturation of the System L transport system at in vivo ranges typical to lactating sows was not evident in mammary tissue explants incubated with increasing Val (Jackson et al., 2000).

Adaptive regulation via mRNA expression beyond stage of lactation is not strong for the highly expressed System L transport system (Shennan and Boyd, 2014). Movement to the membrane is accomplished by glycosylation of a heavy subunit (4F2hc/CD98) that translocate LAT1 and LAT2 transporter proteins (Hundal and Taylor, 2009). Some consider system L transport to be obligatory (Christensen, 1990). The latter assumption infers that mass action kinetics relative to both extracellular and intracellular concentrations dictate the net AA flux rate. Transporters regulated by external hormonal or AA deprivation stimuli such as SNAT2 within System A likely dictate accumulation of System L substrates by driving the exchange driven process (Christensen, 1990, Broer and Broer, 2017, Hoffmann et al., 2018).

System L is of particularly importance given its transport of BCAA which comprise 50% of the EAA content of milk protein (Lapierre et al., 2012b) and the role of Leu as a powerful regulator of mTOR signaling pathway (Wolfson and Sabatini, 2017). Increasing supplies by supplementing individual crystalline AA likely does not infer proportional supply increases intracellular due to the complexity of transport. For example, increasing Leu in blood plasma by 13% in dairy cows fed a low CP diet using supplemental crystalline Leu failed to increase milk protein yield or phosphorylation of mTOR pathway (Arriola Apelo et al., 2014a). Jugular or abomasum infusions in lactating dairy cows with one or more of the BCAA implicated in vitro

for regulating mTOR and increasing milk protein synthesis have largely not shown any benefit in vivo (Korhonen et al., 2002, Appuhamy et al., 2011b, Kassube et al., 2017, Curtis et al., 2018). In vitro trials usually provide extracellular NEAA by using Dulbecco's Modified Eagle medium (DMEM) which may enhance uptake of Leu and other important EAA for signaling via System L exchange (Appuhamy et al., 2012, Arriola Apelo et al., 2014c, Li et al., 2017c). For example, the Gln concentration in DMEM supplemented at manufacture recommendations is 2500  $\mu M$ , which is 568% higher than typical in-vivo Gln concentrations. Glutamine has been implicated with increasing mTOR signaling and the effect might be entirely driven by increasing concentrations of System L substrates (Hundal and Taylor, 2009, Nicklin et al., 2009, Taylor, 2014). Hence, stimulation of milk protein synthesis requires increased intracellular concentrations of EAA. To achieve this EAA concentration buildup requires sodium dependent loaders to drive up concentrations of EAA associated with System L using exchange driven transport by NEAA. One reason for the lack of replication of in vitro experimental findings to in vivo experiments might be due to AA transport as the NEAA concentrations in most in vitro experiments are at supraphysiologic concentrations.

The harmonizing effects of System ASC, L, and  $y^+L$  corrects for intracellular deficiencies by decreasing cellular efflux of whichever AA is limiting (Broer and Broer, 2017). Significant emphasis in ruminant nutrition is placed on balancing the metabolizable AA profile to match milk protein needs to prevent deficits and also to reduce excess AA supplied relative to demand (NRC, 2001, Schwab, 2014). However, if one considers how large the excess of post-absorptive AA supply is to mammary demand, the ability to mitigate any AA profile imbalances by bi-directional transport is strong. Most nutrition models infer fixed efficiency for maintenance on an aggregated basis (NRC, 2001) or individual EAA (CNCPS) and these models suggest Met

and Lys as limiting for milk production in most US based diets. Take CNCPS as an example, the milk protein efficiency from absorbed supply to milk protein at the optimal requirement of 1.12 g/Mcal of ME is 53%. This likely can vary substantially given changes in Met supply. If Met efflux decreased from declining intracellular Met concentrations, then increased Met net uptake efficiency will result and mitigate the Met shortage. Hence, the lack of responses to what are perceived limiting AA should not be unexpected when one realizes the quite complex ability of AA transport to manage homeostasis of intracellular AA supplies.

The effect of AA transport on intracellular AA profile and concentration also necessitates to empirically or mechanistically represent AA transport in milk protein prediction models. For example, the model proposed by Castro et al., (2016) implicated that Leu and Ile extracellular supplies increase mTOR phosphorylation in a curvilinear manner. However, all of the experimental data for deriving this model was cell culture based with supraphysiologic NEAA concentrations. The latter appears to be an important determinant in the intracellular concentrations of Leu and Ile as well as other System L AA (Nicklin et al., 2009). If NEAA would not have been supplied to the mammary epithelial cells, no phosphorylation of mTOR would have occurred as predicted by Castro et al., 2016. Amino acid transport needs to be understood and modeled in order to develop a robust mammary tissue model for predicting milk protein yield responses.

## **2.12. Udder AA Metabolism**

The majority of the AA taken up by the udder on a net basis (i.e. 90%) are destined for milk protein assembly (Cant et al., 2018). The remaining AA are used for cellular protein needs, catabolism (i.e. oxidation), or transamination. The BCAA, Arg, and Lys are taken up well in excess of milk protein needs (Lapierre et al., 2012b). The EAA taken up in excess are utilized for synthesis of NEAA and oxidation for energy. Isotope work has demonstrated the

incorporation of these EAA transformation into most of the NEAA, i.e. Gln, Glu, Asp, Asn, Ser, Ala, Pro (Verbeke et al., 1972, Roets et al., 1979, Roets et al., 1983, Rubert-Aleman et al., 1999). The isotope-labeled EAA carbon transfer to  $^{13}\text{CO}_2$  or  $^{14}\text{CO}_2$  has also been observed for Leu and Val, indicating oxidative metabolism usage of these EAA (Roets et al., 1979, Roets et al., 1983, Raggio et al., 2006). Increasing dietary supplies of these EAA (BCAA, Arg, and Lys) results in linear increases in net uptake by the udder indicating greater catabolism as the ratio of uptake to milk protein output increases linearly (Bequette et al., 1996, Raggio et al., 2006, Doepel and Lapierre, 2011, Guo et al., 2017). The excess uptake is not considered an obligatory requirement for milk protein synthesis for Arg and Leu as their deletion in AA infusates has not reduced milk protein yield (Bequette et al., 1996, Doepel and Lapierre, 2011). One recent study observed decreased milk protein yield from removal of Leu from an EAA infusate (Doelman et al., 2015b). In contrast, removal of lysine from AA infusates consistently decreases milk protein yield (Weekes et al., 2006, Doelman et al., 2015b, Guo et al., 2017).

These results indicate possibly N inefficiencies as the udder will take up increased supplies but not convert them proportionally to milk protein. The udder and milk protein yield appeared insensitive to supplies of BCAA and Arg. Thus, reducing supplies of these EAA should improve N efficiency by reducing excess mammary catabolism. The latter insight seems contrary given that intracellular Leu, Ile, and Arg concentrations have been widely implicated in mTOR regulation of protein translation in vitro (Arriola Apelo et al., 2014c, Hallett and Manning, 2016, Saxton et al., 2016a, Wolfson et al., 2016). In vivo, infusion of these EAA (Leu, Ile, Val, Arg) largely supports the conclusion that milk protein yield is not sensitive and hence, this excess supply produces an inefficiency (Korhonen et al., 2002, Appuhamy et al., 2011b, Kassube et al., 2017, Curtis et al., 2018). More insight into how these EAA that are taken up in

excess affect milk protein synthesis is needed, particularly their effects on regulatory networks related to protein translation.

The net uptake of Phe, Met, His, and in some cases Thr matches precisely the needs for milk protein yield (Lapierre et al., 2012b). This would infer that these AA might be limiting EAA for milk protein synthesis. Phe and Thr are not considered limiting EAA typically (NRC, 2001; Schwab, 2014), however, infusates lacking Phe have resulted in decreased milk protein yield (Doelman et al., 2015a, Doepel et al., 2016). In contrast, supplementing 7 g/d of absorbable crystalline Phe to 596 cows in a field study had no effect on milk protein yield or concentration (Swanepoel et al., 2015). Threonine deficiency has elicited increases in MPF to maintain supplies to the udder but has not decreased milk protein yield (Doepel et al., 2016). Positive milk protein yield responses to Met and His are well documented (Noftsker and St-Pierre, 2003, Chen et al., 2011, Lee et al., 2012, Zanton et al., 2014, Giallongo et al., 2016). However, variable responses and lack of embracement of these AA as limiting EAA is evident as discussed previously. The net uptake being nearly identical to milk protein output does not indicate that unidirectional udder uptake is not in excess of milk protein needs. In lactating goats, 53% of the Met taken up by the udder was effluxed. If this is true in dairy cows, then unidirectional uptake of these EAA (Met, His, Phe, and Thr) likely far exceeds milk protein needs. As discussed earlier, drawdown of any of these EAA intracellular will result in reduced efflux and increased net uptake regardless of blood supplies of these EAA. The efficiency of absorbed supplies of these EAA into milk protein being much less than 100% (e.g. Met at 66%) infers excess available that the mammary gland effluxes to venous blood. In summary, AA taken up by the udder in unity with milk protein demands does not consistently infer substrate supply regulation on milk protein yield. The same appears true for AA taken up in excess by the

udder except for Lys. Examination of regulation of protein synthesis thus consists of more than substrate supplies and is a primary culprit why the single limiting amino acid theory that is characterized by a barrel and shortest stave approach for predicting milk protein output performs so poorly.

### **2.13. Protein Translation and Regulation**

Milk protein synthesis involves transcription of milk protein genes' DNA, translation of mRNA into the respective milk protein polypeptide, and folding, post-translation modification, and packaging into micelles (Akers, 2002). The mechanisms involved in milk protein synthesis are similar to those for general protein synthesis in mammalian cells. Briefly, translation of mRNA consists of three major processes: initiation, elongation, and termination (Wu, 2013a). Initiation consists of binding of the start codon (always Met in eukaryotes) of the mRNA sequence to the 40S subunit of the ribosome that together then is bound to the 60S subunit of the ribosome. Elongation then proceeds along the mRNA sequence using the matching tRNA loaded with the respective AA to create a growing polypeptide chain. Termination occurs when stop codons within the mRNA sequence signal release of the polypeptide and dissociation of the two ribosomal subunits. Following polypeptide release, folding of the milk proteins into the tertiary structure of micelles occurs in the Golgi apparatus. The micelles form secretory vesicles that are exported by the merocrine mechanism (Akers, 2002).

Milk protein synthesis represents an energy and AA demanding event that suggests the need for stringent regulation of milk protein synthesis. Understanding this regulation may provide manipulation strategies for increasing the protein synthesis rate and yield and demand for intracellular AA. The latter function will decrease venous AA concentrations and gut catabolism of AA if occurring independent of changes in absorbed AA supply. The single limiting AA theory represented by barrel and stave visualization predicates that milk protein

synthesis rate is regulated by tRNAs becoming uncharged due to shortage of an AA and thereby halting protein translation (Mitchell and Block, 1946). This theory which is encoded in common nutrition models suggests protein synthesis is extremely sensitive to the AA profile of absorbed protein. If one assumes maintenance and efficiency of use is fixed as encoded in nutritional models, then matching the daily absorbed AA profile to the individual tRNA needs appears to be critical for regulating milk protein yield. This inflexibility of handling varying AA supplies for protein synthesis appears contrary to the requirement for life (Efeyan et al., 2015). The innate ability of organisms to sense and adapt to changes in nutrient supplies to maintain homeostasis (i.e. AA supply and profile) is a requirement for life. Hence, the finding that the single AA substrate limitation theory does not predict milk protein yield well seems obvious as dairy cows possess a variety of mechanisms to manage varying AA profile supply with the precision needed for milk protein translation. The mTORC1 and ISR pathways represent identified well-conserved regulatory networks of protein synthesis across species that enable sensing and adaptation of nutrient supplies to insure optimal survival of eukaryotic cells (Chantranupong et al., 2015, Wolfson and Sabatini, 2017). Evidence supporting regulation of protein translation by these networks in mammary epithelial cells in vitro is well documented (Appuhamy et al., 2011a, Appuhamy et al., 2012, Arriola Apelo et al., 2014c).

#### **2.14. mTORC1 Pathway**

The mTORC1 serine-threonine kinase complex integrates signals arising from intracellular AA, energy (ATP:AMP ratio), and insulin to direct cellular anabolic activity and proliferation. Additionally, studies have shown that signals arising from prolactin, fatty acids, stress, and oxygen act upon mTORC1 (Chantranupong et al., 2015, Osorio et al., 2016).

Activation of mTORC1 through phosphorylation triggers phosphorylation of ribosomal protein S6 kinase 1 at T389 position (S6K1) and eukaryotic translation initiation factor 4E-binding

protein 1 (4EBP1) as well as signaling proteins associated with autophagy, lipid biosynthesis, lipolysis, and cellular microtubular organization (Kanehisa et al., 2017). Activation of S6K1 then spurs phosphorylation of 40S ribosomal protein S6 (rpS6) and eukaryotic translation initiation factor 4B (EIF4B) (Kanehisa et al., 2017). Upregulated rpS6 is thought to spur biogenesis of ribosomes thereby increasing the number of ribosomes available for protein translation (Gentilella et al., 2015, Fyfe et al., 2018). Phosphorylation of EIF4B promotes recruitment of the translation initiation complex. Unphosphorylated 4EBP1 directly inhibits protein translation by binding to eIF4E and antagonizing the attachment of eIF4E to eIF4G (Brunn et al., 1997). Phosphorylation of 4EBP1 stops binding to eIF4E which then allows association of eIF4E to eIF4G. The latter association is necessary for the assembly of the start codon (Met-tRNA) together with ribosomal subunits that occurs during translation initiation. Phosphorylation of S6K1 and 4EBP1 and the corresponding downstream effects increase initiation rate thereby increasing protein translation rate. Activation of these described proteins from EAA supplementation has been positively correlated with casein synthesis rate in mammary tissue slices (Appuhamy et al., 2011a, Appuhamy et al., 2012, Arriola Apelo et al., 2014d).

### **2.15. ISR Pathway**

The ISR pathway integrates a diverse set of stress signals including amino acid deprivation, endoplasmic reticulum stress (unfolded proteins), viral infection, and heme deprivation to regulate a cellular response to reestablish cellular homeostasis (Pakos-Zebrucka et al., 2016a). Four kinases consisting of PKR-like ER kinase (PERK), double-stranded RNA-dependent protein kinase (PKR), heme-regulated eIF2 $\alpha$ , and general control non-derepressible 2 (GCN2) when activated phosphorylate eukaryotic translation initiation factor 2 alpha (eIF2 $\alpha$ ). Of these kinases, GCN2 senses intracellular AA shortages via uncharged tRNAs. The

phosphorylation of eIF2 $\alpha$  inhibits eIF2B-mediated release of GDP from eIF2 and replacement with GTP. This inhibition prevents the previously discussed important initiation step of binding of the start codon to the 40s ribosomal protein subunit. The primary activation of eIF2 $\alpha$  is increased expression and translation of ATF4, a basic leucine zipper transcription factor. ATF4 activates transcription of a plethora of genes related to the ISR response to maintain cellular homeostasis. These ISR activities are related to amino acid transporters, redox status, protein maturation and degradation, autophagy, mitochondrial function, transcription factors, apoptosis, and GADD34 feedback. Specifically, one of these activities is increased expression of Sestrin proteins (importance discussed in later section). Decreased phosphorylation of eIF2 $\alpha$  has not been observed in mammary tissue slices despite addition of EAA and increased casein synthesis rates (Appuhamy et al., 2012, Arriola Apelo et al., 2014c).

## **2.16. Mechanisms for AA Activation of mTOR**

Much of the research of mTOR and ISR signaling has demonstrated correlations with certain extracellular AA, hormone, or energy supplies but not specific mechanisms for intracellular activation. Activation of mTORC1 complex requires two Rheb and Rag guanosine triphosphatases GTPases for translocation to the lysosomal surface (Peng et al., 2014). To activate these GTPases, GATOR1 and GATOR2 interaction is required. One of the Sestrin proteins, Sestrin2 binds to GATOR2 and prevents association with GATOR1 (Wolfson et al., 2016). The apo structure of Sestrin2 contains a binding pocket with affinity for Leu, Ile, and Met (Saxton et al., 2016b). Upon binding of these EAA to Sestrin2, a conformational change occurs that releases GATOR2 from Sestrin2. The  $k_m$ 's for Leu, Ile, and Met binding to Sestrin2 in HEK-293T cells were demonstrated to be 20  $\mu M$ , 616  $\mu M$ , and 354  $\mu M$ , respectively (Wolfson et al., 2016). Leucine has affinity within the in-vivo range whereas Ile and Met binding appears to

only occur at supraphysiologic concentrations. In mammary epithelial cells, addition of AA compared to AA-free media decreased total abundance of Sestrin2 which also correlated to decreased total abundance of GCN2 (Luo et al., 2018). As mentioned previously, activation of ISR network increases expression of ATF4 which increases transcription of Sestrin2. Overexpression of Sestrin2 blocks activation of mTORC1 from the AA stimulation (Luo et al., 2018). In summary, activation of mTORC1 by Leu specifically occurs by binding to Sestrin2 in the cytosol, however, an activated ISR pathway can completely block activation by Leu (Wolfson and Sabatini, 2017).

A similar mechanism exists for Arg and Met sensing and mTORC1 activation. CASTOR1 binds to GATOR2 and inhibits association with GATOR1 thereby preventing recruitment of mTORC1 to the lysosome. The CASTOR1-GATOR2 complex is disassociated by Arg binding to CASTOR1 in the cytosol of HEK-294T cells at a dissociation constant of 30  $\mu\text{M}$  (Chantranupong et al., 2016) which is within the typical in vivo range. Another mechanism for mTORC1 regulation by Arg also exists. The lysosomal transporter SLC38A9 is regulated by lysosomal Arg concentrations and upon increased Arg, SLC38A9 is activated which triggers efflux of EAA, including Leu, out of the lysosome. The outflow of Leu to the cytosol then can activate mTORC1 through Sestrin2 (Wyant et al., 2017).

Methionine does not directly interact with GATOR2 or GATOR1 but through S-adenosylmethionine sensor (SAMTOR) (Gu et al., 2017). Methionine is converted to s-adenosyl methionine (SAM) via one-carbon metabolism pathway. The product SAM binds to SAMTOR complex at a dissociation constant of 7  $\mu\text{M}$  triggering disruption of SAMTOR and GATOR1 association (Gu et al., 2017). Freeing GATOR1 allows interaction with GATOR2 and for mTORC1 activation via recruitment to the lysosome to proceed. One carbon metabolism

therefore appears to be involved in activation of mTORC1, suggesting that methyl donors such as choline may interact with mTORC1 activation. A shortage of methyl donors in the udder may block activation of mTORC1 as SAMTOR will be bound to GATOR1.

Mechanisms for activation of mTORC1 by Arg, Leu, and Met supplies have been identified (Sabatini, 2017). In contrast, for the other EAA, only correlations between extracellular supplies and activated mTORC1 have been demonstrated and often at supraphysiologic concentrations. Changes in activation of mTORC1 signaling from these other EAA also could occur from decreased inhibition from the ISR pathway. For example, feeding MAC-T cells 1.33 mM of Val which is 432% higher than typical cow plasma concentrations did increase casein synthesis and mTORC1 signaling activation (Dong et al., 2018), however, this could be considered irrelevant given the extracellular media Val concentration is supraphysiologic. Similarly, in another study with mammary tissue slices, 420  $\mu$ M of Ile which is 293% higher than in vivo concentrations also increased casein synthesis and mTORC1 signaling (Appuhamy et al., 2012). The latter may have occurred via Sestrin2 binding and supraphysiologic Ile concentrations.

### **2.17. Other Mechanisms for Activation of mTOR**

Energy, oxygen, growth factors, and insulin signals are integrated via tuberous sclerosis complex (TSC) and affect downstream mTOR activation (Sabatini, 2017). Activated TSC blocks G-protein Ras homolog (Rheb) by inhibiting the GTPase activating protein (Tee et al., 2003). Inhibition of Rheb decreases mTORC1 activity. Insulin, upon binding to its receptor and activating insulin receptor substrate 1 (IRS1), causes phosphorylation of Akt which then blocks TSC activation (Garami et al., 2003). Increased AMP/ATP ratio activates AMP-activated protein kinase (AMPK) which activates the TSC complex thereby providing an energy sensing mechanism (Inoki et al., 2003a, Inoki et al., 2003b). Low intracellular oxygen, which in the

udder is delivered by hemoglobin carried with blood flow, activates REDD1 which subsequently inhibits the TSC complex (Katiyar et al., 2009). In summary, these other inputs provide regulation of mTORC1 activity independent of AA supplies on protein translation rate.

### **2.18. mTORC1 Feedback Loops**

Negative feedback from activation of a particular pathway is necessary for homeostasis and to prevent out of control activation. Several mechanisms independent of the ISR pathway provide negative feedback regulation on mTOR. Phosphorylation of S6K1 leads to phosphorylation of IRS1 at sites that promote IRS1 degradation (Haruta et al., 2000, Um et al., 2004) which reduces sensitivity to insulin. Activated S6K1 also reduces transcription of the IRS1 gene (Harrington et al., 2004). S6K1 also phosphorylates Rictor which blocks mTORC2 activation of Akt (Dibble et al., 2009). Mechanisms for minimizing growth factor signaling such as sequestering extracellular IGF-1 also exist (Ding et al., 2016). Increased expression of MAP2K1, MAP2K4, and MAP4K4 occurring from activated mTORC1 pathway also decreases insulin signaling (Bouzakri and Zierath, 2007). mTORC1 also appears to regulate AA transporters and upstream mTORC1 targets such as Sestrin2 via ATF4 independent of eIF2 $\alpha$  phosphorylation (Park et al., 2017). When protein translation and mTORC1 activity is reduced, 4E-binding proteins repressors reduce ATF4 translation and mRNA stability. This provides a mechanism to balance intracellular AA supplies with demand under non-stress conditions via ATF4. In summary, activated mTORC1 from energy, hormones, or AA appears to, over time, decrease sensitivity to the energy and hormone inputs to achieve a new steady state of anabolic signaling. When milk protein production is reduced, blunted mTORC1 activation may reduce intracellular amino acid influx and endoplasmic reticulum protein folding capability by decreasing ATF4 expression.

## 2.19. In Vivo mTOR and ISR Regulation Evidence in Dairy Cows

Evidence correlating increased milk protein yield and activation of mTOR and ISR signaling proteins (S6K1, 4EBP1, rpS6, eIF2 $\alpha$ ) is weak, particularly in long term trials. Substantial increases in milk protein yield of 29% and 16% from infused EAA or replacing forage with corn grain resulted in no phosphorylation state differences of mTOR and ISR signaling proteins (Bajramaj et al., 2017, Nichols et al., 2017). Five-day infusions of all EAA increased milk protein yield and phosphorylation state of S6K1 in one study but not in another study (Doelman et al., 2015a, Doelman et al., 2015b). Subtraction of Lys, His, Met, Phe, or Trp from EAA infusates for 5 d increased phosphorylation state of S6K1 (Doelman et al., 2015a, Doelman et al., 2015b). Subtraction of His and Lys increased phosphorylation state of S6K1 more than the positive control infusate containing all EAA (Doelman et al., 2015a, Doelman et al., 2015b). Protein yield in these AA subtraction treatments was lower than the positive control containing all EAA despite activation of S6K1. Feeding a higher CP diet (16.9 versus 15.0%) or supplementation of Met, Lys, and Leu for 15 d did not change phosphorylation state of S6K1, rpS6, 4EBP1, or eIF2 $\alpha$  compared to the low-protein negative control diet (Arriola Apelo et al., 2014a). The higher CP diet elicited a 9% increase in milk protein yield and a 2-kg/d milk yield increase compared to the negative control. In lactating goats feed-deprived for 24 h and then infused with an AA mixture similar to casein for 9 h, milk protein yield increased by 39% but no changes were observed in phosphorylation state of S6K1, 4EBP1, or eIF2 $\alpha$  (Guo et al., 2017). Infusing casein and starch increased milk protein yield and S6K1 activation (Rius et al., 2010a). Feed deprivation for 22 h and subsequent infusion of EAA and glucose for 9 h increased milk protein yield by 33% and phosphorylation of S6K1 and rpS6 (Toerien et al., 2010). These results provide sparse evidence that mTOR and ISR regulatory pathways regulates milk protein yield in dairy cows, particularly in longer term trials (>5 d).

Leucine, as discussed previously, is well documented activator of the mTOR pathway and increases in protein synthesis. Supplementing cows with Leu or BCAA has not increased milk protein yields (Huhtanen et al., 2002, Korhonen et al., 2002, Appuhamy et al., 2011b, Arriola Apelo et al., 2014a). Hence, the hypothesis that manipulation of certain EAA to increase protein translation rate via activated mTOR pathway, and thereby milk protein yield in dairy cows that is centered on in vitro findings and research outside of dairy science, apparently should be rejected. The mTOR and ISR regulatory pathways integrate a variety of signals, hence variation in responses to the strategy of supplementing EAA to manipulate mTOR activation seems apparent. As previously discussed, increased expression of Sestrin2 can completely block activation of mTORC1 from EAA. Endoplasmic reticulum stress has been correlated to trans-10, cis-12 CLA in mouse adipocytes and activation of eIF2 $\alpha$  and ATF4 which might reduce sensitivity of ISR and mTOR pathways to AA supplies. Negative feedback inhibition may wipe out increases in phosphorylation states of commonly measured mTOR signaling factors when higher milk protein yield is achieved over long-term trials. All of these discussed trials have utilized the western immunoblotting technique for inference on mTOR and ISR pathway regulation changes. This technique has a number of technical issues that introduce variation and should be only considered semi-quantitative (Aebersold et al., 2013). High analytical error of western immunoblotting may also mask more subtle changes that occur in vivo compared to in vitro experiments. These trials also only analyze 3-6 proteins associated with these regulatory pathways whereas more than 100 proteins are associated with the *Bos taurus* mTOR regulator network (Kanehisa et al., 2017). In summary, while evidence is not strong in support of a role for mTOR and ISR in regulating milk protein yield, the highly multivariate nature of these regulatory networks and analytical ability make in vivo experimentation challenging. Further

research is needed to elucidate whether this mTOR and ISR hypothesis should be accepted or rejected.

## **2.20. Other Protein Regulation Possibilities in Dairy Cows**

The discussed mTOR and ISR networks primarily affect the initiation rate of translation and, as stated, do not explain *in vivo* responses well. Other potential regulatory nodes are milk protein gene transcription, protein abundance of translation apparatus machinery, and changes in secretory cell number (Cant et al., 2018). Several studies related to energy restriction, hyperinsulinemic euglycemic clamp, and EAA infusion as reviewed by Cant et al. (2018), all increased milk protein yield but did not change mRNA abundance of milk protein genes (e.g. CSN3, LALBA, CSN1, CSN2). Hence, milk protein gene transcription does not appear to regulate milk protein yield from nutritional interventions.

Evidence supporting changes in translation machinery abundance *in vivo* is mixed, as pointed out by Cant et al. (2018). A 29% increase in milk protein yield did not elicit a change in mRNA expression of genes encoding for ribosomes (Nichols et al., 2017). This would indicate no change in ribosomal number to support greater milk protein yield. Infusion of a complete mix of EAA elicited an increase in milk protein yield but did not change abundance of S6K1 (Doelman et al., 2015a, Doelman et al., 2015b) whereas another study observed an increase in S6K1 abundance (Nichols et al., 2017). The analysis of more proteins (i.e., > 5) associated with mTOR and ISR pathways possibly is necessary to better understand whether changes do occur or not to affect rate of milk protein synthesis.

The number of secretory cells in the udder is affected by proliferation, differentiation, and apoptosis (Cant et al., 2018). Of these factors, differentiation of secretory cells has been proposed to be sensitive to EAA supplies and partially explain increases in milk protein yield (Nichols et al., 2017). Once amino acids are translated into polypeptides, folding into beta-

sheets and  $\alpha$ -helices and then ultimately the tertiary structure of that protein with the help of chaperones occurs in the endoplasmic reticulum. Proper folding then supports casein phosphorylation at specific sites to enable formation of micelles that are packaged into secretory vesicles for export out of mammary epithelial cells.

Differentiation enables fully developed protein folding capability to handle translated protein flow. An increase in misfolded proteins or excess of unfolded proteins will cause ER stress and release of immunoglobulin protein (BiP) from UPR sensors (Hetz et al., 2015). The dissociation of BiP can trigger three arms in response to unfolded proteins through three protein signaling cascades; phosphorylation of PERK, transcription factor 6 (ATF6), or endoribonuclease inositol-requiring enzyme 1 (IRE1) (Hetz et al., 2015). Phosphorylation of PERK triggers the previously discussed IRS pathway by phosphorylating eIF2 $\alpha$  and increasing expression of ATF4 (Pakos-Zebrucka et al., 2016b). When activated by cleavage, ATF6 increases mRNA expression of X-box binding protein (XBP1) and the gene encoding BiP, HSPA5. In concert with upregulated mRNA expression of XBP1, a phosphorylated IRE1 splices the XBP1 mRNA that is then translated to XBP1 protein. This protein then upregulates genes related to ER biogenesis and secretory vesicle maturation. In lactating mice, deletion of XBP1 at the onset of lactation resulted in minor changes in milk composition but an 80% reduction in pup growth (Davis et al., 2016). This reduction in pup growth suggests reduced milk yield. This reduction was partially explained by reduced ER abundance and increased ER stress indicative of a lack of capacity for the ER in the mice udder to fold and package milk components for secretion. One study that involved a 29% increase in milk protein yield demonstrated increased mRNA abundance of the spliced form of XBP1 which indicates greater ER biogenesis and differentiation to accommodate the increased milk protein yield (Nichols et al., 2017). In dairy

cows fed a higher CP diet (14.9 versus 12.6%), milk and milk protein yield increased by 5.6 kg/d and 263 g/d, respectively (Curtis et al., 2018). In this study, the spliced form XBP1 mRNA abundance numerical decreased by 27% in the higher producing cows (Curtis, 2018). This contradicts the hypothesis that milk protein yield is regulated by ER capacity and differentiation.

Conclusive mechanistic evidence on how AA supplies regulate milk protein yield in vivo appears to not be present. Perhaps this finding should not be a surprise given the complex regulation milk protein synthesis which makes study difficult. It seems evident that mammary epithelial cells possess flexibility to react to changing AA supplies via mTOR and ISR pathways (Efeyan et al., 2015). Feedback loops of these pathways as well as analytical challenges with assessing these pathways make inference in lactating dairy cows a challenge. Good evidence supports regulation by mTOR and ISR pathways under short term conditions involving substantial changes in nutrient supply (e.g. fasting and then feeding). Longer term studies involving treatments with substantial differences in milk protein yield have not elicited changes in phosphorylation state of mTOR and ISR pathways and in some cases, in the opposite directions. The theory that increased ER differentiation and capacity for processing and exporting milk protein was demonstrated in a 5-d experiment but not in a 21-d experiment. Further research is needed for more conclusive evidence on how milk protein synthesis regulation occurs in dairy cows.

### **2.21. Conclusion**

Balancing for AA and reducing dietary CP to improve N efficiency has not been widely adopted and is partly predicated on inability of nutrition models to predict success from AA balancing with an adequate frequency. The inflexible post-absorptive metabolism model of nutrition models is likely why low adoption of balancing for AA has occurred. Flexibility in handling varying AA supplies and profile while matching the precision needed for milk protein

translation is a requirement for life. Cell signaling via mTORC1 and ISR networks, AA transport adaptive regulation and high bi-directional transport, blood flow, and liver and gut tissue all act in concert to support milk protein yield. Predicting milk protein yield without representation of these mechanisms will lead to failure in predicting when EAA are deficient.

Of the N losses in dairy cows contributing to the 20-25% observed efficiency, post-absorptive losses represent the largest loss and an opportunity for manipulation. Representing the gut tissue demand as a function of blood flow, AA absorbed supplies, and blood AA concentrations will greatly enhance our ability to predict efficiency of use and maintenance. At the cell level, better understanding of how changes in arterial AA supplies translate into intracellular concentration changes is needed to predict changes in milk protein translation rate. Assuming mass-action kinetics infers that when AA supplies are reduced, intracellular AA supplies will be reduced which will down-regulate milk protein yield. If this is the case, improvements in N efficiency seem fleeting. However, most AA transport studies measure only net uptake, usually only consider one AA at a time, and are short-term. These experimental conditions might not adequately assess AA transport when AA supply is varied. Amino acid transport is highly multivariate, rapid bi-directional transport is evident, and changes in expression of transporters and movement of transporters to the membrane do occur. This flexibility demonstrates how AA transport can manage changes in AA supply while maintaining homeostasis of intracellular AA supplies. The lack of protein yield responses to supplementation of limiting EAA is partly due to the flexibility of AA transport which can simply decrease efflux of a deficient AA thereby improving net uptake to the udder independent of an arterial supply change. More understanding of AA transport in dairy cows is needed to provide a framework for nutrition models.

Substantial *in vitro* and short-term animal trial research has demonstrated how energy, EAA (particularly Leu, Met, and Arg), and hormones activate mTORC1 pathway which triggers a variety of pro-anabolic responses. One of these responses is a change in proteins associated with translation initiation, which enhances the rate of protein translation and thereby increases casein synthesis. However, long-term animal trials have not supported this hypothesis of mTORC1 and ISR regulation of milk protein yield. Long-term regulation to a new anabolic state is quite complex with negative feedback loops and integration from factors beyond EAA that are difficult to control in an animal trial. Additionally, analysis of only a few proteins in this pathway with a semi-quantitative technique may mask accurate inference into how mammary epithelial cells are regulating milk protein yield.

In summary, our understanding of post-absorptive AA metabolism continues to progress in dairy cattle. Incorporation of these findings and additional future research related to protein translation regulation at the udder and AA uptake by the udder into nutrition models should greatly enhance our ability to optimally supplement protein to lactating dairy cows.

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**Table 2. 1.** Metabolizable methionine balance relative to requirements given varying efficiency for a lactating dairy cow producing 45 kg/d milk with 3.10% true protein

Supply, g/d	Balance, g/d	Maintenance, g/d <sup>1</sup>	Milk, g/d	Efficiency %	Met, \$/d
62	-24	11	74	57 <sup>1</sup>	0.56
62	-12	10	64	66 <sup>2</sup>	0.29
62	-3	9	56	75	0.07
62	+5	8	50	85	-
62	+8	7	47	90 <sup>4</sup>	-

<sup>1</sup>Maintenance requirement calculated using CNCPS v6.55 model (VanAmburgh et al., 2015).

<sup>2</sup>The proposed optimal efficiency of use for Met (Tylutki and VanAmburgh, 2015).

<sup>3</sup>CNCPS v6.55 model contains 66% efficiency of use (VanAmburgh et al., 2015).

<sup>4</sup>Observed efficiency at the minimum of the 2<sup>nd</sup> derivative of the logistic function described by Doepel et al. (2004).

**Table 2. 2.** Amino acid transport systems in lactating dairy cows and their description of mechanism and substrates<sup>1</sup>

Transport System	Protein	Gene	Mechanism	Substrates	Notes
System A	SNAT2	SLC38A2	sodium dependent	Gly, Pro, Ala, Ser, Cys, Gln, Asn, His, Met	Loader, Adaptive affinity
System ASC	ASCT1	SLC1A4	exchange	Ala, Ser, Cys	Harmonizer
	ASCT2	SLC1A5	exchange	Ala, Ser, Cys, Thr, Gln	Harmonizer
System Gly	GLyT1	SLC6A9	sodium dependent	Gly	Loader
System L	LAT1	SLC7A5	exchange	His, Met, Leu, Ile, Val, Phe, Tyr, Trp	Harmonizer
	LAT2	SLC7A8	exchange	Ala, Asp, Cys, Gln, Gly, Ile, Leu, Met, Phe, Ser, Thr, Trp, Tyr, Val	Harmonizer
System N	SNAT3	SLC38A3	sodium dependent	Gln, Asn, His	Loader
System X <sub>ag</sub>	EAAT3	SLC1A1	sodium dependent	Asp, Glu, Cys	Loader
	EAAT2	SLC1A2	sodium dependent	Asp, Glu, Cys	Loader
	EAAT1	SLC1A3	sodium dependent	Asp, Glu	Loader
System y <sup>+L</sup>	y+LAT1	SLC7A7	exchange	Arg, Lys, Gln, His, Met, Leu, Ala, and Cys	Harmonizer
System y <sup>+</sup>	CAT-1	SLC7A1	uniporter	Arg, His, Lys,	Loader, Adaptive affinity
	CAT-3	SLC7A3	uniporter	Arg, His, Lys,	Loader, Adaptive affinity

<sup>1</sup>Literature sources: Baumrucker, 1984, Baumrucker, 1985, Wu, 2013, Shennan and Boyd, 2014, Taylor, 2014, Broer and Broer, 2017

## **CHAPTER 3: Short communication: Effects of varying extracellular amino acid profile on intracellular free amino concentrations and cell signaling in primary mammary epithelial cells**

### **3.1. Abstract**

Extracellular amino acid profiles affect intracellular AA concentrations and profile as well as signaling proteins that regulate protein translation rate. The objective of this work was to assess the effects of various extracellular AA profiles and the Lys to Met ratio to determine signaling protein sensitivity. Six AA profiles representing Dulbecco's modified Eagle's medium, blood meal, corn gluten meal, casein, plasma of lactating cows, and a negative control (NEG) represented the first factor (PROFILE) and the Lys to Met ratio (unaltered or set to 3:1) was the second factor (MK). Treatments were arranged in a six by two factorial manner resulting in 12 treatments that were replicated four times. The total AA concentrations for all treatments were set to 659 mg/L (63% of DMEM) except NEG (0 mg/L). Confluent mammary epithelial cells were exposed to the treatment media for 80 minutes (SD = 7.4). Intracellular concentrations of seventeen AA were affected by PROFILE but the sum of AA was not different between the AA profiles (323 mg/L, SD = 20) except for NC (171 mg/L). The Met and Leu percent of total intracellular AA mass, as an example, varied from 0.58 (COW) to 6.94 (NC, +MK) and 0.05 (NC, -MK) to 4.63 (CORN, +MK). Overall, balancing for Lys and Met increased intracellular concentrations by 54% and 71% respectively. Within the mechanistic target of rapamycin (mTOR) pathway, phosphorylation of mTOR (Ser2448), ribosomal protein S6 (Ser235/236), and eukaryotic initiation factor 4 E binding protein 1 (Thr37/46) (4EBP1) were increased for all five AA profiles relative to the NEG control. There were no differences among these five AA profiles, indicating lack of sensitivity to various AA profiles. Only phosphorylation of 4EBP1 was increased for MK, indicating some sensitivity to balancing for

Lys and Met. Phosphorylation of eukaryotic initiation factor 2 alpha subunit (Ser51) was unaffected by PROFILE and MK factors. Regression analyses indicated that intracellular concentrations of Met, Thr, Ile, and Leu predicted phosphorylation of mTOR related proteins with adequate precision and accuracy, suggesting that multiple EAA dictate regulation regardless of AA ratios. Changes in extracellular AA profiles translated to modified intracellular AA profiles, with no single profile uniquely stimulating phosphorylation of the mTOR pathway related proteins indicating that identification of a single optimal AA profile is unlikely.

**Key words:** amino acids, mammalian target of rapamycin (mTOR), translation regulation

### 3.2. Introduction

Protein efficiency of lactating dairy cows is low (25%) (Hristov et al., 2004) and identification of the optimal AA profile for metabolizable protein beyond Met and Lys is not specified by nutrition models (NRC, 2001, VanAmburgh et al., 2015). Overfeeding of N is the typical strategy for meeting individual AA requirements. Cells have requirements for 20 AA, hence diet optimization for a dairy cow could require consideration of up to 20 dimensions (Piper et al., 2017). This can potentially reduce to 10 dimensions if the non-essential AA can be ignored. Ignoring the remaining dimensions beyond Met and Lys, as is done when balancing for MP, will mask the ability to identify limiting EAA or the optimal profile. Use of traditional cell culture media AA concentrations during experimentation may also lead to incorrect inference (e.g. Dulbecco's modified Eagle's medium (DMEM)). Media designed to mimic the organism of interest is preferred. Varying media components or media unlike the organism of interest has been shown to result in varying treatment outcomes despite similarly imposed treatments (Cantor et al., 2017). Mammary cell culture provides a useful experimental tool that is economical compared to in-vivo trials and allows for testing of a high number of treatments on isolated epithelial cells (Clark et al., 1978). However, most in-vitro studies consider a few dimensions of the AA problem while fixing the remaining dimensions to the AA profile of DMEM (Appuhamy et al., 2012, Arriola Apelo et al., 2014c, Dong et al., 2018). Often, some AA are present at supraphysiologic concentrations (e.g. 1614% higher Met than in-vivo concentrations) and ratios (e.g. 1.4 ratio of Lys to Ile) (Dong et al., 2018). Given that specific individual AA are necessary for protein translation, providing a medium similar to that of plasma from lactating dairy cows

should provide more meaningful results when evaluating effects of changing supply or ratios of dietary AA.

Intracellular AA concentrations can change the rate of protein translational machinery via signal transduction of the mammalian target of rapamycin (mTOR) or integrated stress response (ISR) pathways (Appuhamy et al., 2012, Arriola Apelo et al., 2014c). Interestingly, multiple EAA appear to independently possess the ability to upregulate protein translational machinery and casein synthesis both in-vitro and in-vivo (Arriola Apelo et al., 2014d, Liu et al., 2017). The apparent flexibility infers that identification of a single optimal profile for stimulating mTOR as possibly a moot objective. Perhaps, various distinct profiles of AA can upregulate protein translational machinery and milk protein synthesis. Transport competition and the dynamics of efflux driven transport exert regulation on protein translational machinery (Nicklin et al., 2009, Taylor, 2014) which necessitates consideration of all twenty AA when evaluating AA profiles and effects on signal transduction related to mTOR pathway.

We hypothesized that multiple distinct sets of twenty AA could stimulate protein translational machinery. Additionally, we hypothesized that a constant lysine to methionine ratio of 3 to 1 would also stimulate protein translational machinery. The objectives were to assess short term incubations of AA profiles similar to casein, corn protein, DMEM, blood meal, and plasma from lactating dairy cows, and the interaction of lysine and methionine within those profiles on protein synthesis regulation.

### **3.3. Materials and methods**

Bovine primary mammary epithelial cells were obtained from the State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agriculture Sciences, Beijing, China. The cells were isolated from Chinese Holstein dairy cows (Hu et al., 2009). To confirm that the cells were bovine and epithelial in origin, identification of epithelial markers via

RT-PCR and proteomics were conducted (data not shown). Cells were seeded on 6-well plates (35 mm in diameter) treated with polystyrene (Corning, #0197A38) at an average density of 625,000 cells per well and incubated at 37 °C under 5% CO<sub>2</sub> (Thermo Scientific, HERAcCell 150i). The culture medium was DMEM/F-12 (Sigma-Aldrich, #D8900) containing 10% fetal bovine serum, 5 mg/L bovine insulin (Gemini Bio-Products, #700-112P), 5 mg/L prolactin (Sigma-Aldrich, #L6520), 5 mg/L progesterone (Sigma-Aldrich, #P7556), 5 mg/L transferrin (Sigma-Aldrich, #T8158), 0.01 mg/L epidermal growth factor (Sigma-Aldrich, #E4127), 1% of a 100X antibiotic-antimitotic mix (Gibco, #15240062), and 1 mg/L hydrocortisone (Sigma-Aldrich, #H0888). All media were adjusted to a pH of 7.3 and sterile filtered through a 0.20 µm filter (Thermo Scientific Nalgene Rapid-Flow, #595-4520). Cells were cultured to 90% confluency plus 6 d to achieve cellular differentiation (Nan et al., 2014). This was followed with serum-free medium containing all of the other components for 20 h. Cells were subsequently washed 2x with warm PBS (37 °C), and then incubated for 3 h with a starvation of media containing no AA, Earle's Balanced Salt Solution 10% (Sigma-Aldrich, #E7510), sodium bicarbonate (2.2 g/L), D-Glucose (2.151 g/L), 1% of a 100X MEM vitamin solution (Sigma #M6895), and the previously described hormones and antibiotics.

The AA profile and concentrations of treatment medium are described in Table 1 and mimicked six AA profiles (**PROFILE**) that were unbalanced for Lys to Met (**-MK**) or balanced to a mass ratio of 3 to 1 Lys to Met (**+MK**). The AA profiles were those of DMEM (**DMEM**), milk casein (Galindo et al., 2011) (**CASEIN**), blood meal (average of 13 samples reported by University of Illinois ingredient database ([http://nutrition.ansci.illinois.edu/feed\\_database.html](http://nutrition.ansci.illinois.edu/feed_database.html))) (**BM**), corn gluten meal (average of 9 samples reported by University of Illinois ingredient database) (**CORN**), average plasma AA concentrations of lactating dairy cows (average DIM =

75 d, average milk yield, 45.1 kg/d) (Swanepoel et al., 2016) (**PLASMA**), and a negative control containing no AA (**NEG**) Treatment target concentrations were achieved by the addition of the respective individual AA (L-isomer) (Sigma-Aldrich). Individual wells (n=2) across six-well plates (n=4) were randomly assigned to each treatment media (n=12) within each experimental run. The experiment was replicated on 4 different days using four different batches of thawed cells resulting in 4 blocks.

Cells were incubated with the respective treatment medium for an average of 80 min (SD = 7.4) following two washes of warm PBS (37 °C). Following incubation, media were discarded, and cells were washed twice with ice-cold stop buffer (1 mM NaF and 10  $\mu$ M Na<sub>3</sub>VO<sub>4</sub> in PBS) and then 300  $\mu$ l of ice cold RIPA buffer (Cell Signaling, #9806) that contained phosphatase (Halt Phosphatase Cocktail, Thermo Scientific, #78428) and protease inhibitors (Promega, #G6521) was added to the cells and allowed to incubate for 5 min. Then cells were mechanically detached and homogenized with the lysis buffer, inverted for 30 min at 4 °C, and then centrifuged for 20 min at 4 °C and 12,000 rcf. Approximately 200  $\mu$ l supernatant were stored at -80 °C for western immunoblotting and total protein analyses. The remaining supernatant was gravimetrically weighed and then deproteinized using 7% sulfosalicylic acid and centrifuged at 16,000 rcf for 15 min at 4 °C. The supernatant was collected, gravimetrically weighed, combined with a mixture solution of U-[<sup>13</sup>C,<sup>15</sup>N] AA derived from algae (Lot #16824, Product #CNLM-452) supplemented with [<sup>15</sup>N<sub>3</sub>, 99% enriched] His (Lot #15858, Product #NLM-1513), [<sup>15</sup>N, 98% enriched] Cys (Lot #14674, Product #NLM-2295), [<sup>15</sup>N, 99% enriched] Thr (Lot #20756, Product #NLM-742), [methyl-<sup>13</sup>C<sub>3</sub>, 99% enriched] Met (Lot #24031, Product #CLM-206), [Amide-<sup>15</sup>N, 98% enriched] Asn (Lot #12319, Product #NLM-120), and [Amide-<sup>15</sup>N, 98% enriched] Gln (Lot#15592, Product #NLM-557) and subsequently desalted by ion

chromatography (AG 50W-X8 resin; Bio-Rad Life Science), eluted with 2 N ammonium hydroxide, and freeze dried. All isotopes were purchased from Cambridge Isotope Laboratories Inc, Tewksbury, MA. Dried samples were resolubilized in 0.1 N HCl, dried under nitrogen for 20 min at 35°C, solubilized in acetonitrile (J. T. Baker Inc., Phillipsburg, NJ), and converted to *N*-(*tert*-butyldimethyl) AA derivatives by incubation for 1 h at 70°C in *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (SELECTRA-SIL; UCT Inc., Bristol, PA). Amino acid derivatives were separated by gas chromatography (Trace GC Ultra; Thermo Scientific) and quantified for isotopic enrichment by mass spectrometry (DSQII; Thermo Scientific) (Calder et al., 1999). Calibration curves for derivation of AA concentrations from the isotopic ratios were developed using the same labeled AA mixture spiked into the samples gravimetrically combined in varying ratios with an AA standard mix (Sigma-Aldrich, #AAS18) plus L-Asn, L-Gln, L-Trp, and L-Cys (Sigma-Aldrich, St. Louis, MO).

Total protein concentrations were determined in duplicate using a bicinchoninic acid (BCA) assay. Protein concentrations averaged 5.9 mg/μl (SD = 1.09). Aliquots of 30 μg of protein per sample were combined with 4x Laemmli sample buffer (Bio-Rad, #161-0747) containing 10% 2-mercaptoethanol, incubated at 95° C for 5 min, placed on ice for 2 min, centrifuged at 16,000 rcf for 1 min at 20°C, and then separated on 7.5 to 12% SDS-PAGE gels (Bio Rad Mini-PROTEAN TGX Gels) at 100 V for 90 min. The separated proteins were electrotransferred onto Immobilon-FL polyvinylidene fluoride (PVDF) membranes (Sigma-Aldrich, #IPFL00010), stained for visual total protein transfer confirmation using Ponceau S solution (Santa-Cruz Biotechnology, Inc., #sc-301558), washed vigorously with water, and then blocked for 1 h at room temperature with Odyssey blocking buffer (TBS) (Licor, #927-50000). Blots were incubated overnight at 4°C with primary rabbit and mouse antibodies in Odyssey

blocking buffer (TBS) containing 0.2% Tween-20 solution (Bio-Rad, #161-0781). Primary monoclonal antibodies used included total and site-specific phosphorylated mTOR (Ser2448) (mouse #4517 and rabbit #5536), ribosomal protein S6 (Ser235/236) (mouse #2317 and rabbit #4858), eIF2 $\alpha$  (Ser51) (mouse #2103 and rabbit #3597), 4E-BP1 (Thr37/46) (mouse #sc-81149 and rabbit #2855), and the total form only of  $\alpha$ -tubulin (rabbit #2144). All primary antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA) except the total form of 4E-BP1 which was acquired from Santa Cruz Biotechnology Inc. (Dallas, TX). All antibodies were diluted at 1:1,000 (vol/vol) except for the phosphorylated form of ribosomal protein S6, which was 1:2000 (vol/vol). Following incubation, membranes were washed 5 times for 5 min each with Tris buffered saline containing 0.1% Tween-20 solution and then incubated for 1 h at room temperature with goat anti-mouse (IRDye 680RD, LI-COR, #925-68070) and anti-rabbit (IRDye 800CW, LI-COR, #926-32211) secondary antibodies (diluted to 1:10,000 (vol/vol) in Odyssey blocking buffer (TBS), 0.2% Tween-20 solution, and 0.01% sodium dodecyl sulfate.

Membranes were washed 5 times as described previously and scanned using an Odyssey Infrared Imaging System (LI-COR Biosciences). The signal intensity of phosphorylated and total forms of the proteins was quantified using Image Studio software from LI-COR (version 5.2.5).

Statistical analysis was conducted using R version 3.3.2 (R Core Team, 2016) and the *lmer* package (Bates et al., 2015). The intracellular free AA concentrations were tested using a mixed model that contained the fixed effects of PROFILE (df=5), balancing lysine and methionine to 3 to 1 ratio, MK, (df=1), and interaction of PROFILE and MK (df=5), the random effect of block (df=3) and residual error (df=33). For the western immunoblotting data, the phosphorylation intensity was divided by the total protein intensity resulting in a ratio and subsequently log (e) transformed as data were not normally distributed (Shapiro-Wilk test). The

transformed data were then fit to a mixed model that included the previously described fixed and random effects, as well as the random effect of gel and sample as a repeated effect nested within block. The denominator degrees of freedom for all tests were adjusted using the Kenward-Rogers option. Main effects and interactions were deemed significant at  $P < 0.05$  and a tendency at  $0.05 < P < 0.10$ . Post-hoc mean-separation testing was conducted only on significant main effects using the “lsmeansLT” package. The log transformed phosphorylated to total protein ratio was regressed on intracellular AA concentrations while also accounting for the random effects of block, gel, and repeated effects of sample nested within block. All essential AA were tested using a stepwise, backward elimination, regression procedure with variables having significant parameters ( $P < 0.05$ ) retained. Root mean squared errors as a percentage of the mean (RMSE) and mean squared prediction errors partitioned into mean bias, slope bias, and dispersion were calculated (Bibby and Toutenberg, 1977). The Concordance correlation coefficient (CCC) was also calculated to provide a dimensionless evaluation of precision and accuracy (Lin, 1989).

### **3.4. Results and Discussion**

The objective of this experiment was to evaluate various AA profiles as well as the merits of balancing Lys to Met at a 3 to 1 ratio for regulation of protein translational machinery. An optimal profile of AA beyond lysine and methionine has not been put forth (NRC, 2001). The barrel and stave analogy would suggest a single optimal AA profile solution (Mitchell and Block, 1946). Knowledge of precise individual AA requirements will allow lower CP diets to be fed, thereby improving N efficiency without compromising animal performance. Additionally, economic evaluation of feedstuffs is routinely conducted on the aggregated calculations of MP or CP (St-Pierre and Glamocic, 2000, Cabrera et al., 2011), neither of which are required by mammary epithelial cells. For example, feedstuffs with high corn protein concentrations (i.e. corn distillers or corn gluten feed) are routinely termed “bargain” feedstuffs (Cabrera et al.,

2011) while other ingredients such as animal or soy protein are considered “expensive” feedstuffs. Evaluation of feedstuffs on AA content might better match ingredient economic value with nutrient value to dairy cows.

The selection of casein, corn gluten meal, and blood meal profiles of AA for this experiment was meant to mimic extreme variants in AA profile, not what would occur in the plasma of lactating dairy cow. The use of DMEM profile and cow plasma profile was intended to identify if there are differences between these two AA profiles that might suggest DMEM is not an optimal AA profile for experimentation and inference. The concentration of the 5 AA profiles was set at 4.57 mM (i.e. 63% of DMEM concentrations), which represents the concentration that resulted in the maximum stimulation of casein synthesis in a previous cell culture experiment (Arriola Apelo et al., 2014d). Another reason that this total concentration was selected versus a lower concentration was to maximize differences between individual AA, thereby treatments. However, our imposed treatments might mask substrate limitations by targeting a concentration in significant excess of what occurs in-vivo. Total arterial AA concentrations in dairy cattle average 2.30 mM (Swanepoel et al., 2016) with the venous concentrations likely being much less, and the latter representing the concentrations that mammary epithelial cells and their transporters would be exposed to (Hanigan et al., 1998c). While our objective was to compare distinct profiles, one might want to experiment using mammary venous concentrations as the experimental media concentrations in order to better represent transport and substrate dynamics that occur in-vivo.

The extracellular EAA content of the treatments expressed as a mass proportion of total AA varied from 44.5 (CORN) to 57.5% (BM). Individually, Ile and Leu varied from 1.8 for BM to 5.2 (DMEM), 5.6 (DMEM), and 16.2 (PLASMA) percent of total AA. Lysine and methionine

in the unbalanced (-MK) treatments had ratios ranging from 1.8 (CORN) to 9.4 (BM) and 1.2 (COW) to 2.8 (CASEIN) as percent of total AA, respectively. Of the NEAA, Ala and Gln had large differences, ranging from 0.4 (DMEM) to 8.3 (COW) and 6.8 (BM) to 34.9 (DMEM). The AA concentration of the treatments is listed in Table 1 and reflects these distinct AA profiles. Lys and Met ratios as a percent of total AA for the +MK factor met what is described as the optimal profile of absorbed AA (NRC, 2001), i.e. 2.4 and 7.2% of total AA.

Intracellular free AA concentrations are listed in Table 2, and seventeen AA were affected by the main effect of AA profile ( $P < 0.001$ ). Across treatments, most AA were present in greater concentration than the NEG and also differed between BM, CASEIN, CORN, PLASMA, and DMEM treatments despite similar total AA concentrations between the latter 5 treatments. Intracellular AA concentrations are a function of AA transport, protein synthesis demand, protein degradation, synthesis (i.e. NEAA), and oxidation (Bender, 2012). The precision required to support protein translation is achieved by various mechanisms, particularly transport (Christensen, 1990). The treatment profile versus the intracellular profile changed for most AA, which is reflected in most AA having differences in transport efficiency between extracellular and intracellular space (Supplemental Material, Table 1). Amino acid transport has been suggested to approximate mass action kinetics in the in vivo range (Hanigan et al., 1992), hence, a similar proportion of AA in terms of concentration should occur intracellularly. Sixteen AA had varying proportions of AA in the intracellular space as compared to extracellular, which suggests regulation of the underlying mechanisms. The dynamics of transport, i.e. efflux, and competition, and intracellular metabolism, i.e. AA demand for protein synthesis, oxidation, NEAA synthesis, etc., change intracellular profile. For example, Ala intracellular concentrations ranged from 41.6 (CORN) to 303% (DMEM) of extracellular concentrations ( $P < 0.001$ ). The

latter finding may reflect high intracellular synthesis of Ala from EAA occurring with the DMEM treatment (Supplemental Material, Table 1). Met and Lys intracellular concentrations were affected by an interaction of PROFILE and MK effects ( $P < 0.001$ ). This was evident as the Met and Lys concentrations were higher in the NEG+MK treatment compared to the NEG-MK treatment. The interaction effect was present despite similar extracellular concentrations of Met and Lys in the medium which infers other AA affect uptake of Met and Lys. This may reflect uninhibited AA transport as the other eighteen AA were not present in the media for the NEG+MK treatment. Competitive inhibition of transport may occur when high concentrations of extracellular AA are present which might prevent limiting EAA from reaching desired intracellular AA concentrations (Christensen, 1990). The total mass of AA ranged from 159 mg/L to 360 mg/L intracellularly whereas the extracellular ranged from 0 mg/L to 699 mg/L. Intracellular Leucine ranged from 0.05 to 4.63 % of total AA on a mass basis reflecting significant range in ratios across treatments. Excluding the NEG treatment, the range was 1.53 to 4.63 for Leu and 0.18 to 1.52 to Ile as a % of total AA.

The mTOR pathway is an important regulator of anabolic metabolism and its activation has been correlated with milk protein yield in vitro and some in vivo studies (Toerien et al., 2010, Arriola Apelo et al., 2014d, Liu et al., 2017). Phosphorylation of mTOR at Ser2448 and downstream at rpS6 and 4EBP1 are good indicators of activation of this pathway. All five profiles of AA increased phosphorylation of these three targets ( $P < 0.001$ ) relative to the negative control (Table 3). There was no effect of MK on phosphorylation of rpS6 and mTOR ( $P = 0.23$  and  $P = 0.50$ , respectively) but MK increased phosphorylation of 4EBP1 ( $P = 0.03$ ). Interestingly, among the 5 profiles with varying ratios of key EAA, phosphorylation rates did not differ for any of the three proteins measured. This might indicate a precision issue with the

western immunoblotting measurement or that multiple distinct sets of AA can activate the mTOR pathway in an equal manner. Multiple linear regression analyses testing intracellular total AA and individual EAA revealed that several EAA, Met, Thr, Ile, and Leu were significant for predicting the observed phosphorylation changes (Supplemental Material, Table 2). The regression findings confirm that signal transduction regulation related to protein initiation can be stimulated by the same EAA as previously observed by Appuhamy et al. (2012) and Arriola Apelo et al. (2014c). For example, phosphorylation of 4EBP1 was predicted with good accuracy and precision from intracellular concentrations of Met, Thr, Ile, and Leu. Despite distinct AA profiles, adequate supplies of either Met, Thr, Ile, and Leu within the DMEM, CORN, BM, CASEIN, and PLAMSA treatments elicited changes in phosphorylation of mTOR pathway related proteins.

### **3.5. Conclusion**

Distinct extracellular and intracellular AA profiles were able to upregulate key phosphorylation sites related to protein translational machinery. Balancing extracellular Met and Lys according to NRC 2001 had benefits only on phosphorylation of 4EBP1, an important step for translation initiation. There were no interactions between the effects of balancing Met and Lys and the other AA, indicating independence of the effects. In support of previous findings, multiple linear regression indicated that intracellular concentrations of several EAA (Met, Ile, Leu, and Thr) explained phosphorylation of mTOR pathway targets in an additive and independent manner. Our findings suggest that the objective of identifying a single optimal EAA profile as unlikely to be achieved. Instead, as long as several EAA are present in adequate concentration, protein translational machinery can be stimulated. This means nutritionists should consider various formulation options for maximizing milk protein yield and not a single unique EAA profile.

### **3.6. Acknowledgements**

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**Table 3. 1.** Amino acid concentrations ( $\mu M$ ) of the extracellular media provided to mammary epithelial cells

$\mu M$	AA Profile <sup>1</sup>											
	DMEM		CORN		BM		CASEIN		PLASMA		NEG	
	-MK <sup>2</sup>	+MK <sup>2</sup>	-MK <sup>2</sup>	+MK <sup>2</sup>	-MK <sup>2</sup>	+MK <sup>2</sup>	-MK <sup>2</sup>	+MK <sup>2</sup>	-MK <sup>2</sup>	+MK <sup>2</sup>	-MK <sup>2</sup>	+MK <sup>2</sup>
Ala	32	32	606	606	585	585	222	222	615	615	-	-
Arg	440	440	124	124	168	168	136	136	184	184	-	-
Asn	32	32	228	228	366	366	185	185	17	17	-	-
Asp	32	32	63	63	101	101	148	148	5	5	-	-
Cys	126	126	70	70	51	51	15	15	17	17	-	-
Gln	1575	1575	694	694	307	307	581	581	857	857	-	-
Glu	32	32	163	163	72	72	333	333	126	126	-	-
Gly	158	158	239	239	357	357	159	159	822	822	-	-
His	94	94	86	86	261	261	114	114	120	120	-	-
Ile	262	262	209	209	88	88	244	244	247	247	-	-
Leu	284	284	814	814	645	645	456	456	380	380	-	-
<b>Lys</b>	<b>314</b>	<b>251</b>	<b>63</b>	<b>270</b>	<b>331</b>	<b>252</b>	<b>269</b>	<b>252</b>	<b>130</b>	<b>268</b>	-	<b>259</b>
<b>Met</b>	<b>73</b>	<b>105</b>	<b>105</b>	<b>112</b>	<b>53</b>	<b>105</b>	<b>125</b>	<b>105</b>	<b>54</b>	<b>112</b>	-	<b>108</b>
Phe	136	136	254	254	278	278	200	200	119	119	-	-
Pro	95	95	488	488	244	244	591	591	239	239	-	-
Ser	158	158	256	256	256	256	349	349	213	213	-	-
Thr	283	283	177	177	256	256	234	234	252	252	-	-
Trp	28	28	19	19	49	49	38	38	181	181	-	-
Tyr	135	135	191	191	113	113	195	195	134	134	-	-
Val	285	285	255	255	484	484	310	310	574	574	-	-
Total AA, mg/L	659	652	659	699	659	652	659	653	659	693	-	64
Lys:Met, mg/L	5.4	3.0	0.8	3.0	7.9	3.0	2.7	3.0	3.0	3.0	-	3.0
Lys % AA	8.9	7.2	1.8	7.2	9.4	7.2	7.6	7.2	3.7	7.2	-	-
Met % AA	1.7	2.4	2.4	2.4	1.2	2.4	2.8	2.4	1.2	2.4	-	-

<sup>1</sup>AA Profile; DMEM=Dulbecco Modified Eagle's Medium (Dulbecco and Freeman, 1959), CORN=corn gluten meal (n=9), BM=ring dried blood meal (n=13), CASEIN=milk casein (Galindo et al., 2011), PLASMA=average blood plasma of lactating dairy cows (Swanepoel et al., 2016), NEG=negative control of no amino acids.

<sup>2</sup>MK=balancing for methionine and lysine according to NRC (2001), negative sign indicating unbalanced and positive sign balanced.

**Table 3. 2.** Intracellular free AA concentrations ( $\mu M$ ) in mammary epithelial cells following 80 min treatment incubation (treatment main effect means)

$\mu M$	AA Profile <sup>1</sup>						MK <sup>2</sup>		SEM	Effect ( <i>P</i> -value)		
	BM	CASEIN	CORN	PLASMA	DMEM	NEG	-	+		Profile	MK	Profile * MK
Ala	303.1 <sup>a</sup>	155.6 <sup>c</sup>	253.7 <sup>b</sup>	273.1 <sup>b</sup>	110.2 <sup>d</sup>	79.4 <sup>e</sup>	191.4	200.3	23.9	<0.001	0.16	0.45
Arg	13.8 <sup>b</sup>	12.3 <sup>b</sup>	13.0 <sup>b</sup>	16.0 <sup>b</sup>	30.0 <sup>a</sup>	10.6 <sup>b</sup>	15.5	16.4	3.0	<0.001	0.44	0.61
Asn	119.5 <sup>a</sup>	42.1 <sup>b</sup>	52.3 <sup>b</sup>	ND <sup>3</sup>	ND <sup>3</sup>	ND <sup>3</sup>	24.9	21.7	9.3	<0.001	0.60	0.75
Asp	38.2 <sup>a</sup>	20.8 <sup>c</sup>	26.8 <sup>b</sup>	17.2 <sup>c</sup>	10.0 <sup>d</sup>	6.6 <sup>d</sup>	21.0	18.9	3.8	<0.001	0.24	0.82
Gln	49.1 <sup>d</sup>	103.0 <sup>c</sup>	111.7 <sup>c</sup>	130.7 <sup>b</sup>	235.1 <sup>a</sup>	7.8 <sup>e</sup>	106.9	105.5	15.3	<0.001	0.81	1.00
Glu	1040.2 <sup>c</sup>	1075.1 <sup>bc</sup>	1123.5 <sup>b</sup>	1234.3 <sup>a</sup>	1177.4 <sup>ab</sup>	820.8 <sup>d</sup>	1077.8	1079.3	140.4	<0.001	0.95	0.82
Gly	273.1 <sup>b</sup>	255.5 <sup>bc</sup>	235.6 <sup>c</sup>	332.8 <sup>a</sup>	210.7 <sup>d</sup>	164.8 <sup>e</sup>	243.8	247.0	33.3	<0.001	0.58	0.82
His	58.2 <sup>ab</sup>	120.5 <sup>a</sup>	58.3 <sup>ab</sup>	76.5 <sup>ab</sup>	32.0 <sup>b</sup>	21.6 <sup>b</sup>	87.0 <sup>a</sup>	35.4 <sup>b</sup>	29.6	0.10	0.02	0.13
Ile	4.5 <sup>d</sup>	30.8 <sup>b</sup>	22.5 <sup>c</sup>	31.1 <sup>b</sup>	35.3 <sup>a</sup>	ND	20.1	20.9	2.3	<0.001	0.41	0.60
Leu	97.2 <sup>b</sup>	65.8 <sup>c</sup>	114.2 <sup>a</sup>	50.8 <sup>d</sup>	36.2 <sup>c</sup>	1.0 <sup>f</sup>	59.7	62.0	7.5	<0.001	0.42	0.59
Phe	54.4 <sup>a</sup>	36.9 <sup>c</sup>	42.0 <sup>b</sup>	18.4 <sup>c</sup>	24.3 <sup>d</sup>	4.4 <sup>f</sup>	29.7	30.5	2.2	<0.001	0.39	0.21
Pro	195.3 <sup>c</sup>	437.9 <sup>a</sup>	311.6 <sup>b</sup>	195.9 <sup>c</sup>	135.7 <sup>d</sup>	83.3 <sup>e</sup>	227.0	226.2	25.5	<0.001	0.90	0.67
Ser	27.9 <sup>b</sup>	39.7 <sup>a</sup>	25.7 <sup>b</sup>	21.2 <sup>b</sup>	10.8 <sup>c</sup>	6.8 <sup>c</sup>	23.9	20.1	4.7	<0.001	0.08	0.52
Thr	35.9 <sup>a</sup>	26.1 <sup>b</sup>	8.3 <sup>c</sup>	27.0 <sup>b</sup>	27.7 <sup>b</sup>	ND	16.1	17.2	6.0	<0.001	0.39	0.81
Trp	35.0	45.3	38.3	44.0	31.4	32.8	35.0	40.6	6.3	0.19	0.16	0.57
Tyr	5.6 <sup>d</sup>	23.7 <sup>a</sup>	19.3 <sup>b</sup>	9.5 <sup>c</sup>	11.5 <sup>c</sup>	ND	9.7	10.6	2.1	<0.001	0.15	0.44
Val	58.7 <sup>b</sup>	34.5 <sup>c</sup>	24.2 <sup>d</sup>	69.9 <sup>a</sup>	31.5 <sup>c</sup>	1.9 <sup>e</sup>	36.2	37.4	4.6	<0.001	0.48	0.87

<sup>a-c</sup>Means within a row and main effect column with different superscripts differ ( $P < 0.05$ ).

<sup>1</sup>AA Profile; DMEM=Dulbecco Modified Eagle's Medium (Dulbecco and Freeman, 1959), CORN=corn gluten meal, BM=ring dried blood meal, CASEIN=milk casein (Galindo et al., 2011), PLASMA=average blood plasma of lactating dairy cows (Swanepoel et al., 2016), NEG=negative control of no amino acids.

<sup>2</sup>MK=balancing for methionine and lysine according to NRC (2001), negative sign indicating unbalanced and positive sign balanced.

<sup>3</sup>ND= amino acid not detected or estimated to be less than zero.

**Table 3. 3.** Intracellular Lys and Met free AA concentrations ( $\mu M$ ) in mammary epithelial cells following 80 min treatment incubation (least-square treatment means)

Main Effects		Amino acid, $\mu M$	
AA Profile <sup>1</sup>	MK <sup>2</sup>	Lys	Met
Blood	-	27.7 <sup>bc</sup>	15.9 <sup>de</sup>
	+	24.4 <sup>bc</sup>	34.5 <sup>c</sup>
Casein	-	29.4 <sup>b</sup>	51.2 <sup>b</sup>
	+	23.8 <sup>bcd</sup>	30.0 <sup>c</sup>
Corn	-	10.6 <sup>e</sup>	30.7 <sup>c</sup>
	+	28.5 <sup>bc</sup>	32.8 <sup>c</sup>
Cow	-	17.9 <sup>d</sup>	13.2 <sup>e</sup>
	+	28.4 <sup>bc</sup>	28.3 <sup>c</sup>
DMEM	-	26.0 <sup>bc</sup>	22.7 <sup>d</sup>
	+	22.6 <sup>cd</sup>	34.6 <sup>c</sup>
Neg	-	6.5 <sup>e</sup>	9.6 <sup>e</sup>
	+	54.5 <sup>a</sup>	84.8 <sup>a</sup>
SEM		3.6	3.7
		Main Effects ( <i>P</i> -value)	
PROFILE		<0.001	<0.001
MK		<0.001	<0.001
PROFILE*MK		<0.001	<0.001

<sup>a-f</sup>Means within a column with different superscripts differ ( $P < 0.05$ ).

<sup>1</sup>AA Profile; DMEM=Dulbecco Modified Eagle's Medium (Dulbecco and Freeman, 1959), CORN=corn gluten meal, BM=ring dried blood meal, CASEIN=milk casein (Galindo et al., 2011), PLASMA=average blood plasma of lactating dairy cows (Swanepoel et al., 2016), NEG=negative control of no amino acids.

<sup>2</sup>MK=balancing for methionine and lysine according to NRC (2001), negative sign indicating unbalanced and positive sign balanced.

**Table 3. 4.** Phosphorylated-to-total signaling protein ratios of various proteins subjected to varying extracellular AA profiles and balancing lysine and methionine to a three to one ratio.

Main Effects		Signaling protein phosphorylation ratio <sup>3</sup>			
AA Profile <sup>1</sup>	MK <sup>2</sup>	mTOR	rpS6	4EBP1	eIF2a
BM		8.48 <sup>a</sup>	3.49 <sup>a</sup>	7.18 <sup>a</sup>	0.51
	-MK	7.77	3.19	6.97	0.71
	+MK	9.24	3.82	7.41	0.37
CASEIN		7.87 <sup>a</sup>	2.81 <sup>a</sup>	7.18 <sup>a</sup>	0.51
	-MK	7.59	2.71	7.48	0.45
	+MK	8.17	2.90	8.01	0.35
CORN		8.93 <sup>a</sup>	3.02 <sup>a</sup>	8.14 <sup>a</sup>	0.42
	-MK	9.40	2.80	8.83	0.44
	+MK	8.47	3.26	7.51	0.40
COW		8.26 <sup>a</sup>	2.46 <sup>a</sup>	6.77 <sup>a</sup>	0.41
	-MK	7.72	2.12	5.96	0.36
	+MK	8.85	2.85	7.70	0.46
DMEM		7.90 <sup>a</sup>	2.86 <sup>a</sup>	7.79 <sup>a</sup>	0.50
	-MK	7.23	2.69	6.99	0.53
	+MK	8.62	3.04	8.68	0.47
NEG		5.19 <sup>b</sup>	0.02 <sup>b</sup>	1.84 <sup>b</sup>	0.54
	-MK	5.83	0.00	1.53	0.52
	+MK	4.62	0.06	2.21	0.56
Overall (MK)	-MK	7.52	0.91	5.56 <sup>b</sup>	0.49
	+MK	7.80	1.64	6.35 <sup>a</sup>	0.43
SEM		0.36	1.14	0.29	0.26
Main Effects ( <i>P</i> -value)					
AA PROFILE		<0.001	<0.001	<0.001	0.37
MK		0.50	0.23	0.03	0.22
AA PROFILE * MK		0.18	0.57	0.16	0.22

<sup>a,b</sup>Means within main effects with different superscripts differ ( $P < 0.05$ ).

<sup>1</sup>AA Profile; DMEM=Dulbecco Modified Eagle's Medium (Dulbecco and Freeman, 1959), CORN=corn gluten meal (n=9), BM=ring dried blood meal (n=13), CASEIN=milk casein (Galindo et al., 2011), PLASMA=average blood plasma of lactating dairy cows (Swanepoel et al., 2016), NEG=negative control of no amino acids.

<sup>2</sup>MK=balancing for methionine and lysine according to NRC (2001), negative sign indicating unbalanced and positive sign balanced.

<sup>3</sup>rpS6 = ribosomal protein S6, eIF2a = eukaryotic initiation factor 2 a subunit, mTOR = mammalian target of rapamycin, and 4EBP1= eukaryotic translation initiation factor 4E-binding protein

### 3.8. Supplemental Material

**Table 3.S 1.** Intracellular AA concentrations expressed as a percentage of extracellular AA concentrations following 80 min of treatment incubation.

% <sup>3</sup>	AA Profile <sup>1</sup>						MK <sup>2</sup>			Effect ( <i>P</i> -value)		
	BM	CASEIN	CORN	PLASMA	DMEM	NEG	-MK	+MK	SEM	Profile	MK	Profile * MK
Ala	51.4 <sup>b</sup>	70.7 <sup>b</sup>	41.6 <sup>b</sup>	44.1 <sup>b</sup>	302.7 <sup>a</sup>	-	97.7	106.5	14.6	<0.001	0.33	0.49
Arg	7.9 <sup>b</sup>	8.8 <sup>b</sup>	10.8 <sup>a</sup>	8.7 <sup>b</sup>	6.8 <sup>b</sup>	-	8.6	8.5	1.6	0.01	0.87	0.91
Asn	32.3 <sup>a</sup>	23.4 <sup>a</sup>	22.7 <sup>a</sup>	-	-	-	-29.2	-28.7	18.9	<0.001	0.97	1.00
Asp	38.2 <sup>a</sup>	13.9 <sup>b</sup>	44.7 <sup>a</sup>	-	33.3 <sup>a</sup>	-	33.9	31.2	5.8	<0.001	0.38	0.97
Gln	15.8	17.8	16.2	16.2	14.9	-	16.1	16.2	2.3	0.38	0.94	0.98
Glu	1486.1 <sup>b</sup>	325.8 <sup>d</sup>	702.2 <sup>cd</sup>	949.5 <sup>c</sup>	3924.8 <sup>a</sup>	-	1466.6	1488.7	236.8	<0.001	0.87	1.00
Gly	76.5 <sup>d</sup>	160.4 <sup>a</sup>	98.5 <sup>c</sup>	40.5 <sup>e</sup>	133.8 <sup>b</sup>	-	100.5	103.3	15.2	<0.001	0.62	0.99
His	22.6	109.5	63.7	118.8	35.6	-	88.0	52.1	35.9	0.19	0.24	0.29
Ile	5.0 <sup>c</sup>	12.8 <sup>a</sup>	10.7 <sup>b</sup>	12.4 <sup>a</sup>	13.6 <sup>a</sup>	-	10.7	11.1	1.1	<0.001	0.44	0.56
Leu	15.2 <sup>a</sup>	14.3 <sup>ab</sup>	14.1 <sup>b</sup>	13.4 <sup>bc</sup>	12.9 <sup>c</sup>	-	13.7	14.3	1.6	0.001	0.11	0.06
Lys <sup>3</sup>	9.7 <sup>b</sup>	9.4 <sup>b</sup>	10.6 <sup>b</sup>	10.6 <sup>b</sup>	9.0 <sup>b</sup>	21.1 <sup>a</sup>	-	-	1.5	<0.001	-	-
Met <sup>3</sup>	32.9 <sup>b</sup>	28.5 <sup>b</sup>	29.2 <sup>b</sup>	25.4 <sup>b</sup>	33.0 <sup>b</sup>	78.7 <sup>a</sup>	-	-	3.5	<0.001	-	-
Phe	18.7 <sup>a</sup>	18.4 <sup>a</sup>	16.8 <sup>b</sup>	15.3 <sup>c</sup>	17.4 <sup>ab</sup>	-	16.8	17.8	1.3	<0.001	0.03	0.06
Pro	81.4 <sup>b</sup>	74.2 <sup>bc</sup>	63.6 <sup>c</sup>	81.6 <sup>b</sup>	150.8 <sup>a</sup>	-	89.5	91.1	12.2	<0.001	0.77	0.97
Ser	10.7 <sup>a</sup>	11.4 <sup>a</sup>	9.9 <sup>a</sup>	10.1 <sup>a</sup>	6.7 <sup>b</sup>	-	10.8	8.7	1.9	0.01	0.01	0.18
Thr	13.8 <sup>a</sup>	11.3 <sup>b</sup>	4.6 <sup>c</sup>	10.8 <sup>b</sup>	9.9 <sup>b</sup>	-	9.8	10.4	2.5	<0.001	0.48	0.86
Tyr	5.1 <sup>d</sup>	13.6 <sup>a</sup>	10.1 <sup>b</sup>	7.3 <sup>c</sup>	8.9 <sup>bc</sup>	-	8.8	9.2	1.7	<0.001	0.58	0.16
Val	12.2 <sup>a</sup>	11.1 <sup>a</sup>	9.3 <sup>b</sup>	12.3 <sup>a</sup>	11.2 <sup>ab</sup>	-	11.0	11.4	1.3	<0.001	0.27	0.25

<sup>a-f</sup>Means within a row with different superscripts differ ( $P < 0.05$ ).

<sup>1</sup>AA Profile; DMEM=Dulbecco Modified Eagle's Medium (Dulbecco and Freeman, 1959), CORN=corn gluten meal, BM=ring dried blood meal, CASEIN=milk casein (Galindo et al., 2011), PLASMA=average blood plasma of lactating dairy cows (Swanepoel et al., 2016), NEG=negative control of no amino acids.

<sup>2</sup>MK=balancing for methionine and lysine according to NRC (2001), negative sign indicating unbalanced and positive sign balanced.

<sup>3</sup>%: (intracellular AA  $\mu M$  / extracellular AA  $\mu M$ ) \* 100.

<sup>3</sup>No estimates were derived for Met and Lys MK main effects since only the positive MK values were considered for Met and Lys. The NEG treatment has no amino acids extracellular for all treatments except +MK.

**Table 3.S 2.** Multiple linear regression analyses using stepwise backward elimination for parameter selection of intracellular free AA concentrations on phosphorylation ratios of mTOR related signaling proteins following incubation with varying AA profiles and balancing for Lys to Met ratio for 80 min.

Model <sup>1</sup>	Coefficient	<i>P</i> -value	RMSE, % <sup>2</sup>	MB, % <sup>3</sup>	SB, % <sup>4</sup>	CCC <sup>5</sup>
<b>mTOR</b>						
Intercept	1.682	0.02	13.8	0	0.12	0.93
Ile, uM	0.006	0.001				
Leu, uM	0.004	<0.001				
<b>rpS6</b>						
Intercept	3.721	0.01	61.6	0	0.42	0.99
Ile, uM	0.043	0.04				
Leu, uM	0.022	0.005				
Met, uM	0.023	0.07				
Thr, uM	0.057	0.002				
<b>4EBP1</b>						
Intercept	0.759	0.09	11.1	0	0.36	0.96
Ile, uM	0.016	<0.001				
Leu, uM	0.007	<0.001				
Met, uM	0.003	0.08				
Thr, uM	0.012	<0.001				

<sup>1</sup>rpS6 = ribosomal protein S6, mTOR = mammalian target of rapamycin, and 4EBP1= eukaryotic translation initiation factor 4E-binding protein 1.

<sup>2</sup>RMSE, % = root mean square prediction error as a percent of the observed mean.

<sup>3</sup>MB, % = mean bias as a percent of mean square prediction error.

<sup>4</sup>SB, % = slope bias as a percent of mean square prediction error.

<sup>5</sup>CCC = Concordance correlation coefficient.

### 3.8.1. References

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## **CHAPTER 4: An in vitro method for assessment of amino acid bi-directional transport and intracellular metabolic fluxes**

### **4.1. Abstract**

Understanding amino acid (AA) uptake by mammary tissue as AA supply varies is critical for predicting milk component production and understanding tissue function. Our objective was to develop an in vitro method to quantify cellular uptake, efflux, and intracellular metabolism of individual AA that could be implemented for evaluating these factors when AA supply and profile are varied. Bovine primary mammary epithelial cells were grown to confluency and exposed to media with an AA profile and concentration similar to lactating dairy cow plasma for 24 h. Cells were then preloaded in media enriched with  $^{15}\text{N}$  labelled AA for 24 h followed by removal of the  $^{15}\text{N}$  labelled media and incubation with media enriched with  $^{13}\text{C}$  labelled AA for 0, 15, 60, 300, 900, 1800, and 3600 seconds. Extracellular free AA and intracellular free and protein-bound AA were analyzed for concentrations and isotopic enrichment by gas chromatography mass spectrometry. A dynamic, 12-pool model was constructed representing total extracellular and intracellular free, and protein bound pools of an AA and their respective  $^{15}\text{N}$  and  $^{13}\text{C}$  isotopes. The model was fitted to the Ala and Leu data collected in the experiment using maximum likelihood-based methods. Markov chain Monte Carlo simulation ( $n=5000$ ) was conducted to evaluate prediction errors by deriving standard errors and posterior distributions for rate constants, fluxes, and pools. Alanine fluxes ( $\mu\text{mol}/\text{min}$ ) were: influx 51.2 (+/-2.1), efflux 44.7 (+/-2.1), turnover 0.6 (+/-0.3), transamination 37.2 (+/-1.9), synthesis 17.4 (+/-1.1) and oxidation 24.5 (+/-3.1). Leu fluxes ( $\mu\text{mol}/\text{min}$ ) were: influx 18.8 (+/-1.2), efflux 13.6 (+/-1.1), turnover 3.0 (+/-0.3), transamination 1.3 (+/-0.1), synthesis 0.0, and oxidation 5.1 (+/-0.2). The Ala turnover rates were 181, 580, and 74 percent

per hour for extracellular, intracellular, and fast protein-bound pools, respectively. Leu turnover rates were 95, 857, and 157 percent per hour for extracellular, intracellular, and fast protein-bound pools, respectively. The average root mean squared prediction error across the isotope enrichments, pools, and concentrations was 9.7 and 14.1% for Ala and Leu, respectively, and collinearity among parameters was low, indicating adequate fit and identifiability. The described model provides insight on individual AA transport kinetics and a method for future evaluation of AA transport and intracellular metabolism when subjected to varying AA supplies.

**Key words:** amino acids, transport, isotope, Ala, Leu

#### **4.2. Introduction**

Udder AA uptake from arterial supplies and conversion into milk protein is relatively low. Only 22% of arterial AA daily influx although it varies considerably across AA, is transformed into milk protein (Arriola Apelo et al., 2014b). If more AA could be taken up by the udder, then venous blood exiting would have lower AA concentrations. Lower AA venous concentrations will reduce splanchnic catabolism since these tissue-beds extract AA by mass-action (Hanigan et al., 2004). This splanchnic catabolism of AA is the primary culprit of the low 25% N efficiency in dairy cows (Hristov et al., 2004, Arriola Apelo et al., 2014b).

Mammary amino acid transport in dairy cows is largely influenced by milk protein synthesis rate (Cant et al., 2018). Increased milk protein translational rate can be achieved by increasing intracellular AA concentrations, which activates the mammalian target of rapamycin pathway (Wolfson and Sabatini, 2017). Activation of this pathway has corresponded to increased milk protein synthesis (Arriola Apelo et al., 2014d) which would increase cellular demand for AA.

Increased AA demand by the udder without changing post-absorptive supplies might lower venous concentrations and increase the proportion of absorbed AA converted into milk protein. However, these changes, i.e. decreased AA venous concentrations, require increased uptake to achieve greater intracellular concentrations of certain EAA. If linear (mass action) net AA uptake is assumed by the udder, decreasing or increasing dietary metabolizable protein (MP) would change intracellular AA concentrations and thus milk protein synthesis rate. To make a sizeable change in N efficiency (increase to 30%), maintaining or increasing udder intracellular AA concentrations via better udder AA transport when MP supplies are reduced via better udder AA transport is necessary. Elucidating how AA transport responds to changing supplies and profile is needed to identify if manipulation of AA transport is possible. Additionally, production responses to what are considered limiting AA are not predicted with good accuracy (Hanigan et al., 2000, Sinclair et al., 2014). Amino acid transport may play an important role in maintaining intracellular AA homeostasis in the face of daily AA supply changes (Broer and Broer, 2017), thereby mitigating model perceived AA shortages.

Amino acids can enter mammary epithelial cells via three mechanisms, passive diffusion, sodium-driven gradient, or amino acid exchange (Taylor, 2014). Active transporters lack high specificity in most scenarios. Some transporters work with up to a dozen AA and AA can use multiple transporter systems (Christensen, 1990). The active transporters can build up steep intracellular concentration gradients relative to extracellular concentrations (“loaders”) while others act as exchangers (“harmonizer”) to catalyze transport of other AA (Broer and Broer, 2017). In the mammary glands of dairy cows, these “loader” transporters consist of system A, system Gly, system N, system X<sub>ag</sub>, and system y<sup>+</sup> (Shennan and Boyd, 2014). The “harmonizer” transporters consist of system ASC, system L, and system y<sup>+L</sup>. Another class of

transporters react to short-term AA deprivation by increasing expression and activity. These are referred to as “rescue” transporters, i.e. SNAT2 within system A and CAT-1 within system y+ (Broer and Broer, 2017). Amino acid transporters likely have evolved to manage the variation in daily influx of AA so that intracellular concentrations were matched to protein synthesis needs (Christensen, 1990). Net flux of an AA often can be quite different than influx and efflux, and the bi-directional flows confer more flexibility than could be achieved with a single, unidirectional flux. Thus changes in blood concentrations can be buffered to provide a more stable intracellular environment (Christensen, 1982).

Previously, net AA fluxes were observed to exhibit mass action responses to arterial AA supplies in lactating dairy cows and pig mammary tissue explants (Hanigan et al., 1992, Hurley et al., 2000, Jackson et al., 2000). Many experimental protocols have evaluated a single AA with various competitor AA or inhibiting-molecules on kinetics and transporter activity (Tovar et al., 2000). However, the AA concentrations evaluated might be quite different from in-vivo, and the relationships with the transport of the other 19 AA has not been assessed. Studies of AA transport should consider that individual AA uptake is not univariate, but likely a twenty dimension problem (Christensen, 1990). Additionally, measuring the enrichment of an isotope intracellularly will provide some information on turnover and net uptake, but it does not allow derivation of unidirectional uptake or intracellular metabolism.

The objectives of this study were to develop an in-vitro method for simultaneous measurements of bidirectional transport and intracellular metabolism of multiple AA. We hypothesized that use of three unique isotopic labels per AA and the construction and fitting of a dynamic 12-pool model to the data will address our objectives.

### **4.3. Methods**

### 4.3.1. Experimental Design

Bovine primary mammary epithelial cells (BPME) were obtained from the State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, China. These cells were harvested using a method previously described (Hu et al., 2009). Cells were verified to be mammary epithelial cells of bovine origin by proteomic, PCR, and western immunoblotting analyses. Proteins associated with epithelial cells, such as KRT18 and KRT8, were identified, and casein synthesis was confirmed using western immunoblotting.

The BPME cells were thawed and seeded into 2 89.4-mm by 19.2-mm TC-treated polystyrene cell culture dishes (Corning, Product #353003) at  $1.9 \times 10^6$  cells per dishes. Cells were cultured in growth media containing 10% fetal bovine serum, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (Gibco, #12400-024), sodium bicarbonate, antibiotic-antimycotic (Gibco, #15240062), 5 mg/L of bovine insulin (Gemini Bio-Products, #700-112P), 5 mg/L Helo transferrin (Sigma-Aldrich, #T8158), 5 mg/L prolactin (Sigma-Aldrich, #L6520), 5 mg/L progesterone (Sigma-Aldrich, #P7556), 1 mg/L hydrocortisone (Sigma-Aldrich, #H0888), and 0.01 mg/L epidermal growth factor (Sigma-Aldrich, #E4127) at 37°C in 5% CO<sub>2</sub> for 5 days (Nan et al., 2014). Cells were subsequently transferred to 27 culture dishes (89.4 mm by 19.2 mm, (Corning, Product #353003)) and incubated in growth media that was changed daily. Culture dishes were weighed prior to addition of cells and media. Upon reaching >90% confluency 3 days post-seeding, the growth media was replaced with a steady-state media that consisted of 10% Earle's balanced salts solution (Sigma-Aldrich, #E7510) containing sodium bicarbonate, D-Glucose (2.151 g/L), minimal essential media vitamin solution 100X (Gibco, #11120052), antibiotic-antimycotic 100X, 5 mg/L of bovine insulin, 5 mg/L bovine Helo

transferrin, 5 mg/L prolactin, 5 mg/L progesterone, 1 mg/L hydrocortisone, and 0.01 mg/L bovine epithelial growth factor. Individual AA were added to the steady-state media at concentrations to mimic the profile and concentration of the blood plasma of lactating cows producing on average 45.1 kg/d of milk (Swanepoel et al., 2016) (Table 1). Cells remained in this steady-state media for 24 h (media changed every 12 h) prior to the experiment.

Universally  $^{13}\text{C}$  labeled AA derived from algae (U- $^{13}\text{C}$ ], 97-99% enriched, Lot #30287, Product #CLM-1548), universally  $^{15}\text{N}$  labeled AA derived from algae (U- $^{15}\text{N}$ ], 98% enriched, Lot #24163, Product #NLM-2161), and  $^{13}\text{C}^{15}\text{N}$  universally labeled AA derived from algae (U- $^{13}\text{C}^{15}\text{N}$ ], 98% enriched, Lot #16824, Product #CNLM-452) were purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA). Amino acid concentrations and profiles of the three labeled algae were determined (Supplemental Material, Table 1) by measurement of the isotope dilution associated with the addition of a known amount of a mix of 18 unlabeled amino acids (AAS18; Sigma Aldrich, St. Louis, MO) using gas chromatography-mass spectrometry. The enriched algae underwent acid hydrolysis, hence, Gln, Asn, Cys, and Trp were not present in the algae. The  $^{13}\text{C}^{15}\text{N}$  enriched algae also was analyzed by HPLC at the University of Missouri for comparison purposes (Supplemental Material, Table 1).

Media mimicking the steady-state media components and concentrations, except that the AA were enriched with  $^{13}\text{C}$  labeled AA or  $^{15}\text{N}$  labeled AA, were formulated resulting in two media. These media will be referred to as  $^{13}\text{C}$  enriched medium or  $^{15}\text{N}$  enriched medium (Table 1).

Upon removal of the steady-state media, cells were washed once with warm PBS and 10 mL of the  $^{15}\text{N}$  enriched media were added per plate. Cells were incubated 24 h in  $^{15}\text{N}$  enriched media that was changed every 8 hours to minimize the effects of depletion of AA concentrations.

During the  $^{15}\text{N}$  incubation, 0.4 mL of media was sampled at 8, 16, 22, and 23 from 3 plates and stored at  $-20^{\circ}\text{C}$  in order to assess extracellular AA concentrations and enrichment. Following 24 h of  $^{15}\text{N}$  incubation, cells were washed twice with warm PBS ( $37^{\circ}\text{C}$ ), and 6 mL of warm  $^{13}\text{C}$  enriched media ( $37^{\circ}\text{C}$ ) was added to the cells. Cells were incubated with the  $^{13}\text{C}$  enriched media for 0 s, 15 s, 1 min, 5 min, 15 min, 30 min, and 60 min. The zero time point was utilized to correct for extracellular media contamination of intracellular AA during cell lysis (Darmaun et al., 1988). Other selected time points were based on preliminary experiments (data not shown). At each of the time points, 1 mL of media was removed from the plate, placed into ice-cold 1.5-mL tubes, and stored at  $-20^{\circ}\text{C}$ . Cells from each of those plates were rinsed 3 times with ice cold PBS and the culture dish was weighed. Pre-weighed ice-cold 50% sulfosalicylic acid was then added and the plate was vigorously agitated to lyse the cells. Cell debris were mechanically detached using a cell scraper, and the homogenate was pipetted into an ice-cold 2-mL tube and stored at  $-20^{\circ}\text{C}$ . Upon removal of cells, the culture dish was weighed again to assess cell mass.

Viable live cells from 3 culture dishes were counted following 60 min of incubation with the  $^{13}\text{C}$  enriched media. Culture dishes were washed twice with warm PBS ( $37^{\circ}\text{C}$ ) and each plate was weighed. Next, 1 mL of 0.05% Trypsin EDTA was added to the plate and incubated at  $37^{\circ}\text{C}$  for 5 min. Upon visible detachment of cells, cells were removed in warm PBS and centrifuged for 5 minutes at 500 rcf. The supernatant was decanted, and the pellet resuspended in PBS and centrifuged for another 5 min at 500 rcf. The supernatant was then discarded. The pellet was resuspended in PBS, the weight was recorded, and 100  $\mu\text{L}$  of the suspensions was removed for cell counting using a hemacytometer and Trypan Blue solution, 0.4% (Amresco, #K940). The remaining sample was centrifuged at 2,500 rcf for 5 min, the supernatant decanted, and the cells were lysed by addition of 1 mL of 2% SDS lysis buffer containing phosphatase

(ThermoFisher, #78426) and protease (Promega, #G6521) inhibitors. The samples were constantly agitated for 30 min at 4°C and centrifuged for 1 h at 21,130 rcf at 4°C to remove insoluble material. The supernatant was stored at -80°C. Total protein in the lysate was determined using a bicinchoninic acid assay (BCA) (Smith et al., 1985).

#### **4.3.2. Amino Acid Analysis**

The extracellular media contents were gravimetrically weighed, deproteinized using 7% sulfosalicylic acid and centrifuged at 16,000 rcf for 15 min at 4°C. The intracellular lysates plus sulfosalicylic acid were gravimetrically weighed and centrifuged at 16,000 rcf for 15 min at 4°C. Supernatants of the extracellular media and intracellular contents were gravimetrically weighed and combined with  $^{13}\text{C}^{15}\text{N}$  universally labeled AA algae (97-99% enriched, Cambridge Isotope Laboratories Inc., Tewksbury, MA). The intracellular contents pellet were gravimetrically weighed and combined with 250  $\mu\text{l}$  of a mixture containing  $^{13}\text{C}^{15}\text{N}$  universally labeled AA algae (97-99% enriched, Cambridge Isotope Laboratories Inc., Tewksbury, MA) and 33.3  $\mu\text{l}$  per protein mg of 6 M HCl containing 0.1% phenol and hydrolyzed for 20 h at 97.5°C. The acid hydrolyzates were desalted by ion chromatography (AG 50W-X8 resin; Bio-Rad Life Science), eluted with 2 N ammonia hydroxide into silanized glassware, and freeze-dried. Dried samples were resolubilized in 0.1 N HCl and derivatized in acetonitrile (J. T. Baker Inc., Phillipsburg, NJ) and to form a *N*-(*tert*-butyldimethyl) AA derivatives by incubation for 1 h at 70°C in *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (SELECTRA-SIL; UCT Inc., Bristol, PA). Amino acid derivatives were separated by gas chromatography (Trace GC Ultra; Thermo Scientific) and quantified by mass spectrometry (DSQII; Thermo Scientific) for  $^{15}\text{N}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}^{13}\text{C}$  enrichments of ions derived from each AA as previously described (Calder et al., 1999). Calibration curves for derivation of AA concentrations from the isotopic ratios were developed

using the same labeled-AA mixture spiked into the samples gravimetrically combined in varying ratios with an AA standard mix (Sigma-Aldrich, #AAS18) plus L-Asn, L-Gln, L-Trp, and L-Cys (Sigma-Aldrich, St. Louis, MO).

### 4.3.3. Model Development

A dynamic system of differential equations and state variables was constructed to represent the transport and metabolism of a single AA within mammary epithelial cells. The described model was coded in R (version 3.3.2 and RStudio version 1.0.13; Team, 2016). Primary-state variables were extracellular free AA ( $Q_{xAA(i)}$ ), intracellular free AA ( $Q_{nAA(i)}$ ), intracellular fast turnover protein-bound AA ( $Q_{tfAA(i)}$ ), and intracellular slow turnover protein-bound AA ( $Q_{tsAA(i)}$ ) with replication 3 times to represent the mass of  $^{12}\text{C}$  amino acids,  $^{13}\text{C}$  amino acids, and  $^{15}\text{N}$  amino acids resulting in 12 pools (Table 2). Rate constants and fluxes are described in Table 3. Mass ( $Q$ ), flux ( $F$ ), concentration ( $C$ ), and volume ( $V$ ) had units of  $\mu\text{mol}$ ,  $\mu\text{mol}/\text{min}$ ,  $\mu\text{M}$ , and  $\text{mL}$ , respectively. Construction of the model and fluxes were governed by known physiological mechanisms (Waterlow, 2006). Additionally, fast and slow turnover protein pools were used to represent the variable return of protein-bound label to the intracellular free pool with respect to time. Such a representation is a simplification, as each protein has its own respective protein turnover pool, and likely ranges from nanoseconds to years in time. However, it has previously been demonstrated that, to adequately represent isotopic movement within the pool over 30-h time periods, specification of fast and slow protein turnover pools is needed (Hanigan et al., 2009, Estes et al., 2018b). The inclusion of the slow turnover pool was not required for the short experimental times represented in the current work, and thus it could be excluded if isotope exposure times are less than 60 minutes.

The volume of the extracellular free AA pool ( $V_x$ ) was assumed to be 6 mL of media whereas the intracellular free AA pool ( $V_n$ ) and the intracellular protein-bound pool ( $V_t$ ) were calculated from measurements of their respective weights. As mentioned previously, 3 measurements of intracellular volume were conducted: volume observed prior to removal, volume of cells removed, and volume of cells mechanically harvested. For our purposes, we designated the latter as the volume of the intracellular free AA pool because of the high variation in the other 2 measurements.

Following removal of the free AA during deproteinization, the pellet was weighed, and it was weighed again prior to acid hydrolysis. The weight of the pellet was assumed to be the average of these 2 measurements and designated as the intracellular protein-bound pool. The mass of the extracellular free AA, intracellular free AA, and total-bound protein pools for the  $i^{\text{th}}$  AA ( $i = \text{Ala and Leu}$ ) were determined as:

$$Q_{xAA(i)} = V_{x(i)} * C_{xAA(i)} \quad (1)$$

$$Q_{nAA(i)} = V_{n(i)} * C_{nAA(i)} \quad (2)$$

$$Q_{tAA(i)} = V_{t(i)} * C_{tAA(i)} \quad (3)$$

where  $Q_{xAA(i)}$ ,  $Q_{nAA(i)}$ , and  $Q_{tAA(i)}$  represents the mass of extracellular AA, intracellular AA, and protein bound AA of only  $^{12}\text{C}$  amino acids. The volume and concentration of the intracellular and protein-bound pools were measured at each time point ( $n=24$ ). The mass of each isotope was calculated from the pool size and isotopic enrichment:

$$Q_{xAA(i)}^{13\text{C}} = Q_{xAA(i)} * \left( \frac{C^{13}_{xAA(i)}}{C^{12}_{xAA(i)}} \right) \quad (4)$$

$$Q_{xAA(i)}^{15\text{N}} = Q_{xAA(i)} * \left( \frac{N^{15}_{xAA(i)}}{C^{12}_{xAA(i)}} \right) \quad (5)$$

$$Q_{nAA(i)}^{13\text{C}} = Q_{nAA(i)} * \left( \frac{C^{13}_{nAA(i)}}{C^{12}_{nAA(i)}} \right) \quad (6)$$

$$Q_{nAA(i)}^{15N} = Q_{nAA(i)} * \left( \frac{N^{15}_{nAA(i)}}{C^{12}_{nAA(i)}} \right) \quad (7)$$

$$Q_{tAA(i)}^{13C} = Q_{tAA(i)} * \left( \frac{C^{13}_{tAA(i)}}{C^{12}_{tAA(i)}} \right) \quad (8)$$

$$Q_{tAA(i)}^{15N} = Q_{tAA(i)} * \left( \frac{N^{15}_{tAA(i)}}{C^{12}_{tAA(i)}} \right) \quad (9)$$

The initial proportion of the cell-bound pool resident in the fast and slow protein pools for each AA was estimated as that yielding the best model fit when varying the fast protein pool size by a fraction of 0.5 to 10%. The range was arbitrarily selected by observing how the model fit was changing. The fast protein pool fraction was denoted  $f_{tf}$ :

$$Q_{tfAA(i)} = Q_{tAA(i)} * f_{tf(i)} \quad (10)$$

$$Q_{tsAA(i)} = Q_{tAA(i)} - Q_{tfAA(i)} \quad (11)$$

where  $Q_{tAA(i)}$ ,  $Q_{tfAA(i)}$ , and  $Q_{tsAA(i)}$  represent the total protein turnover pool, fast protein turnover pool, and slow protein turnover pool respectively.

$$Q_{tfAA(i)}^{13C} = Q_{tfAA(i)} * \left( \frac{C^{13}_{tfAA(i)}}{C^{12}_{tfAA(i)}} \right) \quad (12)$$

$$Q_{tsAA(i)}^{13C} = Q_{tAA(i)}^{13C} - Q_{tfAA(i)}^{13C} \quad (13)$$

$$Q_{tfAA(i)}^{15N} = Q_{tfAA(i)} * \left( \frac{N^{15}_{tfAA(i)}}{C^{12}_{tfAA(i)}} \right) \quad (14)$$

$$Q_{tsAA(i)}^{15N} = Q_{tAA(i)}^{15N} - Q_{tfAA(i)}^{15N} \quad (15)$$

Mass action kinetics were assumed for fluxes among pools that included AA influx and efflux between the extracellular media and intracellular space, transamination, catabolism or use for intracellular protein synthesis, and influx and efflux from fast and slow protein pools.

Additionally, for Ala, intracellular synthesis of the respective was considered. Descriptions are provided in Table 2 and 3. The differential equation describing the extracellular media pool for the  $i^{\text{th}}$  AA ( $i=$  Ala and Leu) was:

$$\frac{dQ_{xAA(i)}}{dt} = F_{nAAxAA(i)} - F_{xAA nAA(i)} \quad (16)$$

where  $F_{nAAxAA(i)}$  represents efflux of AA from intracellular free pool to extracellular pool and  $F_{xAA nAA(i)}$  the uptake of AA by the cell. The isotopic fluxes were calculated using the identical form as described above and for the remaining fluxes as follows:

$$E_a = \frac{Q_{a^*}}{Q_a} \quad (17)$$

$$F^*_{a,b} = F_{a,b} \times E_a \quad (18)$$

where  $E_a$ ,  $Q_{a^*}$ , and  $Q_a$  represents the isotope ratio, mass of the isotope, and mass of  $^{12}\text{C}$  respective of a particular amino acid pool. A particular flux of a  $^{12}\text{C}$  amino acid from pool A to B is represented as  $F_{a,b}$  and the flux of the isotope amino acid from pool A to B is represented as  $F^*_{a,b}$ . The AA influx was represented by the following for amino acids and the respective isotopes:

$$F_{xAA nAA(i)} = k_{xAA nAA(i)} \times Q_{xAA(i)} \quad (19)$$

where  $k_{xAA nAA(i)}$  represents the mass action rate constant for uptake of amino acids by the cells.

The AA efflux was:

$$F_{nAAxAA(i)} = k_{nAAxAA(i)} \times Q_{nAA(i)} \quad (20)$$

where  $k_{nAAxAA(i)}$  represents that mass action rate constant for AA efflux by the cells.

The differential for the intracellular free AA was also considered for the  $i^{\text{th}}$  AA and the respective isotopes:

$$\begin{aligned} \frac{dQ_{nAA(i)}}{dt} = & F_{xAAnAA(i)} + F_{tfAAnAA(i)} + F_{tsAAnAA(i)} + F_{nKAnAA(i)} + F_{nSynAA(i)} + F_{nKAnAAu(i)} - \\ & F_{nAAxAA(i)} - F_{nAAOx(i)} - F_{nAAtfAA(i)} - F_{nAAtsAA(i)} - F_{nAAnKA(i)} \end{aligned} \quad (21)$$

where  $F_{tfAAnAA(i)}$  represents the flux from degradation of the fast protein pool,  $F_{tsAAnAA(i)}$  from the degradation of the slow protein pool,  $F_{nKAnAA(i)}$  reamination of keto-acids to form AA in the intracellular free AA pool,  $F_{nSynAA(i)}$  synthesis of AA,  $F_{nAAOx(i)}$  oxidative loss,  $F_{nAAtfAA(i)}$  AA incorporation into the fast turnover protein pool synthesis,  $F_{nAAtsAA(i)}$  AA incorporation into the slow turnover protein pool,  $F_{nAAnKA(i)}$  deamination of loss, and  $F_{nAAnKAu(i)}$  represents deamination flux from the pool of carbon that was previously enriched with  $^{13}\text{C}$  but underwent decarboxylation. Synthesis of fast and slow protein is described as:

$$F_{nAAtfAA(i)} = k_{nAAtfAA(i)} \times Q_{nAA(i)} \quad (22)$$

$$F_{tfAAnAA(i)} = F_{nAAtfAA(i)} \quad (23)$$

$$F_{nAAtsAA(i)} = k_{nAAtsAA(i)} \times Q_{nAA(i)} \quad (24)$$

$$F_{tsAAnAA(i)} = F_{nAAtsAA(i)} \quad (25)$$

$$F_{nAAnKA(i)} = k_{nAAnKA(i)} \times Q_{nAA(i)} \quad (26)$$

$$F_{nKAnAA(i)} = F_{nAAnKA(i)} \quad (27)$$

where  $k_{nAA_{tfAA}(i)}$  and  $k_{nAA_{tsAA}(i)}$  represent the mass action rate constants for protein synthesis. The mass of both protein pools was considered fixed, therefore, entry into each pool was set equal to the exit from each. The differential for the total protein-bound AA pool was the sum of the fast and slow pools:

$$\frac{dQ_{tAA(i)}}{dt} = Q_{tfAA(i)} + Q_{tsAA(i)} \quad (28)$$

The differentials for the fast and slow protein-bound pools were calculated and the pool size was assumed to be fixed.

$$\frac{dQ_{tfAA(i)}}{dt} = F_{nAA_{tfAA}(i)} - F_{tfAA_{nAA}(i)} \quad (29)$$

$$\frac{dQ_{tsAA(i)}}{dt} = F_{nAA_{tsAA}(i)} - F_{tsAA_{nAA}(i)} \quad (30)$$

#### 4.3.4. Parameter Estimation

The FME package of R (Soetaert and Petzoldt, 2010) was utilized to conduct local and global sensitivity, parameter identifiability, Markov chain Monte Carlo analysis, and model fitting as previously described (Wu et al., 2014). The time step was 15 seconds and the Runge-Kutta 4<sup>th</sup> order was utilized for numerical integration. Initial intracellular pool sizes were set to the mean observed pool mass (n=6) from time 0 and time 15 seconds (Table 2). Both time points were used to minimize variance associated with this setting. The respective enrichments and calculated isotope pool masses were calculated from the mean enrichment at time 0 for extracellular and intracellular free AA pools (n=3). The observed enrichment at time 0 for the intracellular free AA pool represented natural background abundance of <sup>13</sup>C AA and any <sup>13</sup>C AA media contamination of the out-facing cellular membrane surface (Darmaun et al., 1988). The  $Q_{xAA(i)}$ ,  $Q_{nAA(i)}$ , and  $Q_{tAA(i)}$ , were fit to the measured mass of the respective <sup>12</sup>C AA which represented 24, 21, and 21 observations, respectively. Additionally, these pools  $Q_{xAA(i)}$ ,  $Q_{nAA(i)}$ ,

and  $Q_{tAA(i)}$  were fit to the measured mass of the respective  $^{13}\text{C}$  and  $^{15}\text{N}$  AA, which represented an additionally 24, 21, and 21 observations each, respectively.

The initial total protein pool mass was set equal to the mean across all samples (n=21) whereas the isotope enrichment was calculated from the observed isotope ratio at time 0 and time 15 seconds (n=6). The fast protein turnover pool was originally set to 5% of the total protein pool, however, this was varied between 0.5 and 10% and set to maximize the best log likelihood function. The initial enrichment of the fast protein turnover pool was set to equal the intracellular free AA pool assuming the pool would have achieved isotopic equilibrium with the free pool over a 24-h period. The enrichment of the slow protein turnover pool was calculated by difference from the mass of isotopes in the total protein and fast turnover protein pools.

Parameter bounds were set to a minimum of zero and maximum of 1 for all parameters except AA synthesis, which was set to 100. Prior to parameter estimation, the linear dependence of the parameters was evaluated to determine collinearity using the *collin* function within the FME R package. A score of one indicates orthogonality between parameters (Brun et al., 2001). The maximum allowable score for a set of parameters was set to fifteen for collinearity as higher scores indicate high collinearity and poor identifiability (Brun et al., 2001).

Model parameters were derived using the entire time series of measurements for pool size, volume, and enrichment using *modCost* and *modFit* functions within the FME package (Soetaert and Petzoldt, 2010). The model residuals were standardized based on the overall standard deviation of each respective variable when calculating residuals for model minimization:

$$Residual_a = \frac{Predicted_a - Observed_a}{SD_a} \quad (31)$$

where  $Predicted_a$ ,  $Observed_a$ , and  $SD_a$  are the modeled, observed, and standard deviation of the observed data, respectively, of an individual variable within the model. Weighting of residuals is important given some variables (isotope ratio) were measured with good precision versus others (AA mass) were measured with lesser precision (Soetaert and Petzoldt, 2010). Initial parameter estimates were derived using the Nelder-Mead algorithm, and final estimates using the initial estimates as starting values and the Marq algorithm, which in nearly all cases, resulted in identical parameter values. Parameters retention in the model was based on  $P$ -values ( $P < 0.10$ ), collinearity, and biological relevance.

Standard errors for fluxes and rate constants were derived by simulation to provide a more robust assessment of uncertainty versus standard errors derived during model parameter estimation (Efron and Tibshirani, 1986). The hierarchical structure of the data, i.e. time-series, small sample size, high dimensionality, and desire for assessment of predictive power of more than parameter sampling distribution necessitated the Bayesian approach over traditional bootstrapping from residuals for derivation of standard errors (Alfaro et al., 2003, Cronin et al., 2010, Efron, 2011). Uncertainty estimates were derived using Markov chain Monte Carlo (MCMC) with the delayed rejection and adaptive Metropolis algorithm (DRAM) (Haario et al., 2006, Laine, 2008). For prior specification, the distribution of parameters  $\theta$  were assumed to be Gaussian, parameters must be between 0 and 1 except for synthesis, which was 100, and within this range, a uniform noninformative probability was specified. A prior error variance must be specified for each parameter (Soetaert and Petzoldt, 2010). This prior variance was derived from the mean of the unweighted squared residuals from the model previously solved by maximum likelihood and the distribution was assumed to be normal. Additionally, the prior error variance is weighted based on the number of observations and a user specified value, which we set to 0.1,

which is quite low. For efficient burn-in, the initial parameter values were set to the maximum likelihood solution. The initial random walk-search-jumping distribution was specified using the variance matrix of the parameters when solved by maxima likelihood and scaled according to (Gelman et al., 2014) as follows;

$$J(\theta^*|\theta^{t-1}) = N(\theta^*|\theta^{t-1}, c^2\Sigma) \quad (32)$$

$$c = \frac{2.4}{\sqrt{d}} \times v \quad (33)$$

where  $J$ ,  $c$ ,  $d$ , and  $v$  represent the target distribution kernel for the Metropolis algorithm, the scaled covariance matrix, the number of parameters, and the unscaled covariance matrix. The proposal jumping distribution was set to update every 100 iterations. The burn-in length was set to one-half of the iterations (Gelman et al., 2014) and the total number of iterations for Markov chain sampling of the probability distribution was 10,000. The Delayed Rejection and Adaptive Metropolis procedure (DRAM) (Haario et al., 2006) was implemented if efficiency was low for the MCMC algorithm search and rejection process (Malve et al., 2007). The targeted acceptance rate by the Metropolis algorithm was 23% given six parameters that were being sampled at once (Gelman et al., 2014). Fine-tuning to achieve this acceptance rate was conducted by varying the jumping rule scale (i.e. number of iterations per jump) and implementation of DRAM. Upon completion of the MCMC analysis, the observed standard deviation of the parameter posterior was considered the standard error of the estimates (Erixon et al., 2003) with the assumption that the posterior distribution was normal and asymptotic. The parameter posterior from the MCMC analyses (n=5000 rows) were then collected into a matrix and randomly sampled 1000 times (R seed = 123). Using the randomly drawn parameters, the model was executed for each sample draw, and the distribution of the model predictions evaluated for the mean, standard deviation, and 95% confidence intervals, thereby, providing estimates of prediction power that accounts for

parameter uncertainty, random error, and measurement error. Root mean squared errors as a percentage of the mean (RMSE) and mean squared errors partitioned into mean bias, slope bias, and dispersion were calculated using the residuals for the maximum likelihood and MCMC solutions (Bibby and Toutenberg, 1977). The Concordance correlation coefficient (CCC) was also calculated to provide a dimensionless evaluation of precision and accuracy (Lin, 1989).

#### **4.4. Results and Discussion**

The objective of this experiment was to develop a method to evaluate AA transport by assessment of AA influx, efflux, and intracellular metabolism. For purposes of demonstration of this technique, one NEAA (Ala) and one EAA (Leu) were arbitrarily selected. This method is applicable to all AA that are enriched separately with  $^{13}\text{C}$  and  $^{15}\text{N}$ . The solved Ala and Leu models with fluxes, mass, and concentrations are provided in Figure 1.

##### **4.4.1. Model Development**

Development of a dynamic model to represent isotopic movement was necessary as isotopic equilibrium and steady-state across pools could not be assumed as has been done in previous transport kinetic experiments (Biolo et al., 1995, Bequette et al., 2000, Miller et al., 2004). An advantage to our approach is the ability to delineate fluxes such as oxidation, transamination, synthesis, and degradation, all of which are difficult to define using steady-state modeling approaches (Biolo et al., 1995, Bequette et al., 2000). Fluxes mimicked biological expectations (Waterlow, 2006) and a previous described AA transport and metabolism model (Hanigan et al., 2009).

Model parameters for uptake and efflux were well defined with SE that were less than 8% of the estimated values, whereas transamination, oxidation/protein export, turnover, and synthesis were derived with less confidence, SE were up to 32% of estimated values (Table 4). While the correlations between uptake and efflux were high, 0.79 and 0.84 for Ala and Leu

respectively, the collinearity score indicated parameters were identifiable (Brun et al., 2001) (Figure 2 and 3). All other parameter correlations were less than 0.63 for Leu, indicating strong ability to uniquely derive the parameters (Figure 3). More problematic, rate constants for oxidation or protein export, incorporation into the fast turnover protein pool, and de-novo synthesis for Ala were highly correlated, ranging from 0.88 to 0.90 (Figure 2). However, the collinearity score was 10, lower than the maximum of 15 recommended by Soetaert and Petzoldt, (2010).

The maximum likelihood solution derived by FME reflected a deterministic model solution, whereas MCMC addresses the probability distribution of parameters (Wu et al., 2014). The uncertainty of predictions which is derived from posterior distributions is considered by some to be more important than derivation of a single set of parameters, as predictive power should account for parameter error, prediction random error, and non-random system error (Laine, 2008). Simulating the prior parameter values using MCMC resulted in different rate constant estimates in most cases (Table 4). The Bayesian fitting process searches through the posterior space, which we specified as uniform within specified lower and upper bounds and seeks solutions that represent improved fit values compared to the prior by the Metropolis algorithm. Given the high dimensionality of the model, high probability fit solutions usually represent a low proportion of specified prior search space. The observed mean of the posterior might reflect the high dimensionality of this problem, poor starting prior specification, and highly contoured nature of the solution space. Parameters with low correlations tended to have lower SE compared to maximum likelihood whereas, highly correlated parameters had higher SE with MCMC, which represents the uncertainty around these parameters.

The posterior distribution and correlation parameter matrices are provided in Figures 2 and 3. The Ala rate constants for synthesis, fast protein turnover, and oxidation are highly correlated (Figure 2) which was reflected in high uncertainty in these rate constants and respective fluxes.

The oxidation rate constant was derived considering net loss of the AA as well as the labeled AA, i.e.  $^{13}\text{C}$  and  $^{15}\text{N}$ . In contrast, transamination rate constant considers no loss of the AA but only loss of  $^{13}\text{C}$  and/or  $^{15}\text{N}$ . For specific AA, return of labeled C or N can occur and was specified using biological inference. The enrichment of these carbon and nitrogen precursor pools for reamination (return) was set to the fast turnover pool enrichment as it improved model fit, reflecting a slight lag in equilibration with the intracellular free AA enrichment, and this lag was previously observed with casein enrichment (Hanigan et al., 2009)

The fraction of total protein that is fast protein constant ( $f_{tf}$ ) was iteratively varied between 0.5 and 10% and selection was based on best model fit. The greatest likelihood ratio was observed at 2.5 and 1% for Ala and Leu respectively. At that setting, the fast protein pools for Ala and Leu reflected proteins that turned over within 128 and 34 minutes, respectively. Hanigan et al. (2009) observed the fast protein proportion to be 6% for Leu when fitting to in vivo data from a 30-h infusion, which is slightly higher than our derived estimates.

Protein turnover has high heterogeneity across the several thousand proteins present in a cell; hence simplification to a 2-pool model is a major assumption. A minimum of 2 protein pools, i.e. fast and slow, are required to account for the changes in isotopic enrichment over time during the experiment. Intracellular free  $^{13}\text{C}$ -Ala enrichment started decreasing after 15 minutes despite extracellular enrichment still being much higher, hence significant Ala entry to the intracellular pool other than from extracellular pool is occurring (Figure 4). In contrast,

intracellular  $^{15}\text{N}$ - Ala enrichment appears to reach equilibrium with extracellular  $^{15}\text{N}$  enrichment within 30 min (Figure 5). This also implies that  $^{15}\text{N}$  enrichment of the fast turnover protein pool and the ammonia pool have apparently reached enrichment equilibrium. It is more difficult to determine if the slow turnover protein pool has reached equilibrium as the contribution to isotope dilution in the intracellular space is small, but such is highly unlikely based on the size of that pool and the turnover rate.

For Leu, de-novo synthesis was not identifiable which was expected given its classification as an essential AA. Transamination to  $\alpha$ -keto-isocaproate was observed as indicated by positive estimates for  $k_{nAA\rightarrow KA}$ . The return of labeled-N during reamination improved model fit for Leu, hence was included, but a more encompassing model should account for labeled N across all AA, not just Leu. The decarboxylation of Leu is irreversible, hence, no carbon tracer should return during reamination and thus the return flux was set to zero.

For Ala, de-novo synthesis was significant and different from zero, which reflects its importance for transporting N. The primary transamination product of Ala is pyruvate and this reaction is reversible. The return of labeled N and C improved model fit for Ala, which reflects use of carbon and nitrogen precursor pools that are enriched during reamination of Ala.

Model fit was evaluated by several methods to assess the accuracy, precision, and robustness of the proposed model (Table 6 and 7). For Ala and Leu, the RMSE was 12% or less for isotopic enrichment of media and intracellular free AA, indicating good fit relative to the observed values. The CCC values generally indicated model appropriateness in terms of accuracy and precision, however, the model poorly represented isotopic enrichment in the protein pool. This was likely because data variation was minimal and only one pool was characterized with inputs and outputs, the fast turnover pool. Evaluation of MCMC derived

parameters indicated similar fit quality in terms of RMSE and CCC despite different parameter estimates. The fitting of isotopic enrichment of Phe in an in vivo assessment of AA transport resulted in an average RMSE of 28.1% (Hanigan et al., 2009) which is higher than the average RMSE of 5.9 and 11.6% for Ala and Leu isotopic enrichments in our study. Other AA transport models have not reported how well proposed parameters represent observed movement of AA (Biolo et al., 1995, Bequette et al., 2000, Miller et al., 2004).

#### **4.4.2. AA Concentration, Transport and Metabolism**

The observed extracellular Ala and Leu concentrations were similar to in-vivo, being within 3.7 and 6.1% (Table 1). Intracellular concentrations were 10.9 and 3.3-fold higher than extracellular for Ala and Leu respectively, indicating a steep concentration gradient (Table 6 and 7). The vast majority of AA transport is mediated by active transport; hence, the observation of a significant gradient supports this theory. In human muscle, Ala and Leu concentrations were found to be 5.5 and 1.5-fold greater in the intracellular space than in the extracellular space (Miller et al., 2004). In lactating dairy goats, intracellular Leu concentrations were 3.3 fold higher than extracellular, exactly what we observed (Hanigan et al., 2009). The much higher ratio of Ala relative to Leu likely reflects de-novo synthesis, importance for providing trans-stimulation to drive System L transport, and its role as a N carrier and mediator (Bender, 2012). Also, the ratio of Ala to Leu changed dramatically between extracellular and intracellular, 1.6 to 5.3, hence, assumptions that blood AA profiles match the intracellular AA profile is incorrect. The intracellular profile represents intracellular metabolism and the dynamics of AA transport which greatly modify blood AA profile.

Within the protein bound pool, our results indicate Leu was present 3-fold higher than Ala which is somewhat unexpected given previous estimates of cattle (Wu, 2013a) and

mammary tissue AA profiles (Shennan et al., 1997) (Table 6 and 7). However, neither of these studies specifically examined bovine epithelial cell protein.

The prediction of AA fluxes (i.e. influx, efflux, and metabolism) represents the primary objective of this method (Table 5). Standard errors based on the MCMC simulation indicated that entry, exit, and net uptake of Ala and Leu from the cell was predicted with 8.1% error or less (Table 5). Metabolic fluxes were predicted with slightly less confidence with the exception of the fast turnover protein synthesis which had a CV of 61.8%. The high turnover rates of the Ala and Leu extracellular (i.e. 37 and 76 min) and intracellular free pools (i.e. 11 and 8 min) illustrate just how rapid exchange is and the inherent flexibility present to meet needs for protein synthesis. Extracellular Ala turnover was approximately twice as high as Leu, which likely reflects its role in driving System L transport, and the high capacity of System ASC, a sodium-dependent transporter, for taking up Ala (Bender, 2012, Broer and Broer, 2017). In lactating goats, approximately 55% of Leu that entered mammary tissue was released which is lower than our observation of 72%. In human muscle, 104% of Ala and Leu entering muscle tissue was released (Miller et al., 2004). An early study in human muscle observed that 136% of Ala that entered muscle cells was released (Biolo et al., 1995).

Whether this high level of exchange occurs in lactating dairy cows is unknown but if confirmed, might provide a strong mechanism for matching intracellular specific AA needs in the face of daily variations in AA supply. High AA exchange rates do occur in the udder of lactating goats, with an estimated 69, 55, 53, and 69% of uptake of Phe, Leu, Met, and Val being effluxed (Hanigan et al., 2009). Competitive inhibition of transport systems is well-documented (Baumrucker, 1984, Hurley et al., 2000, Jackson et al., 2000), hence whether the AA profile or concentration affects exchange of intracellular AA by competing for transporters warrants

investigation. Another question is why such a high exchange of AA which requires energy expenditure exists? Perhaps this is to rapidly modify intracellular AA profile to match translation needs. Is there a specific profile and concentration of AA that best optimizes transport (e.g. minimize competitive inhibition, drive exchange by increasing NEAA) to meet the needs of intracellular protein synthesis?

The turnover rates of the fast protein pool were quite rapid and likely reflect proteins with very short half-lives (i.e. 30 min). The finding that Leu turnover was nearly 4 times faster than Ala turnover confirms a previous study of high turnover rates of BCAA (Hanigan et al., 2009) which reflects that proteins with high turnover rates are more highly enriched in BCAA. In that previous study, the Leu fast turnover rate was 151% h, which is close to what we observed (i.e. 179% h) (Hanigan et al., 2009).

#### **4.4.3. Media Concentrations and Enrichment**

Extracellular AA profile and concentrations were set to mimic plasma concentrations of lactating dairy cows (Swanepoel et al., 2016). Evaluation of AA transport should consider all AA (Christensen, 1990). Transporter specificity is weak as AA share transporters and AA can use multiple transporters. Competition for transporter sites and the synchrony of exchange driven transport ensures considerable interactions among AA with respect to transport. Transport kinetic experiments therefore should not be conducted using standard cell culture media (i.e. DMEM): they should be conducted using media that more closely replicates in-vivo AA concentrations and profile.

The measured AA concentration and profile of the  $^{13}\text{C}$  and  $^{15}\text{N}$  enriched experimental medias were similar for most AA to the targeted concentrations and profile typical of lactating dairy cows (Table 1, Supplemental Material Table 2). The discrepancies in some observed AA concentrations versus target concentrations stems from the inability to simultaneously achieve

desired concentrations and enrichments and error. For Asp, Glu, and Phe, the concentrations in the algae ( $^{13}\text{C}$  enriched and  $^{15}\text{N}$  enriched) and the amount of algae needed to achieve adequate enrichment of the other AA resulted in slightly higher concentrations in the experimental media, particularly for Asp. For these AA, no  $^{12}\text{C}$  was added to the media which resulted in very low assayed abundance of  $^{12}\text{C}$  for concentration determination, particularly for Phe. Three forms of enriched algae from Cambridge Isotope Laboratories were utilized for isotope enrichment but the AA concentration and profile of these algae were unknown prior to analysis. The observed differences between HPLC and isotope dilution measurements likely reflect methodology differences and sample size, with the latter method having 9 replicates (Supplemental material, Table 1). Our results indicate in some cases >25% differences in CP concentration and AA profile between the enriched algae, which might reflect different growing, harvesting, or acid hydrolysis recovery conditions of the enriched algae for each specific production lot. Future research should determine AA content of the algae used as it can be expected to differ from the reported values herein.

Four AA were present in very low to immeasurable concentrations in the respective algae utilized, which is related to algae composition and loss during acid hydrolysis (i.e., tryptophan, cysteine, asparagine, and glutamine). In our experiment, these four AA as well as histidine were added entirely in  $^{12}\text{C}$  form to meet their targeted concentration in the  $^{15}\text{N}$  media. In the  $^{13}\text{C}$  media, individual  $^{15}\text{N}$  enriched Asn, Cys, Gln, and His were added to achieve isotope enrichment, however, only enrichment of Gln and His were observed indicating the Asn and Cys amount were either too low or lost during the derivatization process. The AA concentration for amino acids not enriched in the  $^{13}\text{C}^{15}\text{N}$  algae that were added as an external tracer were not determined as the addition of  $^{15}\text{N}$  or  $^{13}\text{C}$  tracers were not possible since cell and media contents

already contained these isotopes. Hence, this method is not applicable for five of the twenty amino acids, however these amino acids were added to the media at quantities as planned.

Atom percent excess (APE) values were not utilized as reliance on only three background samples and integration of low abundance ions in a background sample on a quadrupole mass spectrometry increases variance of these measurements. Given that we measured enrichment in all pools at time zero, the enrichment change over time as an input for the model yields the same answer as using atom percent excess. The resulting  $^{15}\text{N}$  experimental media achieved atom percent excess values of 42.1 and 52.9 for Ala and Leu, indicating significant enrichment over background (Supplemental Material, Table 4). For the  $^{13}\text{C}$  media, APE values were 46.3 and 68.3 for Ala and Leu (Supplemental Material, Table 4). Interestingly,  $^{13}\text{C}$  enrichment of cellular intracellular free AA but not protein bound AA at time zero was different than the background samples, which indicates extracellular attachment of AA (Supplemental Material, Table 4). Extracellular AA attachment was expected, underscoring the importance of time zero measurements (Darmaun et al., 1988).

Cells were incubated for 24 h with  $^{15}\text{N}$  enriched media in an attempt to achieve near stable enrichment of intracellular free AA and the fast turnover protein bound pool of AA (Supplemental Material, Table 5). Enrichment assessment of Ala and Leu in the media over time indicates that within 16 h, near steady state was achieved within the media. Additionally, the observed turnover rate for the fast turnover protein pool indicates complete turnover within 128 and 34 minutes for Ala and Leu respectively (Table 5). Incubation for 24 hours thus easily achieved a stable plateau within the fast turnover protein and intracellular free AA pools.

A shortcoming of this method was the inability to solve for a slow turnover protein rate

constant, which was reflected by high collinearity and high standard errors when attempting to derive this constant. A longer-term incubation of particularly the  $^{13}\text{C}$  tracer would greatly enhance our ability to derive a slow turnover rate constant. Isotopic equilibrium of media, intracellular, and fast protein pools appeared to be reached within 60 minutes for Ala and Leu  $^{15}\text{N}$  enrichment (Figures 5 and 7). In contrast,  $^{13}\text{C}$  stable plateau enrichment was not achieved and likely reflected intracellular dynamics of protein turnover, particularly the Ala fast pool, still increasing in enrichment (Figures 4 and 6). However, this was apparently inadequate to define the turnover rate of the slow protein pool. This problem should be resolvable by the addition of one or more sampling timepoints with much longer exposure to the  $^{13}\text{C}$  labelled media to allow more time for accumulation of label in the slow turnover pool.

#### **4.5. Conclusion**

We have demonstrated the validity of a method to evaluate AA transport and metabolism when subjected to varying AA supply. This method provides estimates of transport of multiple AA (n=15) which can be modified to measure all 20 AA. The dynamic 12-pool model demonstrated adequate fit and prediction power for assessments of all fluxes except for the slow protein turnover rate given the data collected. In general, observed concentration gradients and fluxes matched biological expectations, suggesting validity of the method and model. The observed fluxes illustrate the rapid exchange of Ala and Leu across the cell membrane, which likely provides harmonization and buffering of extracellular supplies of AA to ensure that cellular supply and use of protein translation are aligned. Application of this method to varying levels of AA supply should provide more insight into AA transport activity and regulation and to the role it plays in regulating milk protein synthesis.

#### 4.6. References

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**Table 4. 1.** Amino acid concentrations and atom percent enrichment of the  $^{13}\text{C}$  and  $^{15}\text{N}$  media before exposure to the cells.

Amino Acid Concentration and Enrichment						
AA	$^{15}\text{N}$ enriched media			$^{13}\text{C}$ enriched media		
	Mean, $\mu\text{M}$	SD, $\mu\text{M}$	AP <sup>1</sup>	Mean, $\mu\text{M}$	SD, $\mu\text{M}$	AP <sup>2</sup>
Ala	324	0.6	55	307	5.9	54
Arg	66	2.8	27	81	2.7	36
Asp	50	1.1	84	-	-	99
Glu	80	4.2	87	42	31.7	89
Gly	375	5.3	29	322	2.1	10
Ile	124	1.4	62	128	2.1	67
Leu	187	2.2	68	202	3.4	68
Lys	96	7.8	74	59	8.8	52
Met	19	1.5	68	19	0.7	65
Phe	70	3.5	73	59	6.2	99
Pro	115	1.7	54	117	1.1	34
Ser	190	17.5	37	139	8.8	32
Thr	195	0.6	38	168	0.4	58
Tyr	57	1.3	56	37	0.7	38
Val	285	1.2	42	274	5.9	41

<sup>1</sup>AP: Atom percent =  $\left(\frac{^{15}\text{N}}{^{12}\text{C}+^{15}\text{N}}\right) \times 100$ .

<sup>2</sup>AP: Atom percent =  $\left(\frac{^{12}\text{C}}{^{12}\text{C}+^{13}\text{C}}\right) \times 100$ .

**Table 4. 2.** Description of initial estimates for volume, concentrations, pool sizes, and isotopic ratios for Alanine and Leucine

Item <sup>1</sup>	Description	Unit	Ala <sup>2</sup>	Leu <sup>2</sup>
$V_x$	Extracellular	mL	6.0	6.0
$V_n$	Intracellular	mL	0.32	0.32
$V_p$	Protein bound volume	mL	0.079	0.079
$C_{xAA}$	Extracellular, <sup>12</sup> C free AA	$\mu M$	114	54
$C_{nAA}$	Intracellular, <sup>12</sup> C free AA	$\mu M$	1,376	237
$C_{tAA}$	Protein bound, <sup>12</sup> C AA	$\mu M$	18,456	54,476
$Q_{xAA}$	Extracellular, <sup>12</sup> C free AA	$\mu mol$	684	322
$Q_{xAA}^{13C}$	Extracellular, <sup>13</sup> C free AA	$\mu mol$	976	818
$Q_{xAA}^{15N}$	Extracellular, <sup>15</sup> N free AA	$\mu mol$	161	58
$Q_{nAA}$	Intracellular, <sup>12</sup> C free AA	$\mu mol$	440	76
$Q_{nAA}^{13C}$	Intracellular, <sup>13</sup> C free AA	$\mu mol$	97	33
$Q_{nAA}^{15N}$	Intracellular, <sup>15</sup> N free AA	$\mu mol$	378	64
$Q_{tAA}$	Protein bound, <sup>12</sup> C AA	$\mu mol$	1458	4304
$Q_{tAA}^{13C}$	Protein bound, <sup>13</sup> C AA	$\mu mol$	153	8.7
$Q_{tAA}^{15N}$	Protein bound, <sup>15</sup> N AA	$\mu mol$	506	1928
$Q_{tfAA}^{13C}$	Fast Protein bound, <sup>12</sup> C AA	$\mu mol$	36.5	43.0
$Q_{tfAA}^{15N}$	Fast Protein bound, <sup>13</sup> C AA	$\mu mol$	3.82	0.09
$Q_{tfAA}$	Fast Protein bound, <sup>15</sup> N AA	$\mu mol$	31.3	36.5
$Q_{tsAA}^{13C}$	Slow Protein bound, <sup>12</sup> C AA	$\mu mol$	1422	4261
$Q_{tsAA}^{15N}$	Slow Protein bound, <sup>13</sup> C AA	$\mu mol$	149.1	8.7
$Q_{tsAA}^{13C}$	Slow Protein bound, <sup>15</sup> N AA	$\mu mol$	475	1891
$E_{xAA}^{13C}$	Extracellular isotope ratio of <sup>13</sup> C to <sup>12</sup> C		1.43	2.54
$E_{xAA}^{15N}$	Extracellular isotope ratio of <sup>15</sup> N to <sup>12</sup> C		0.24	0.18
$E_{nAA}^{13C}$	Intracellular isotope ratio of <sup>13</sup> C to <sup>12</sup> C		0.22	0.43
$E_{nAA}^{15N}$	Intracellular isotope ratio of <sup>15</sup> N to <sup>12</sup> C		0.86	0.85
$E_{tAA}^{13C}$	Protein bound isotope ratio of <sup>13</sup> C to <sup>12</sup> C		0.10	0.002
$E_{tAA}^{15N}$	Protein bound isotope ratio of <sup>15</sup> N to <sup>12</sup> C		0.35	0.45

<sup>1</sup>Model abbreviations for volume, concentration, pools, and isotope ratios.

<sup>2</sup>Alanine and Leucine initial values represent average of 6 plates of cells for volume, concentration, and mass of <sup>12</sup>C whereas mass of <sup>13</sup>C and <sup>15</sup>N and isotope ratios average of 3 plates of cells.

**Table 4. 3.** Descriptions, abbreviations, and units of rate constants and fluxes

Variable	Description	Unit
$k_{xAAnAA}$	Rate constant for uptake	min
$k_{nAAxAA}$	Rate constant for efflux	min
$k_{nAAtfAA}$	Rate constant for fast turnover protein synthesis	min
$k_{nAAtsAA}$	Rate constant for slow turnover protein synthesis	min
$k_{nAAnKA}$	Rate constant for transamination	min
$k_{nAAOx}$	Rate constant for oxidation	min
$f_{synthesis}$	Fraction constant for NEAA synthesis	min
$f_{tf}$	Fast turnover fraction of protein bound pool	%
$f_{carbon}$	Fraction of $^{13}C$ carbon used for reamination or synthesis	%
$f_{nitrogen}$	Proportion of $^{15}N$ nitrogen used for reamination or synthesis	%
$F_{xAAnAA}(i,j,k)$	Flux AA from extracellular to intracellular	$\mu\text{mol}/\text{min}$
$F_{nAAxAA}(i,j,k)$	Flux of AA from intracellular to extracellular	$\mu\text{mol}/\text{min}$
$F_{nAAOx}(i,j,k)$	Flux of AA from intracellular to oxidation	$\mu\text{mol}/\text{min}$
$F_{nAAtfAA}(i,j,k)$	Flux of AA from intracellular to fast protein synthesis	$\mu\text{mol}/\text{min}$
$F_{tfAAnAA}(i,j,k)$	Flux of AA from fast protein degradation to intracellular	$\mu\text{mol}/\text{min}$
$F_{nAAtsAA}(i,j,k)$	Flux of AA from intracellular to slow protein synthesis	$\mu\text{mol}/\text{min}$
$F_{tsAAnAA}(i,j,k)$	Flux of AA from slow protein degradation to intracellular	$\mu\text{mol}/\text{min}$
$F_{nAAnKA}(i,j,k)$	Flux of AA from intracellular to transamination	$\mu\text{mol}/\text{min}$
$F_{nKANAA}(i,j,k)$	Flux of AA from transamination to intracellular	$\mu\text{mol}/\text{min}$
$F_{nSynAA}(i,j,k)$	Synthesis flux of AA to intracellular	$\mu\text{mol}/\text{min}$
$F_{nKANAAu}(j,k)$	Flux of $^{12}C$ AA from transamination of $^{13}C$ AA to intracellular	$\mu\text{mol}/\text{min}$

<sup>1</sup>Subscripts  $i$ ,  $j$ , and  $k$  represent the flux of  $^{12}C$ ,  $^{13}C$ , or  $^{15}N$  enriched amino acid respectively.

**Table 4. 4.** Rate constant estimates derived by maximum likelihood and Markov Chain Monte Carlo simulation

Parameter	ML <sup>1</sup>				MCMC <sup>2</sup>		
	Estimate	SE	CV % <sup>3</sup>	<i>P</i> -value	Estimate	SE	CV % <sup>3</sup>
<i>Alanine</i>							
$k_{xAA_nAA}$	0.032	0.002	7.6	<0.001	0.034	0.001	4.0
$k_{nAA_xAA}$	0.053	0.004	8.1	<0.001	0.065	0.004	6.1
$k_{nAAOX}$	0.029	0.004	12.8	<0.001	0.034	0.005	14.3
$k_{nAA_tfAA}$	0.001	0.000	31.8	<0.01	0.001	0.001	42.1
$k_{nAA_nKA}$	0.044	0.003	6.6	<0.001	0.043	0.002	5.4
$f_{synthesis}$	8.914	1.662	18.6	<0.001	8.699	1.845	21.2
$f_{tf}$	2.50	-	-	-	2.50	-	-
$f_{carbon}$	100	-	-	-	100	-	-
$f_{nitrogen}$	100	-	-	-	100	-	-
<i>Leucine</i>							
$k_{xAA_nAA}$	0.018	0.001	6.7	<0.001	0.021	0.001	6.6
$k_{nAA_xAA}$	0.087	0.007	8.3	<0.001	0.099	0.006	6.4
$k_{nAAOX}$	0.033	0.002	5.4	<0.001	0.030	0.002	6.4
$k_{nAA_tfAA}$	0.019	0.002	12.0	<0.001	0.016	0.004	22.0
$k_{nAA_nKA}$	0.008	0.001	13.7	<0.001	0.006	0.001	8.7
$f_{tf}$	1.00	-	-	-	1.00	-	-
$f_{carbon}$	-	-	-	-	-	-	-
$f_{nitrogen}$	100	-	-	-	100	-	-

<sup>1</sup>ML: Model solution obtained by maximum likelihood using nonlinear optimization.

<sup>2</sup>MCMC: Model solution represents the parameter posterior mean derived by Markov chain Monte Carlo simulation (n=5000 runs).

<sup>3</sup>CV %: percent coefficient of variation, standard error divided by parameter estimate.

**Table 4. 5.** Cellular flux predictions for Alanine and Leucine, umol/min

Item	Ala			Leu		
	Mean <sup>1</sup>	SE <sup>2</sup>	CV % <sup>3</sup>	Mean <sup>1</sup>	SE <sup>2</sup>	CV % <sup>3</sup>
Entry	51.2	2.1	4.1	18.8	1.2	6.2
Exit	44.7	2.1	4.6	13.6	1.1	8.1
Net Uptake	6.4	0.3	5.1	5.2	0.1	2.8
Fast Turnover Protein Synthesis	0.6	0.3	61.8	3.0	0.3	9.6
Fast Turnover Protein Degradation	0.6	0.4	60.0	2.4	0.3	11.1
Loss to Transamination	37.2	1.9	5.1	1.3	0.1	9.3
Synthesis	17.4	3.2	18.7	-	-	-
Loss to Oxidation or Protein Export	24.5	3.1	12.6	5.1	0.2	3.2
Extracellular Turnover of Free AA, % h	164.0			79.2		
Intracellular Turnover of Free AA, % h	548.7			776.3		
Fast Turnover Protein bound AA, % h	47.0			179.1		

<sup>1</sup>Average flux over 60 minutes using rate constants derived by maxima likelihood.

<sup>2</sup>SE: standard error represents the standard deviation of 1000 simulations randomly drawing parameters from the respective parameter posterior.

<sup>3</sup>CV %: standard error divided by mean flux value.

**Table 4. 6.** Model fit statistics for Ala predictions

Item	ML <sup>1</sup>							MCMC <sup>2</sup>	
	Obs <sup>3</sup>	Pred <sup>4</sup>	RMSE <sup>5</sup>	Mean	Slope	Dispersion	CCC <sup>6</sup>	RMSE	CCC
	<i>Mean</i>		<i>% of Mean</i>	<i>% of Mean square prediction error</i>				<i>% of Mean</i>	
Isotope Ratio									
E <sub>nAA</sub> <sup>13C</sup>	0.31	0.31	11.6	1.7	8.6	89.7	0.91	15.4	0.86
E <sub>xAA</sub> <sup>13C</sup>	1.01	1.01	4.9	1.1	34.5	64.4	0.99	3.8	0.99
E <sub>nAA</sub> <sup>15N</sup>	0.62	0.60	6.3	8.4	6.5	85.1	0.98	6.2	0.98
E <sub>xAA</sub> <sup>15N</sup>	0.34	0.33	5.9	19.1	0.2	80.7	0.96	4.4	0.98
E <sub>tAA</sub> <sup>13C</sup>	0.11	0.11	3.0	1.5	0.3	98.2	0.13	3.1	0.18
E <sub>tAA</sub> <sup>15N</sup>	0.35	0.35	3.8	2.3	0.1	97.6	0.03	3.8	0.04
Pool mass, μmol									
Q <sub>nAA</sub>	445	451	15.5	0.4	22.2	77.4	0.17	19.8	-0.17
Q <sub>xAA</sub>	712	716	6.6	0.8	2.2	97.0	0.44	6.5	0.46
Q <sub>tAA</sub>	1,458	1,458	4.8	0.0	-	-	-	4.8	-
Q <sub>nAA</sub> <sup>13C</sup>	136	138	13.4	3.5	21.8	74.7	0.85	12.5	0.86
Q <sub>nAA</sub> <sup>15N</sup>	268	268	17.0	7.2	11.5	81.3	0.84	17.7	0.85
Q <sub>xAA</sub> <sup>13C</sup>	709	708	5.2	0.1	33.9	66.0	0.99	4.7	0.99
Q <sub>xAA</sub> <sup>15N</sup>	246	241	11.1	3.5	0.7	95.8	0.90	9.6	0.93
Q <sub>tAA</sub> <sup>13C</sup>	154	154	3.1	0.7	1.7	97.6	0.08	3.2	0.11
Q <sub>tAA</sub> <sup>15N</sup>	507	504	3.3	1.8	0.1	98.1	0.04	3.3	0.06
Concentration, μM									
C <sub>xAA</sub>	278	278	5.5	0.0	0.5	99.5	0.85	5.4	0.86
C <sub>nAA</sub>	3036	2677	43.0	5.9	7.6	86.5	-0.04	46.6	-0.10
C <sub>tAA</sub>	26,929	26,624	10.4	1.2	0.0	98.8	0.00	10.4	0.00

<sup>1</sup>ML: Model solution obtained by maxima likelihood using nonlinear optimization.

<sup>2</sup>MCMC: Model solution represents the parameter posterior mean derived by Markov chain Monte Carlo simulation (n=5000 runs).

<sup>3</sup>Obs: Measured mean isotope ratio, pool size, and concentration.

<sup>4</sup>Pred: Model predicted mean isotope ratio, pool size, and concentration.

<sup>5</sup>RMSE: Root mean square error as a percentage of the observed mean.

<sup>6</sup>CCC: Concordance correlation coefficient.

**Table 4. 7.** Model fit statistics for Leu predictions

	ML <sup>1</sup>							MCMC <sup>2</sup>	
	Obs <sup>3</sup>	Pred <sup>4</sup>	RMSE <sup>5</sup>	Mean	Slope	Dispersion	CCC <sup>6</sup>	RMSPE	CCC
			<i>% of Mean</i>	<i>% of Mean square prediction error</i>				<i>% of Mean</i>	
Isotope Ratio									
E <sub>nAA</sub> <sup>13C</sup>	0.97	1.10	9.7	49.7	13.6	36.7	0.98	16.7	0.95
E <sub>xAA</sub> <sup>13C</sup>	2.22	2.24	2.9	15.4	0.4	84.2	0.97	2.6	0.98
E <sub>nAA</sub> <sup>15N</sup>	0.56	0.53	8.3	0.1	41.5	58.4	0.98	7.3	0.98
E <sub>xAA</sub> <sup>15N</sup>	0.24	0.24	4.0	40.4	26.2	33.4	0.98	2.6	0.99
E <sub>tAA</sub> <sup>13C</sup>	0.01	0.01	29.1	0.0	3.2	96.8	0.94	29.2	0.93
E <sub>tAA</sub> <sup>15N</sup>	0.47	0.45	15.9	11.7	0.3	88.0	0.00	15.9	0.00
Pool mass, μmol									
Q <sub>nAA</sub>	64	62	12.5	0.1	6.7	93.2	0.73	12.5	0.72
Q <sub>xAA</sub>	303	307	4.2	12.1	0.0	87.8	0.80	4.2	0.81
Q <sub>tAA</sub>	4,304	4,304	22.7	0.0	NA	NA	NA	22.7	NA
Q <sub>nAA</sub> <sup>13C</sup>	58	63	17.0	15.9	3.5	80.6	0.90	22.5	0.85
Q <sub>nAA</sub> <sup>15N</sup>	38	35	16.6	1.4	12.5	86.1	0.95	15.8	0.96
Q <sub>xAA</sub> <sup>13C</sup>	676	694	4.6	35.2	1.2	63.6	0.97	4.3	0.97
Q <sub>xAA</sub> <sup>15N</sup>	73	73	4.8	3.9	7.3	88.8	0.95	4.6	0.96
Q <sub>tAA</sub> <sup>13C</sup>	23	29	19.8	33.9	28.9	37.2	0.97	17.0	0.98
Q <sub>tAA</sub> <sup>15N</sup>	1,985	1,921	16.6	3.6	28.2	68.2	0.02	16.6	0.02
Concentration, μM									
C <sub>xAA</sub>	175	179	4.1	26.0	1.9	72.1	0.94	4.0	0.95
C <sub>nAA</sub>	571	503	41.7	6.1	3.7	90.3	-0.01	40.3	0.03
C <sub>tAA</sub>	79,817	78,670	19.6	0.6	24.5	74.9	0.01	19.6	-0.01

<sup>1</sup>ML: Model solution obtained by maxima likelihood using nonlinear optimization.

<sup>2</sup>MCMC: Model solution represents the parameter posterior mean derived by Markov chain Monte Carlo simulation (n=5000 runs).

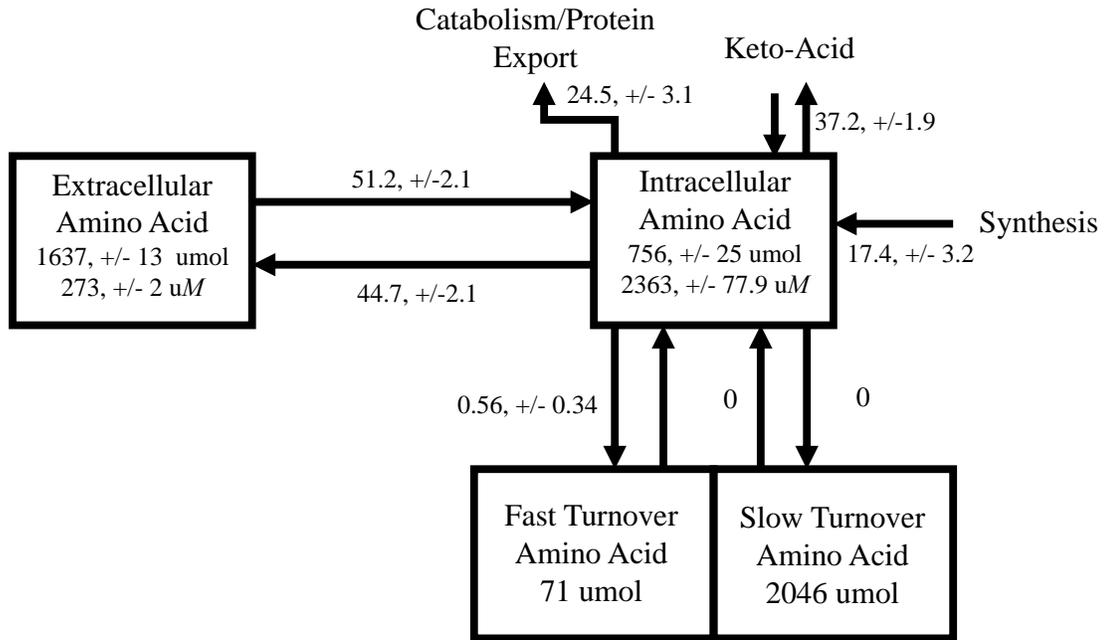
<sup>3</sup>Obs: Measured mean isotope ratio, pool size, and concentration.

<sup>4</sup>Pred: Model predicted mean isotope ratio, pool size, and concentration.

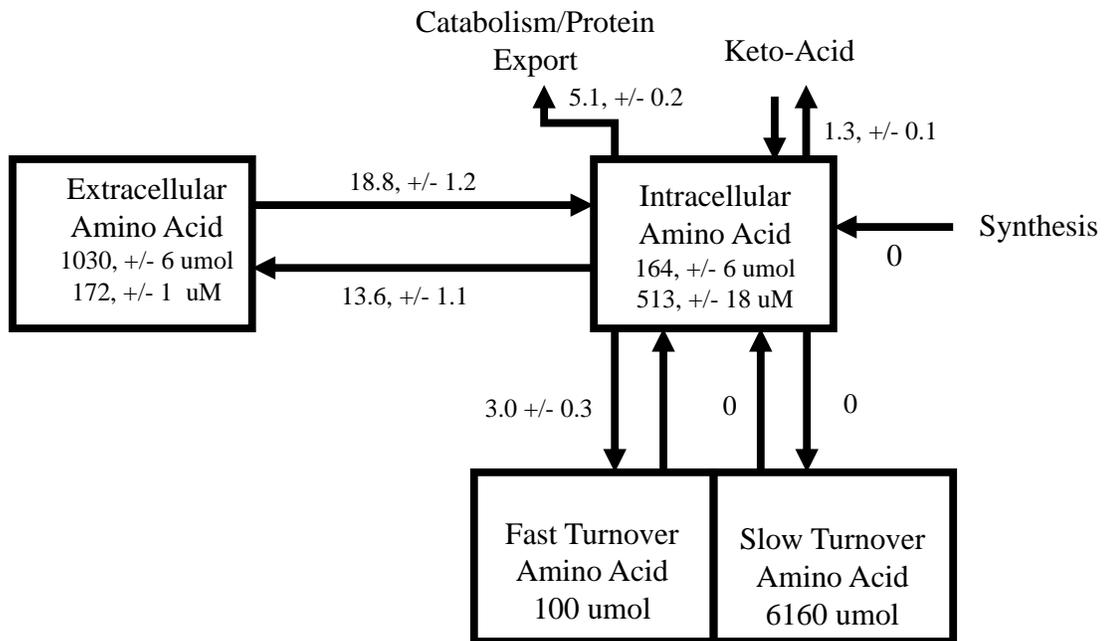
<sup>5</sup>RMSE: Root mean square error as a percentage of the observed mean.

<sup>6</sup>CCC: Concordance correlation coefficient.

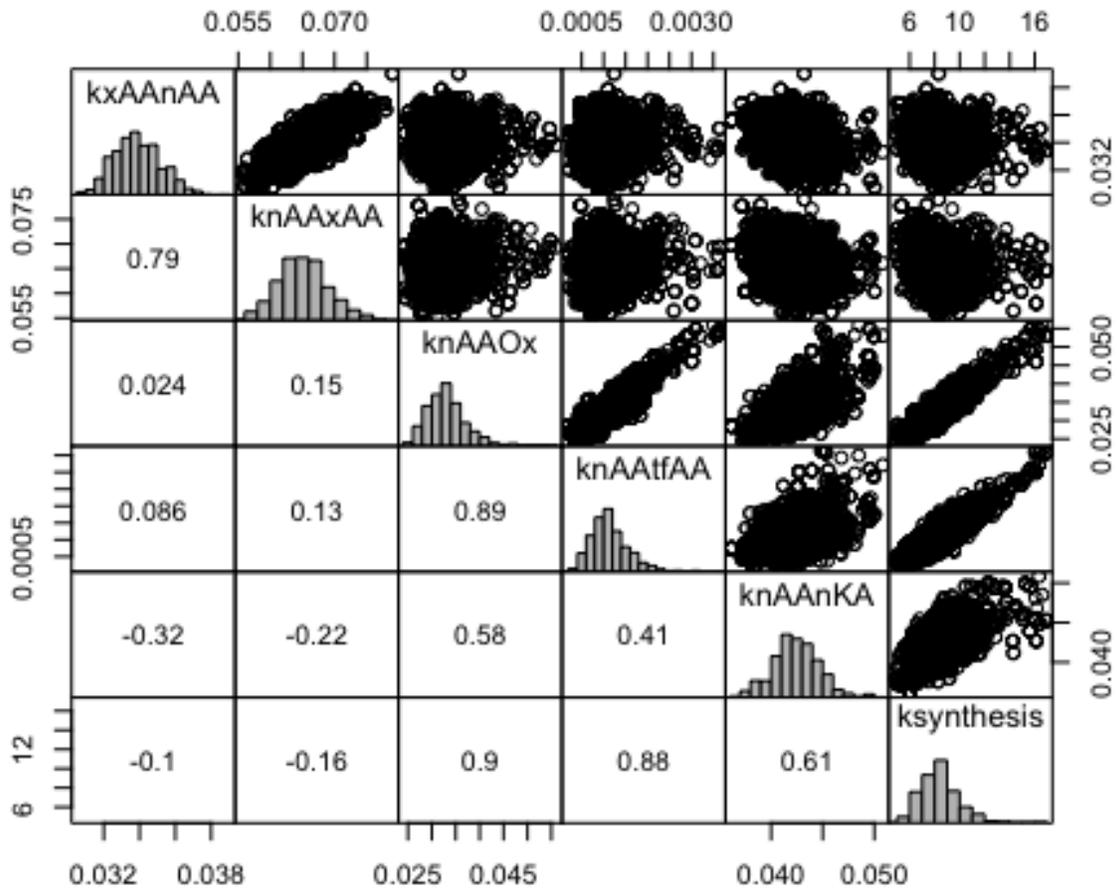
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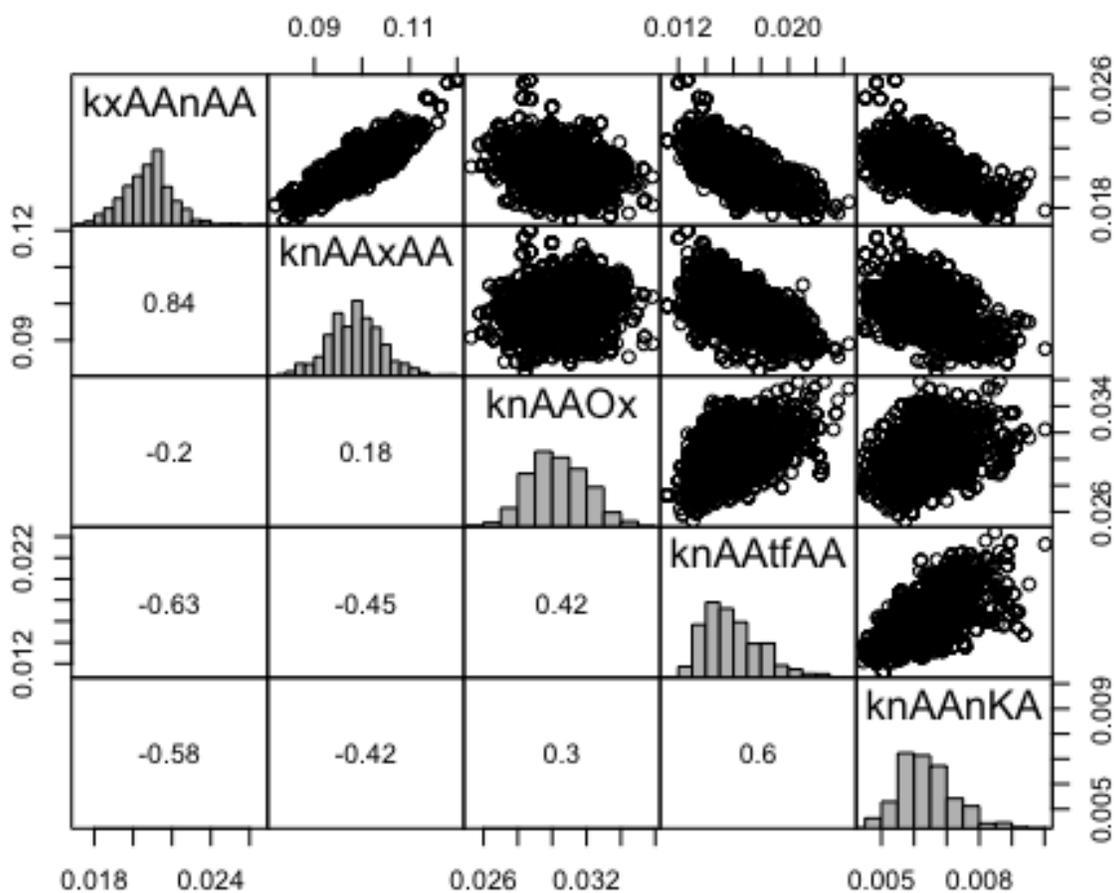
B



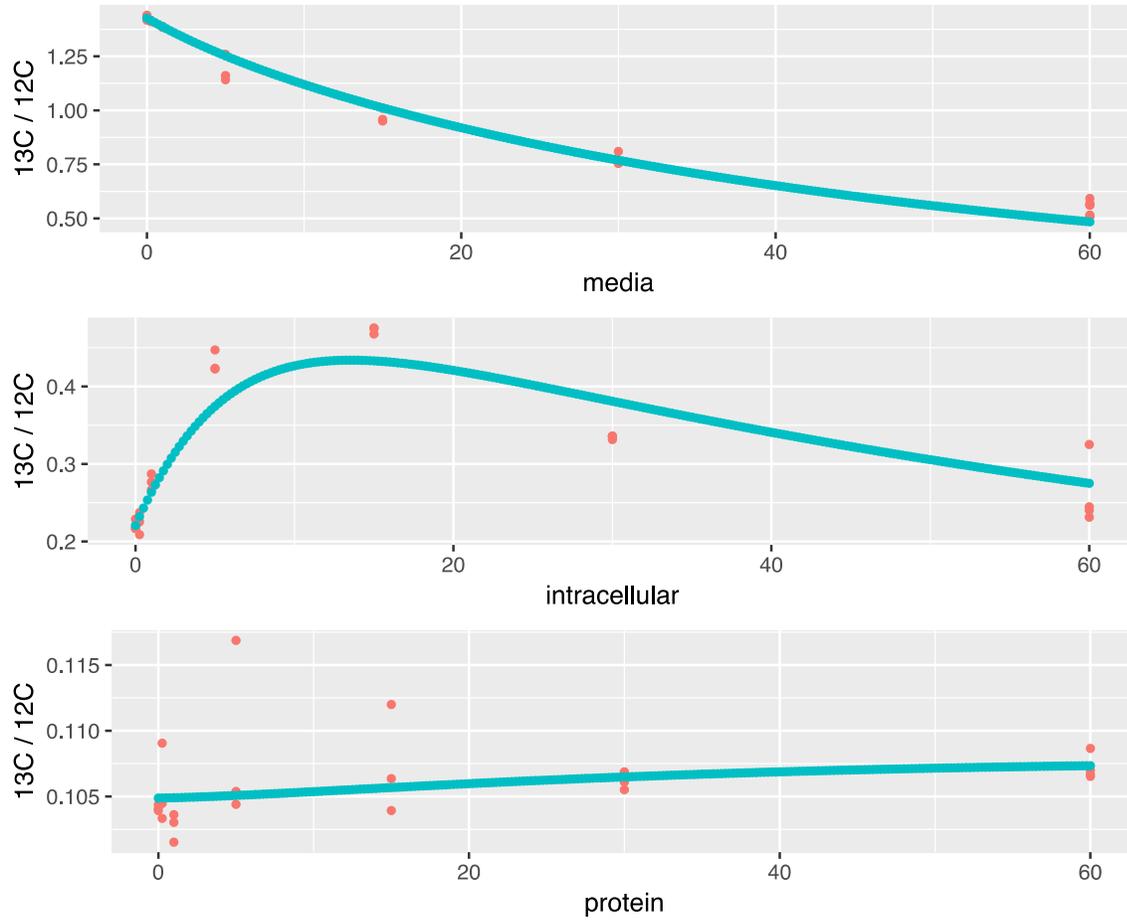
**Figure 4. 1.** Alanine (A) and Leucine (B) pool fitted pool masses ( $\mu\text{mol}$ ), concentration ( $\mu\text{M}$ ), and fluxes ( $\mu\text{mol}/\text{min}$ ). The extracellular AA concentrations was similar to in vivo blood plasma concentrations.



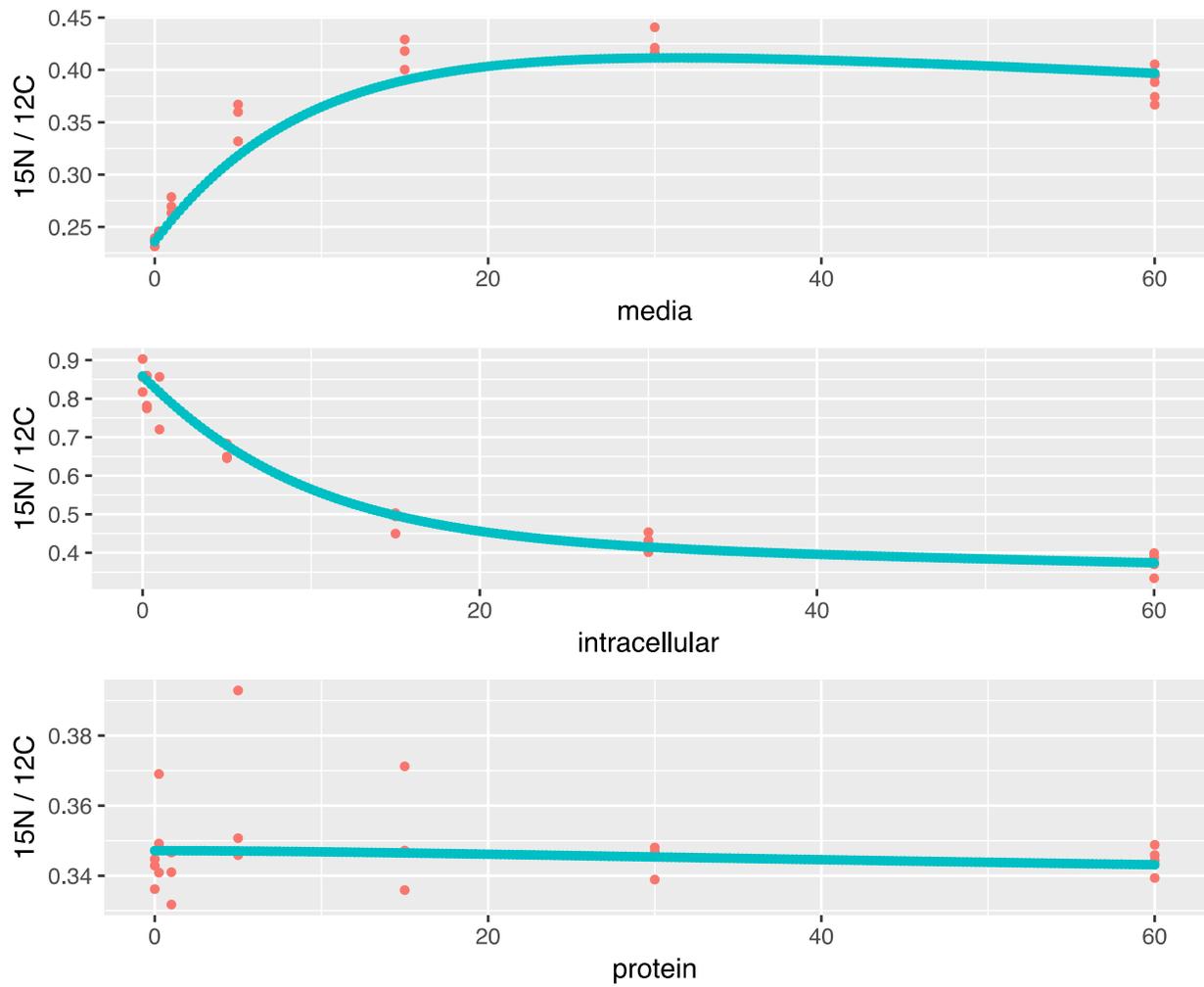
**Figure 4. 2.** Alanine parameter correlations derived from Markov Chain Monte Carlo simulations ( $n=5000$ ). The pairwise relationship is presented in plots above the diagonal, the correlation coefficients in the cells below the diagonal, and the posterior distribution of each parameter on the diagonal. Abbreviations indicate the following rate and fractional constants:  $k_{xAAAnAA}$  = uptake,  $k_{nAAxAA}$  = efflux,  $k_{nAAOx}$  = oxidation,  $k_{nAAtfAA}$  = fast turnover synthesis,  $k_{nAAAnKA}$  = transamination, and  $k_{synthesis}$  = Ala synthesis.



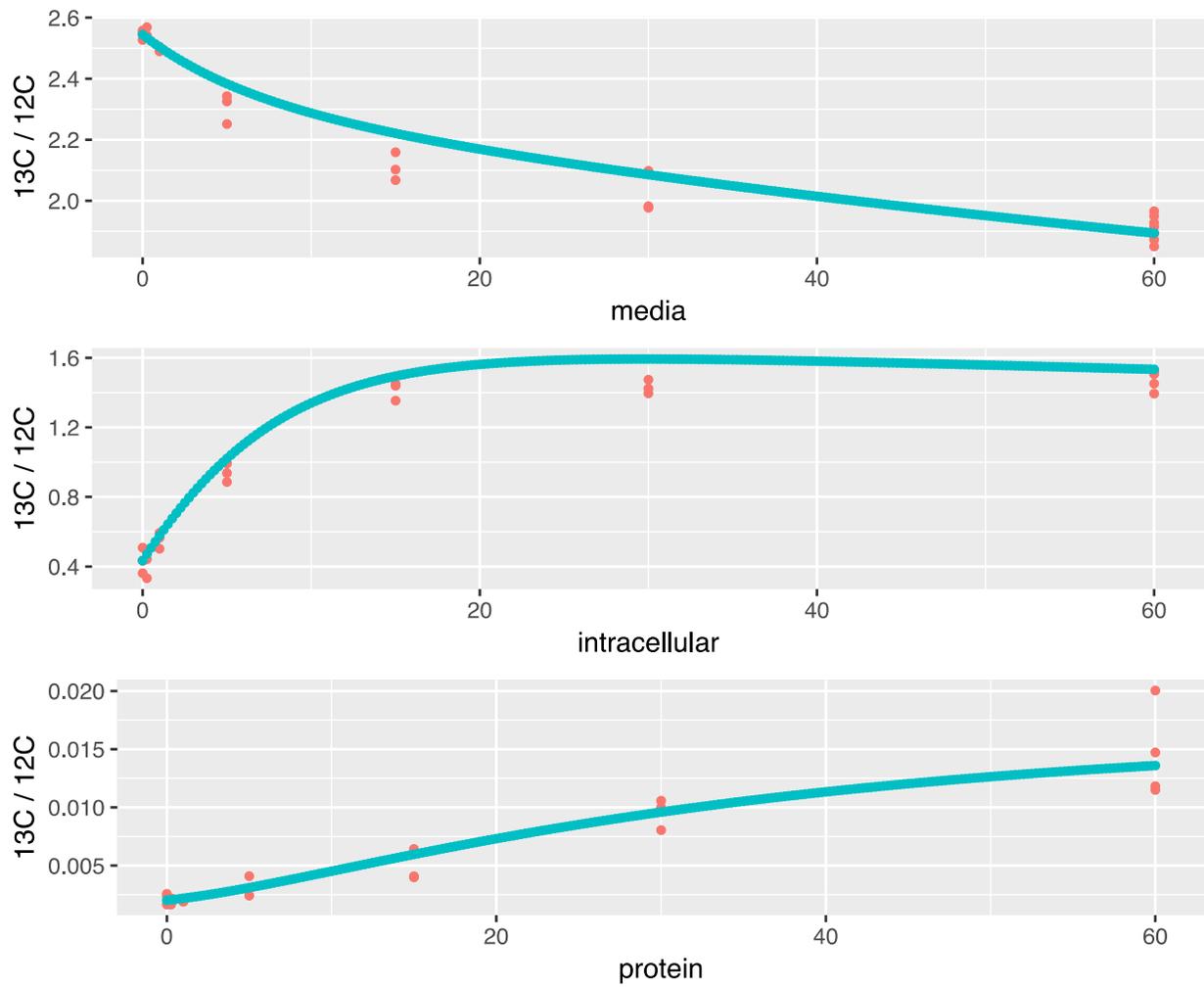
**Figure 4. 3.** Leucine parameter correlations derived from Markov Chain Monte Carlo simulations (n=5000). The pairwise correlation plots are displayed above the diagonal, the correlation coefficients are presented below the diagonal, and the posterior distribution of each parameter on the diagonal. Abbreviations indicate the following rate and fractional constants:  $k_{xAA_nAA}$  = uptake,  $k_{nAA_xAA}$  = efflux,  $k_{nAA_{Ox}}$  = oxidation,  $k_{nAA_{tfAA}}$  = fast turnover synthesis, and  $k_{nAA_nKA}$  = transamination.



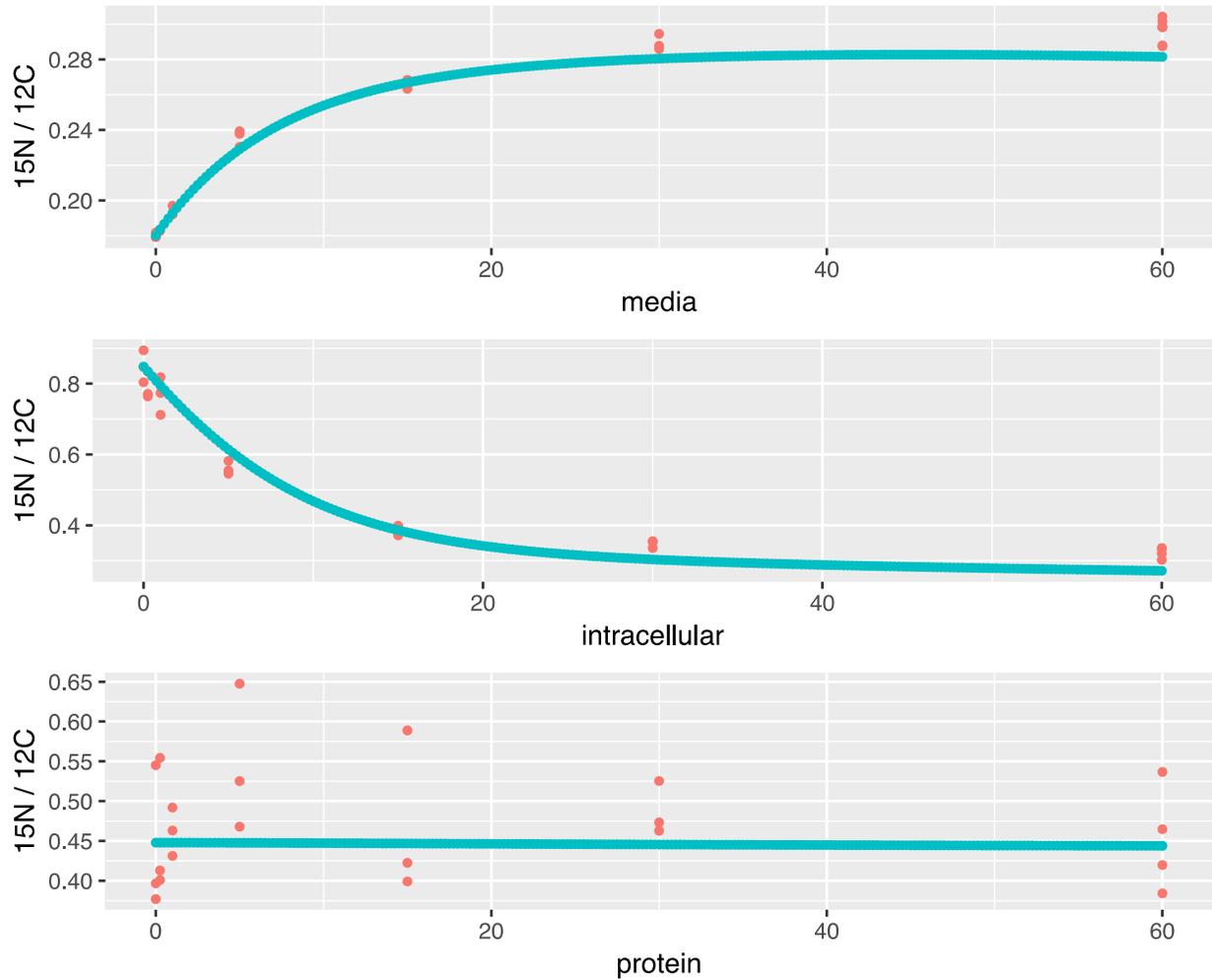
**Figure 4. 4.** Observed and predicted alanine isotope ratios for the media and intracellular free alanine pools, and the protein-bound alanine pool. The y-axis of the three plots indicate the isotope ratio of  $^{13}\text{C}$  mass to  $^{12}\text{C}$  mass. The x-axis labels refer to the following: media = extracellular free AA, intracellular=intracellular free AA, and protein=intracellular protein bound AA.



**Figure 4. 5.** Alanine observed and predicted isotope ratio of  $^{15}\text{N}$  to  $^{12}\text{C}$  within the media, intracellular, and protein-bound pools. The y-axis of the three plots indicate the isotope ratio of  $^{15}\text{N}$  mass to  $^{12}\text{C}$  mass. The x-axis labels refer to the following: media = extracellular free AA, intracellular=intracellular free AA, and protein=intracellular protein bound AA.



**Figure 4. 6.** Leucine observed and predicted isotope ratio of  $^{13}\text{C}$  to  $^{12}\text{C}$  within the media, intracellular, and protein-bound pools. The y-axis of the three plots indicate the isotope ratio of  $^{13}\text{C}$  mass to  $^{12}\text{C}$  mass. The x-axis labels refer to the following: media = extracellular free AA, intracellular=intracellular free AA, and protein=intracellular protein bound AA.



**Figure 4. 7.** Leucine observed and predicted isotope ratio of  $^{15}\text{N}$  to  $^{12}\text{C}$  within the media, intracellular, and protein-bound pools. The y-axis of the three plots indicate the isotope ratio of  $^{15}\text{N}$  mass to  $^{12}\text{C}$  mass. The x-axis labels refer to the following: media = extracellular free AA, intracellular=intracellular free AA, and protein=intracellular protein bound AA.

#### 4.7. Supplemental Material

**Table 4.S 1.** Observed total AA concentration and amino acid profiles of three isotope labelled, (97-99% enriched) algae<sup>1</sup>

	Method			
	Mass Spectrometry			HPLC
	<sup>13</sup> C <sup>2</sup>	<sup>15</sup> N <sup>3</sup>	<sup>13</sup> C <sup>15</sup> N <sup>4</sup>	<sup>13</sup> C <sup>15</sup> N <sup>4</sup>
Total AA, % as fed basis	61.6	68.8	79.9	82.6
AA, % of Total AA				
Ala	10.4	8.7	6.6	9.4
Arg,	3.6	3.0	5.3	2.9
Asn	-	-	-	-
Asp	6.0	7.6	6.3	5.4
Cys	-	-	-	-
Glu	11.6	12.4	6.9	10.4
Gln	-	-	-	-
Gly	6.5	4.8	4.1	5.6
His	1.8	-	-	0.9
Ile	8.1	6.1	5.5	7.0
Leu	12.2	10.5	9.8	10.4
Lys	2.7	3.2	2.5	2.7
Met	2.6	1.8	2.1	1.2
Phe	7.6	5.3	6.9	6.2
Pro	3.3	4.0	1.7	3.1
Ser	2.7	2.1	2.3	2.5
Thr	6.4	5.1	5.4	4.9
Trp	-	-	-	-
Tyr	1.6	4.9	3.9	1.7
Val	9.1	6.9	6.3	8.3

<sup>1</sup>All algae purchased from Cambridge Isotope Laboratories, Andover, MA.

<sup>2</sup>U-[<sup>13</sup>C] 97-99% enriched AA derived from algae (Lot #30287, Product #CLM-1548).

<sup>3</sup>U-[<sup>15</sup>N] 98% enriched AA derived from algae (Lot #24163, Product #NLM-2161).

<sup>4</sup>U-[<sup>13</sup>C, <sup>15</sup>N] 98% enriched AA derived from algae (Lot #16824, Product #CNLM-452).

**Table 4.S 2.** Amino acid concentration of DMEM and the experimental media

AA, $\mu M$	DMEM <sup>1</sup>	Experiment media <sup>2</sup>
Ala	50	268
Arg	699	80
Asn	50	7*
Asp	50	2
Cys	-	2*
Glu	50	55
Gln	2500	374
Gly	250	358
His	150	52
Ile	416	107
Leu	451	165
Lys	499	57
Met	116	24
Phe	215	52
Pro	150	104
Ser	250	93
Thr	449	110
Trp	44	79
Tyr	214	59
Val	452	250

<sup>1</sup>DMEM: Dulbecco's Modified Eagle's medium (Dulbecco and Freeman, 1959).

<sup>2</sup>Planned experimental media that represented the average plasma AA concentrations in lactating cows (~45 kg/d milk yields) (Swanepoel et al., 2016). Asp and Cys concentrations represented results from a lactating cow experiment (Haque et al., 2015).

**Table 4.S 3.** Volume and cell associated measurements

Item	Mean	SD	CV <sup>1</sup>
Starting plate weight, g	19.37	0.25	1.3
Plate weight before lysis, g	20.07	0.82	4.1
Ending plate weight, g	19.49	0.33	1.7
Tube weight, g	1.37	0.03	2.5
SSA weight, g <sup>2</sup>	1.14	0.06	5.1
Cell weight prior to lysis, g	0.81	0.61	75.9
Cell weight following lysis, g	0.68	0.61	89.5
Cell weight by difference, g	0.32	0.11	35.7
Pellet weight by difference, g	0.09	0.01	9.6
Pellet weight, g	0.07	0.01	12.9
Cells per mL	1,200,000	176,777	14.7
Total number of cells	1,870,654	256,689	13.7
Total Protein, µg/mL	4,055.6	554.9	13.7
Total Protein, mg	3.3	-	-
Cell Protein, ng/cell	1.8	-	-

<sup>1</sup>CV = coefficient of variation.

<sup>2</sup>SSA = sulfosalicylic acid (50% vol/vol).

**Table 4.S 4.** Observed isotope ratio and atom percent of natural background, experimental media, and the initial time zero. Time zero represents natural background enrichment and extracellular matrix enrichment.

Item	Ala				Leu				
	Isotope Ratio		Atom Percent <sup>1</sup>		Isotope Ratio		Atom Percent <sup>1</sup>		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
<b>BACKGROUND<sup>2</sup></b>									
Media, free AA									
$E_{xAA}^{13C}$	0.09	0.00	7.3	0.19	0.00	0.00	0.00	0.00	
$E_{xAA}^{15N}$	0.16	0.01	12.8	0.37	0.18	0.01	15.4	0.49	
Intracellular, free AA									
$E_{nAA}^{13C}$	0.09	0.00	7.4	0.07	0.00	0.00	0.09	0.16	
$E_{nAA}^{15N}$	0.16	0.00	12.5	0.01	0.18	0.00	15.1	0.04	
Intracellular, protein AA									
$E_{tAA}^{13C}$	0.08	0.00	6.3	0.14	0.00	0.00	0.02	0.00	
$E_{tAA}^{15N}$	0.16	0.01	12.7	0.54	0.24	0.06	19.0	3.94	
<b>EXPERIMENTAL MEDIA<sup>3</sup></b>									
<sup>15</sup> N Media									
$E_{xAA}^{13C}$	0.41	0.00	13.1	0.08	0.65	0.02	12.5	0.33	
$E_{xAA}^{15N}$	1.71	0.01	54.9	0.10	3.56	0.03	68.3	0.32	
<sup>13</sup> C Media									
$E_{xAA}^{13C}$	1.43	0.01	53.6	0.18	2.54	0.02	68.3	0.12	
$E_{xAA}^{15N}$	0.24	0.00	8.9	0.13	0.18	0.00	4.8	0.03	
<b>TIME 0<sup>4</sup></b>									
Intracellular, free AA									
$E_{nAA}^{13C}$	0.22	0.01	10.6	0.14	0.43	0.07	19.01	2.98	
$E_{nAA}^{15N}$	0.86	0.04	41.3	1.09	0.85	0.05	37.2	2.44	
Intracellular, protein AA									
$E_{tAA}^{13C}$	0.10	0.00	7.2	0.02	0.00	0.00	0.15	0.03	
$E_{tAA}^{15N}$	0.34	0.00	23.6	0.24	0.44	0.09	30.3	4.29	

$$^1\text{Atom Percent} = \left( \frac{^{12}C}{^{12}C + ^{13}C} \right) \times 100.$$

<sup>2</sup>Background represents the average of three plates of cell media free AA, intracellular free AA, and protein bound AA incubated without <sup>13</sup>C or <sup>15</sup>N enriched AA.

<sup>3</sup>Experimental media represents average of three plates prior to incubation with cells. The <sup>13</sup>C media also represents Time 0 media.

<sup>4</sup>Time 0 represents the average of three plates of intracellular free AA, and protein bound AA incubated for 24 hr with <sup>15</sup>N enriched media.

**Table 4.S 5.** Observed Alanine and Leucine concentrations, <sup>15</sup>N enrichment, and <sup>15</sup>N atom percent in media over the 25-h period. Media was replenished at 0, 480, 960, and 1440 min respectively.

Time, min	$\mu M$		<sup>15</sup> N Isotope Ratio <sup>1</sup>		Atom Percent <sup>2</sup>	
	Mean <sup>3</sup>	SD <sup>3</sup>	Mean <sup>3</sup>	SD <sup>3</sup>	Mean <sup>3</sup>	SD <sup>3</sup>
Ala						
<0 <sup>4</sup>	224	2.0	0.16	0.00	13	0.1
0	324	0.6	1.71	0.01	55	0.1
480	315	9.1	0.83	0.01	40	0.2
960	313	14.2	1.05	0.00	44	0.1
1320	288	9.3	1.11	0.01	45	0.2
1380	283	15.0	1.07	0.02	44	1.5
1440	307	5.9	0.24	0.00	8.9	0.1
1440.25	299	10.7	0.24	0.00	9.2	0.0
1441	288	5.3	0.27	0.01	10	0.3
1445	299	25.4	0.35	0.02	14	1.0
1455	299	23.7	0.42	0.00	17	0.7
1470	264	13.2	0.43	0.01	19	0.6
1500	240	15.0	0.39	0.01	20	0.8
Leu						
<0 <sup>4</sup>	88	2.0	0.18	0.00	11	0.2
0	187	2.2	3.56	0.03	68	0.3
8	107	3.6	0.81	0.02	35	0.3
960	117	3.9	1.04	0.01	41	0.4
1320	118	4.1	1.08	0.01	41	0.5
1380	116	13.5	0.98	0.09	38	2.6
1440	202	3.4	0.18	0.00	4.8	0.0
1440.25	198	7.3	0.18	0.00	4.9	0.0
1441	189	3.7	0.19	0.00	5.3	0.1
1445	186	6.6	0.23	0.00	6.7	0.2
1455	181	5.0	0.27	0.00	7.9	0.2
1470	162	7.0	0.29	0.00	8.8	0.2
1500	147	5.0	0.30	0.01	9.3	0.2

$$^1\text{Isotope Ratio} = \frac{\text{Isotope Area}}{^{12}\text{C Area}}$$

$$^2\text{Atom Percent} = \left( \frac{^{12}\text{C}}{^{12}\text{C} + ^{13}\text{C}} \right) \times 100.$$

<sup>3</sup>Mean and standard deviation represent 3 samples

<sup>4</sup>Time <0 represents media obtained following 12-h incubation with a steady-state media that contained no enrichment of amino acids

## **CHAPTER 5: Effects of varying extracellular amino acid concentrations on bi-directional amino acid transport and intracellular fluxes in mammary epithelial cells**

### **5.1. Abstract**

Understanding the regulation of cellular AA uptake as protein supply changes is critical for predicting milk component yields since intracellularly supplies partly regulate protein synthesis. Our objective was to evaluate cellular uptake and kinetic behavior of individual amino acids (AA) when cells are presented with varying extracellular AA supplies. Bovine primary mammary epithelial cells were grown to confluency and transferred to media with an AA profile and concentration similar to that of plasma from dairy cows for 24 h. Treatments were four AA concentrations, 0.36, 2.30, 4.28, and 6.24 mM, which represented 16, 100, 186, and 271% of typical plasma AA concentrations in dairy cows. Twenty-four plates of cells (89.4 x 19.2 mm) were assigned to each treatment. Cells were first subjected to treatment media enriched with <sup>15</sup>N labelled AA for 24 h and then incubated with treatment media enriched with <sup>13</sup>C labelled AA for 0, 15, 60, 300, 900, 1800, and 3600 sec. Intracellular free AA, intracellular protein-bound AA, and extracellular media free AA were analyzed for concentrations and isotopic enrichment using gas chromatography mass spectrometry. A dynamic, 12-pool model was fitted to the data for 14 AA to derive unidirectional uptake and efflux, protein turnover, transamination, oxidation, and synthesis. The derived  $k_m$  for uptake indicated no saturation of AA uptake at typical in-vivo concentrations for 11 of the 14 AA. Arg, Pro, and Val appeared to exhibit saturation kinetics. Net uptake of all EAA except Phe was positive across treatments. Most NEAA exhibited negative net uptake values. Efflux of AA was quite high with several AA exhibiting greater than 100% efflux of the respective influx. Intracellularly pool turnover was rapid for most AA, e.g. 2 min for Arg, demonstrating plasticity in matching needs for protein

translation to supplies. Intracellular AA concentrations increased linearly in response to treatment for most AA while nine AA exhibited quadratic responses. Amino acid uptake is responsive to varying extracellular supplies to maintain homeostasis. No saturation of uptake was evident for most AA, indicating that transporter capacity is likely not a limitation for most AA except possibly Arg, Val, and Pro.

**Key words:** amino acids, transport, flux

## **5.2. Introduction**

The efficiency of conversion of metabolizable protein to milk protein is not fixed (Doepel et al., 2004) as assumed by common nutrition models (NRC, 2001; CNCPS v6.55). Varying economic and environmental factors influence the optimum supply of protein required to maximize income over feed costs (Yoder et al., 2016). However, this point cannot be readily determined if nutrition models do not accurately represent the supply response relationship. The inability of nutrition models to predict responses to varying nutrient supplies (St-Pierre and Thraen, 1999) is partly due to the empirical description of post-absorptive AA metabolism.

Increasing intracellular concentrations of some EAA stimulate translation rates resulting in increased milk protein synthesis (Arriola Apelo et al., 2014c, Arriola Apelo et al., 2014d, Cant et al., 2018). Low intracellular AA concentrations of some AA leads to uncharged tRNAs which reduces protein translation and downregulates anabolic signals associated with the general control nonderepressible 2 complex (GCN2) (Ye et al., 2015). Hence, knowing how the daily absorbed AA supply translates to intracellular AA concentrations is influential in predicting milk protein yield.

Net cellular uptake of AA generally exhibits mass action kinetics within in vivo concentration ranges (Hanigan et al., 1992). Amino acid tracer studies in pig mammary tissue

explants also indicated linear uptake kinetics within typical in-vivo concentration ranges for Val and Lys (Hurley et al., 2000, Jackson et al., 2000). Linear uptake within in-vivo concentration ranges was also observed in mammary tissue explants for Arg and Lys (Baumrucker, 1984), and linear intracellular EAA concentration responses to increasing EAA supplies (Pocius and Baumrucker, 1980). mRNA expression of AA transporters related to Arg and Lys transport (System y<sup>+</sup>, y<sup>+</sup>L) was unaffected in lactating sows despite large changes in dietary CP concentrations of 9.5, 13.5, and 17.5% (Manjarin et al., 2012). If net uptake kinetics are linear and somewhat inflexible, then reducing dietary CP intake should result in proportional reductions in intracellular AA concentrations and milk protein yield.

Amino acid transporter function has evolved to manage daily AA influx to the translation level precision required for intracellular protein synthesis (Christensen, 1990). System A transporter expression (i.e., SNAT2) was upregulated by 25-fold when rat mammary cells were incubated in AA free media (Lopez et al., 2006). The regulation appears to be driven by phosphorylation of eukaryotic initiation factor 2 (Gaccioli et al., 2006). Excess AA appeared to downregulate expression of System A transporters in incubated sow mammary tissue (Chen et al., 2018). Sensing of extracellular AA supplies and transmitting anabolic signals via mTOR was demonstrated to affect System L transporter SLC7A5 (Nicklin et al., 2009, Taylor, 2014). These evaluations of mRNA expression have not evaluated rates of transport of individual AA. Most AA kinetic studies also were conducted over such a short time frame (10 to 30 min) that changes in transport protein activity driven by changes in protein expression would have been insignificant (Pocius and Baumrucker, 1980, Baumrucker, 1984, Hurley et al., 2000, Jackson et al., 2000).

The general promiscuity of AA transporters makes linkage of functional changes in transport to changes in the expression of specific transporters difficult. Some transporters are able to transport up to a dozen AA (System L); most AA can be transported by multiple transporter systems; and transport of some AA is driven by sodium gradients while others are driven by the exchange of sodium dependent transported AA (Christensen, 1990). Incubation of pig mammary tissue with Leu at in-vivo concentrations reduced Val uptake by 47% compared to medium without Leu (Jackson et al., 2000). In vivo, additional AA would be competing with Val for System L transporters. The combination of multiple transport systems each transporting multiple AA and potentially independently regulated in expression, competitive interactions among AA, and the use of some AA as exchangers results in tremendous complexity which would not be captured in the simple linear, univariate uptake equations used in current mammary models or the fixed efficiency equations used in field software.

The objectives of this study were to assess the kinetics of individual AA uptake using the method described in Chapter 4 with AA concentrations varying from below to above the normal in-vivo range for lactating dairy cows. Additionally, we aimed to assess efflux and intracellular metabolism of each AA. We hypothesized that unidirectional uptake of AA would exhibit mass action kinetics for all amino acids within the range of concentrations studied.

### **5.3. Material and Methods**

#### **5.3.1. Experimental Design**

Bovine primary mammary epithelial (BPME) cells were obtained from the State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, China. These cells were harvested using as previously described (Hu et al., 2009) and verified as described in chapter 4. The BPME cells were thawed and plated in fourteen 89.4 mm by 19.2 mm TC-Treated Polystyrene cell culture dishes (Corning, Product

#353003) at a density of  $1.9 \times 10^6$  cells/plate. Cells were cultured in growth media as described in Chapter 4. Using trypsin (Sigma-Aldrich, #59428C), cells were detached from the 2 plates, transferred to 50 mL conical tube, centrifuged at 500 rcf for 5 min at 4°C, resulting supernatant discarded, and then pellet suspended in media. Cells were then plated in 99 cell culture dishes (89.4 mm by 19.2 mm TC-Treated Polystyrene) and incubated with the growth media. Culture dishes were weighed prior to addition of cells and media.

Upon reaching >90% confluency, approximately 3 days after plating, cells were incubated for 24 h in a steady state media as described in Chapter 4 with AA at treatment concentrations provided in Table 1. The media was changed every 12 h. Following 24 h incubation in the steady state media, cells were randomly assigned to one of the four treatments (n=24 plates/treatment). Three plates were retained for background enrichment measurements. Treatments consisted of a constant profile of AA at four concentrations of total AA representing 16, 100, 186, and 271% of that observed in lactating cows (Swanepoel et al., 2016) (Table 1). The resulting total AA concentrations were 0.36, 2.30, 4.28, and 6.24 mM and are abbreviated as Trt1, Trt2, Trt3, and Trt4. All other metabolites were held constant. The target concentrations of AA were achieved using a mix of unlabeled crystalline AA,  $^{13}\text{C}$  universally labeled AA derived from algae (U- $^{13}\text{C}$ , 97-99% enriched), and  $^{15}\text{N}$  universally labeled AA derived from algae (U- $^{15}\text{N}$ , 97-99% enriched) (Cambridge Isotope Laboratories Inc., Tewksbury, MA) as described in chapter 4.

Upon removal of the steady state media, cells from 24 plates per treatment were washed once with warm PBS and placed in 12 mL of  $^{15}\text{N}$  enriched treatment media. Cells were incubated 24 h with  $^{15}\text{N}$  enriched treatment media and fed every 8 h in order to minimize the effects of the low AA concentration, particularly in Trt1. Following 24 h of incubation, the  $^{15}\text{N}$

treatment media was removed, cells were washed twice with warm PBS and 6 mL of  $^{13}\text{C}$  enriched treatment media was added. Cells and treatment media were harvested at 0 s, 15 s, 1 min, 5 min, 15 min, 30 min, and 60 min. The zero time point was utilized to correct for extracellular media contamination of the cell and plate surfaces (Darmaun et al., 1988). The remaining time points were determined from preliminary experiments. At each harvest time point, the treatment media was removed and a 1 mL subsample was placed in an ice-cold 1.5 mL tube and stored at  $-20\text{ }^{\circ}\text{C}$ . Cells were rinsed 3 times with ice cold PBS, the dish plus cells was weighed, 1 mL of pre-weighed ice-cold 50% sulfosalicylic acid was added, the plate was gently shaken to ensure rapid cellular lysing, and cell debris was mechanically removed from the plate surface using a cell scraper. The cell lysate was removed to an ice-cold 2 mL tube and stored at  $-20\text{ }^{\circ}\text{C}$ , and the empty culture dish was weighed.

Viable live cells from three culture dishes per treatment were counted and weighed to derive weight per cell. Culture dishes were washed twice with warm PBS, the plate was weighed, and 1 mL of 0.05% Trypsin EDTA was gravimetrically added to the plate. Upon visible detachment of cells, cells were removed, washed 2X in PBS, weighed, resuspended in PBS, a subsample was removed, incubated 60 min in 0.4% Trypan Blue (Amresco, #K940), and counted using a hemacytometer.

The remaining cells from the cell counting plates were centrifuged at 2500 rcf for 5 min, the supernatant removed, and the cells were lysed in 1 mL of 2% SDS lysis buffer containing phosphatase (ThermoFisher, #78426) and protease (Promega, #G6521) inhibitors. The resulting homogenate was agitated for 30 min at  $4\text{ }^{\circ}\text{C}$  and centrifuged for 1 h at 21,130 rcf at  $4\text{ }^{\circ}\text{C}$  to remove insoluble material. The resulting supernatant was assessed for protein content using a bicinchoninic acid assay (BCA) (Smith et al., 1985) and stored at  $-80\text{ }^{\circ}\text{C}$ .

### 5.3.2. Amino Acid Analyses

The extracellular media samples were thawed and gravimetrically weighed, acidified with 7% sulfosalicylic acid, and centrifuged at 16,000 rcf for 15 min at 4 °C. Samples of extracellular media and intracellular supernatant were gravimetrically combined with  $^{13}\text{C}^{15}\text{N}$  universally labeled AA (derived from algae, 97-99% enriched, Cambridge Isotope Laboratories Inc., Tewksbury, MA). The cell pellet was gravimetrically combined with  $^{13}\text{C}^{15}\text{N}$  universally labeled AA and 6 mol HCl containing 0.1% phenol at 33.3  $\mu\text{l}$  per mg of protein. The sample was blanketed with nitrogen gas, sealed, and acid refluxed at 97.5 °C for 20 h to hydrolyze the peptide bonds. Acid hydrolyzed and deproteinized samples were desalted by ion chromatography (AG 50W-X8 resin; Bio-Rad Life Science), eluted with 2 N ammonia hydroxide into silanized glassware, and freeze dried. Samples were resolubilized in 0.1 N HCl, dried under nitrogen for 20 min at 35°C, solubilized in acetonitrile (J. T. Baker Inc., Phillipsburg, NJ), and converted to *N*-(*tert*-butyldimethyl) AA derivatives at 70°C in *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (SELECTRA-SIL; UCT Inc., Bristol, PA) for 1 h. Amino acid derivatives were separated by gas chromatography (Trace GC Ultra; Thermo Scientific) and enrichment of  $^{15}\text{N}$ ,  $^{13}\text{C}$ , and  $^{13}\text{C}^{15}\text{N}$  ions were determined by mass spectrometry (DSQII; Thermo Scientific). The sample amounts analyzed by the mass spectrometry for extracellular and intracellular samples were 0.20, 0.09, 0.05, and 0.03  $\mu\text{l}$  for Trt1, Trt2, Trt3, and Trt4 respectively. For all protein-bound samples, the amount was 0.01  $\mu\text{l}$ . This was done to prevent detector overload resulting in chromatographic peaks that are not Gaussian in shape. Calibration curves for the labeled AA mixture were generated gravimetrically using an AA standard (Sigma-Aldrich, #AAS18).

### 5.3.3. Statistical Analysis

The 12-pool, dynamic system of state variables described in chapter 4 was fit to the resulting data for each AA to derive unidirectional rates of uptake and efflux, catabolism, and incorporation into protein. Initial conditions and starting values were set as described in chapter 4. The *modCost* and *modFit* functions of the FME package (Soetaert and Petzoldt, 2010) were utilized for fitting to the observed isotope enrichment, AA concentrations, and volume measurements. Residuals were weighted by the overall standard deviation of each variable as previously described. Determination of model structure (i.e. parameters) and subsequent evaluation was completed first for Trt2. Model structure did not change for evaluation of other treatments with an AA unless a parameter was no longer significant ( $P < 0.10$ ). Standard errors for parameters and fluxes were determined by Markov Chain Monte Carlo (MCMC) with delayed rejection and adaptive Metropolis algorithm (DRAM) (Haario et al., 2006) as described previously (Chapter 4). The MCMC generated posterior was sampled 1000 times (R seed = 123), and the model solved 1,000 times to generate a population of parameters and fluxes which were used to derive standard errors. Root mean squared errors as a percentage of the mean (RMSE) and mean squared prediction errors partitioned into mean bias, slope bias, and dispersion were calculated for each AA and treatment (Bibby and Toutenberg, 1977). Concordance correlation coefficients (CCC) were also calculated to provide a dimensionless evaluation of precision and accuracy.

Hypothesis testing was conducted on concentration and volume associated measurements with a model that considered the fixed effect of treatment (4 df), time (6 df), and time by treatment interaction (24 df) and least square derived means were evaluated using linear and quadratic contrast tests. Rate constants and fluxes were statistically evaluated across treatment based on 95% confidence intervals. The mean uptake flux by treatment was standardized to

average protein contained in the plate by treatment and AA. This was done by dividing the mean uptake flux by the mean protein bound AA mass. The standardized AA uptake fluxes were regressed on extracellular AA concentrations using linear or Michaelis-Menten equation forms to derive rate parameters. The latter form was:

$$F_{xAA_nAA(i)}/Q_{tAA(i)} = \frac{V_{m(i)} \times C_{xAA(i)}}{k_{(i)} + C_{xAA(i)}}$$

where  $F_{xAA_nAA(i)}/Q_{tAA(i)}$  represents the unidirectional uptake of the  $i^{\text{th}}$  amino acid standardized to the protein bound pool of the  $i^{\text{th}}$  amino acid ( $\mu\text{mol/s/mmol}$  protein bound AA( $i$ )),  $C_{xAA(i)}$  is the extracellular concentration ( $\mu\text{M}$ ) of the  $i^{\text{th}}$  amino acid,  $V_{m(i)}$  the  $V_{\text{max}}$  of uptake ( $\mu\text{mol/s/mmol}$  protein bound AA( $i$ )), and  $k_{(i)}$  is the  $k_m$  ( $\mu\text{M}$ ). Model fit was evaluated for parameter significance, RMSE, CCC, and Bayesian information criterion. If the CCC scores were below 0.50 or RMSE greater than 40%, the fits were deemed of poor quality and not reliable. Parameters from such models were discarded. The log likelihood was calculated for each model fit, and likelihood-ratio tests were conducted with a chi-square distribution to derive  $P$ -values for model comparisons.

#### 5.4. Results

Target treatment AA concentrations are listed in Table 1 and represent the AA profile observed in a field study of lactating dairy cows located in the high cow pens on twenty dairy farms in California, USA (Swanepoel et al., 2016). Trt2 represents the total AA concentration observed in-vivo (i.e. 2.30 mM) whereas Trt1 represents 16% of in-vivo, Trt3 at 186% of in-vivo, and Trt4 at 271% of in-vivo (Table 1). The total AA concentration differences between treatments were equidistant, being approximately 1.96 mM apart. The extracellular AA concentrations for the  $^{15}\text{N}$  enriched treatment media and  $^{13}\text{C}$  enriched treatment media prior to

incubation with cells were in general as expected and differed by treatment ( $P < 0.001$ ) (Supplemental Material, Table 1 and 2).

#### **5.4.1. Amino Acid Pool Size**

Derivation of model parameters required inputs of pool mass which was determined by multiplication of pool concentration and volume. Extracellular AA concentrations were all linearly affected by treatment ( $P < 0.001$ ) (Table 2). A quadratic fit was observed for 12 of the 14 AA ( $P=0.01$ ) with the only two AA not exhibiting a quadratic effect being Arg and Asp. Time of exposure to the cells affected the concentration of all AA except Ser ( $P < 0.01$ ). Some AA declined in concentration across the 60 min while others increased (data not shown). In general, all EAA except Phe exhibited a decline in media AA concentration over the 60 min exposure whereas all NEAA except Asp increased in media AA concentrations with Trt4 and for some, across all treatments (i.e. Gly and Tyr).

Harvested cell weight tended to be affected by treatment ( $P = 0.09$ ), and those effects were linearly related to extracellular AA concentrations ( $P = 0.01$ ) (Supplemental Materials, Table 3). The increased weight corresponded to differences in cell number ( $P=0.04$ ) with a linear effect also being observed ( $P=0.01$ ). Isolated protein weight was affected by treatment ( $P < 0.001$ ) and linearly increased in response to increased extracellular AA supplies ( $P < 0.001$ ). Individual cell weight tended to be affected by treatment ( $P=0.07$ ) and linearly decreased as extracellular AA supplies increased ( $P=0.03$ ).

Intracellular free AA concentrations were affected by treatment for all AA except Glu and Gly ( $P < 0.01$ ) (Table 3). Thirteen AA increased linearly in concentrations ( $P=0.05$ ) as extracellular total AA concentrations increased. Intracellular concentrations of Ala, Asp, Ile,

Leu, Phe, Pro, Thr, Tyr, and Val also exhibited quadratic responses ( $P < 0.01$ ), with the apparent plateau occurring between Trt3 and Trt4 for Ala, Asp, Glu, Gly, Ile, Leu, Phe, Pro, Tyr, and Val.

Based on examination of ratios of intracellular to extracellular AA concentrations, the gradient between intracellular and extracellular AA pools varied greatly from a low of 0.5 for Arg to 367 for Glu across treatments. Within Trt2, the average ratio of the concentration gradient was 19.8 for NEAA and 4.2 for EAA, indicating a much higher intracellular concentration of NEAA relative to extracellular than for EAA. The intracellular free AA mass is also reported in the Supplemental Material, Table 4. All AA linearly increased in mass ( $P < 0.001$ ), whereas only Arg, Gly, Met, and Ser did not exhibit quadratic responses to increased extracellular concentrations of AA.

Intracellular, protein bound, AA concentrations varied for some AA, i.e. Ala, Asp, Gly, Phe, Ser, Thr, and Tyr ( $P < 0.05$ ) in response to the changing extracellularly AA supplies (Table 4). In contrast, Arg, Ile, Leu, Met, Pro, and Val concentrations in protein lysate were unchanged. Intracellular protein bound AA mass is reported in Supplemental Materials, Table 5. All AA were different in mass except Arg and Asp ( $P < 0.02$ ), reflecting the varying volume of the protein lysate pool.

#### **5.4.2. Model Derivation and Fit**

Amino acid uptake, efflux, and fast protein turnover rate constants were derived for fourteen AA (Table 5, Supplemental Material, Table 6). Transamination, oxidation, slow protein turnover, and fractional synthesis rate constants were derived for some AA. The average CV across all treatments, rate constants, and AA was 17.1%. The reported 95% confidence intervals were derived from the posterior distribution generated by Markov chain Monte Carlo simulation (Table 5, Supplemental Material, Table 6). In some cases, the CV exceeded 100%, reflecting

very low precision of that parameter, however, all parameter estimates were significantly different from 0 ( $P < 0.10$ , data not shown).

The collinearity scores across all treatments and AA were 10 or less indicating adequate identifiability of parameters with respect to collinearity of the parameters (Soetaert and Petzoldt, 2010). Consistent with scores of 5 and greater, correlations between some parameters were reasonably high, i.e.  $> 0.90$  for influx and efflux coefficients for some AA (data not shown). However, the use of dual isotopes, i.e.  $^{13}\text{C}$  and  $^{15}\text{N}$  to simultaneously assess both fluxes enabled adequate parameter identification.

The accuracy and precision of predicted isotope ratios, pool masses, and pool concentrations were assessed. Fit statistics for only the isotope ratio data are shown in Table 7 and Supplemental Materials Table 8. The RMSE in general was greater for Trt1 than the other treatments. Isoleucine, Val, Ala, Gly, Ser, and Tyr had RMSE less than 25% across treatments. In general, the RMSE ranged from 10 to 25%. The CCC ranged even more widely, indicating no correlation or explanatory power for some predictions to 0.99 (almost perfect concordance). The low CCC values were particularly evident for predictions of isotope ratios in the slow protein turnover pools, whereas intracellular and extracellular free AA isotope ratios appeared to be explained with good precision and accuracy.

### **5.4.3. Model Parameters**

In general, the rate constants for AA uptake decreased from Trt2 to Trt4 (Figure 2) (Table 5, Supplemental Material, Table 6). Treatment differences for AA uptake rate constant were observed for all AA except Ser and Tyr ( $P < 0.05$ ). The efflux rate constant was affected by treatment for all AA except Asp ( $P < 0.05$ ). Treatment influenced the fast turnover rate constant in all EAA but only Glu and Pro among the NEAA ( $P < 0.05$ ). Oxidation rate constants were

derived for all AA except Phe, and found to be affected by treatment for all AA except Asp ( $P<0.05$ ). Transamination rate constants were derived for at some of the treatments for EAA and for only Ala and Pro of the NEAA. Treatment differences were only observed for Ile, Phe, and Ala ( $P<0.05$ ). The AA fractional synthesis rate constant reflecting de-novo synthesis was not identifiable with any EAA but was for all NEAA except Pro. Treatment affected the synthesis rate of Asp, Glu, Gly, and Tyr ( $P<0.05$ ).

#### **5.4.4. Amino Acid Fluxes**

Fluxes are reported in Table 6 (EAA) and Supplemental Materials, Table 7 (NEAA). The net uptake per minute ranged from -8.4 for Gly in Trt1 to +6.4  $\mu\text{mol}/\text{min}$  for Ala in Trt2. In general, all essential AA exhibited positive net uptake across treatment except for Phe. In contrast, most NEAA had negative net uptake values for several treatments. Net uptake was affected by treatment for all AA except Asp ( $P<0.05$ ). Unidirectional AA uptake ranged from a low of 0.3  $\mu\text{mol}/\text{min}$  for Met in Trt1 to a high of 82.9  $\mu\text{mol}/\text{min}$  for Ala in Trt4. Most AA exhibited increases in uptake as extracellular concentrations of AA increased. AA uptake was affected by treatment for all AA except Glu ( $P<0.05$ ).

Efflux of AA followed a similar pattern to influx and treatment affected all AA ( $P<0.05$ ). Fast protein synthesis flux was low compared to influx rates and ranged from 0.1 with Met Trt1 to 25.6  $\mu\text{mol}/\text{min}$  for Glu Trt3. Treatment affected fast turnover protein flux of all EAA except Thr and only Asp, Glu, and Pro of the NEAA ( $P<0.05$ ). Transamination fluxes were affected by treatment for only Ile, Leu, Phe, Ala, and Pro ( $P<0.05$ ). Oxidation fluxes differed by treatment

for all AA except Asp and Ser ( $P<0.05$ ). Lastly, the fractional AA synthesis flux was affected by treatment for only Asp, Glu, Gly, and Tyr ( $P<0.05$ ).

#### **5.4.5. Amino Acid Uptake Kinetics**

The results from regressing AA uptake on extracellular concentrations using linear or Michaelis-Menten functions are presented in Table 9. Arg, Pro, and Val had  $k_m$  values that were less than their respective mean in-vivo plasma concentrations. The  $k_m$  for Ile was slightly greater than the mean of in-vivo concentrations. Of the EAA, Val had by far the lowest  $k_m$ , at 49% of mean in-vivo concentrations. The flux data from 11 of the AA were described by the Michaelis-Menten function as well as or better than by the linear function based on RMSE and CCC values. The log-likelihood ratio test indicated the Michaelis-Menten was the more appropriate function for all AA except Asp.

#### **5.4.6. Amino Acid Pool Turnover**

The percent turnover per h for each pool within treatment and AA is listed in Table 8 and Supplemental materials, Table 9. The extracellular pool completely turned over within the 1 h observation period for Thr, Ala, Gly, Pro, Ser, and Tyr for some treatments. Efflux of Met from the cells was not different than 0 for Trt1, and thus the turnover rate was 0 for that AA and treatment. Compared to the other pools, the intracellular pool had the fastest turnover rate across all AA and treatments in general. For some AA, that pool turned over every 2 min (Arg, Trt1) whereas for other AA, it approached 2.3 h (Glu, Trt1). The fast protein turnover pool was slower ranging from 3.4% per h for Gly on Trt3 to 423% per h for Glu on Trt3.

### **5.5. Discussion**

The primary objective of this experiment was to assess cellular AA entry rates given varied extracellular supplies of AA. Intracellular free AA availability partly regulates protein translation (Arriola Apelo et al., 2014d, Sabatini, 2017, Cant et al., 2018), and this availability is

greatly influenced by AA transport. The proportional transfer of AA from blood to intracellular space has been found to be linear within the in vivo range (Hanigan et al., 1992, Hanigan et al., 2002). This infers that when feeding a lower CP diet, intracellular concentrations of EAA will decrease if blood flow remains constant, which should down-regulate translational initiation, and increase uncharged tRNAs. The efficiency of converting absorbed AA into milk protein increases at an increasing rate when protein intake by a lactating dairy cow is decreased (Doepel et al., 2004, Moraes et al., 2018).

If AA uptake is completely regulated by demand, then our experimental model is questionable as milk protein demand was likely substantially different than in vivo. Cells are expected to employ several mechanisms to maintain homeostasis of intracellular AA concentrations within a range and profile to accommodate tRNA needs, cell volume, and metabolic demand purposes (Souba and Pacitti, 1992, Shennan and McNeillie, 1994, Broer and Broer, 2017). Experimental evidence however would suggest cellular uptake of AA in short term experiments occurs independent of intracellular demand. Increasing Ile, Leu, Met, or Thr in mammary tissue slices by 420, 450, 170, and 220  $\mu M$  respectively linearly increased these AA concentrations suggesting that changing concentrations extracellularly translates into increased intracellular AA concentrations (Arriola Apelo et al., 2014a). These increases are larger than the treatment-imposed changes between Trt1 and Trt4. Another experiment with tissue slices increased intracellular Val, Thr, and Phe by 192, 244, and 73  $mM$  between treatments (Appuhamy et al., 2012), also much larger concentration changes than we imposed. Other in-vitro work using amino acid concentrations and profile similar to in-vivo cow observations showed substantial uptake over a 10-min period in mammary tissue slices while achieving steep intracellular gradients (up to 5.3 fold higher than extracellular concentrations) (Pocius and

Baumrucker, 1980). Increasing extracellular Val or Lys for pig mammary tissue explants by 20 mM and 5 mM, respectively, increased intracellular concentrations. Again, these are supraphysiologic extracellular concentrations compared to our imposed treatment changes (Hurley et al., 2000, Jackson et al., 2000). We expected our treatments to elicit changes in intracellular free AA concentrations partially independent of what intracellular protein synthesis demand over the short-term experiment, however, caution should be exercised as general expectation would be that net uptake would be regulated by intracellular demand, not extracellular supplies.

A major advantage of using a cell culture model to explore cellular function is the ability to independently manipulate the cellular environment including extracellular AA concentrations in the absence of confounding factors. The manipulations achieved in the current work would be essentially impossible to achieve *in vivo*. However, significant potential drawbacks exist with cell culture model including the following: 1) the cells were derived from a single source which may or may not be representative of the larger population of dairy cattle, 2) the epithelial cells are removed from the tissue matrix thus eliminating the potential effects of cross-talk with other cell types, 3) there may be systemic control (neural or hormonal) of transport activity that is missing from the culture system, 4) transporter activity may evolve or decay over time in culture, and 4) intracellular metabolism likely does not reflect *in vivo* conditions which may affect transport rates (Souba and Pacitti, 1992). The cellular environment in culture is also different than that of the secretory alveolus where the nutrient supply is adjacent to the basal lateral membrane as opposed to bathing the apical surface in culture (Shennan et al., 1997). Prolactin has been shown to affect expression and activity of System A transporters (Peters and Rillema, 1992), and while prolactin was added to the media, the hormone would primarily be in contact

with the apical membranes at supraphysiologic concentrations rather than the basolateral membrane at in vivo concentrations. Much of the active transport is driven by sodium gradients, and changes in buffering or media pH when new media that is not conditioned is added to cells might lead to transient changes in sodium and potassium gradients independent of AA effects. Lastly, volume regulation is partially regulated by shuttling of amino acids across the cell membranes (Chamberlin and Strange, 1989), which would be independent of extracellular AA concentrations.

To address media pH, osmolarity, and sodium concentrations changing between conditioned and non-conditioned media, using large volumes of media prior to the experimental measurements (12 mL) and changing at 8 h intervals should have minimized those potential problems in our study. Diffusion of large molecules such as prolactin and insulin to the basolateral surface likely is slow, but is overcome by supraphysiologic concentrations present in our media. Diffusion of AA and other small molecules to the basolateral surface would be more rapid and should at least partially replicate diffusion from the capillaries through the extracellular space in vivo. Cellular cross-talk and other potential systemic regulators are not replicated, but this is not necessarily a problem as such effects can be explored with other models given knowledge of isolated epithelial cell responses. Thus, although the culture system is an artificial environment, it can be expected to fairly represent the mechanisms of transport in mammary epithelial cells, and it is likely a close approximation of epithelial cell affinity for each of the AA. However, maximal rates per unit of cell mass are likely less than in vivo due to reduced metabolic use in vitro, and the system will not necessarily be fully representative of the effects of other systemic factors. These potential limitations do not prevent forward progress as the mechanisms and kinetics derived from the in vitro work can be assessed for validity and rescaled

using mammary arterio-venous difference data and integrative modelling techniques (Baldwin, 1995).

### **5.5.1. Amino Acid Uptake Kinetics**

Saturation or near-saturation of uptake within the physiological range is likely for Arg, Pro, and Val, where the  $k_m$  was less than typical in-vivo concentrations. This suggests that transport capacity may become limiting for milk protein synthesis at greater production levels, and that increasing extracellular supplies likely results in diminishing marginal uptake of these AA.

Valine is transported by System L transporters, primarily the sodium independent SLC7A5 and SLC7A8 transporters (Shennan and Boyd, 2014). In a meta-analysis, increasing valine supplies were negatively related to milk protein production (Hanigan et al., 2018). In our study, increasing total AA supplies decreased cellular Val uptake between Trt3 and Trt4, net uptake, and numerically decreased intracellular free Val concentrations. In pig mammary explants, increasing Val to very high concentrations resulted in increased uptake, and the calculated  $k_m$  was 0.64 mM, much higher than typical in-vivo concentrations (Jackson et al., 2000). However, this study to our knowledge, did not include other AA in the media, and thus competition among AA for transporters was not present. The study did separately evaluate competitors and found Leu to strongly inhibit Val uptake as it shares the same transporters as Val, and that physiological concentration of Leu (320  $\mu M$ ) inhibited Val uptake by 47%. In our experiment, all twenty AA were present and up to fourteen AA are known to utilize System L transporters respectively (Wu, 2013a, Shennan and Boyd, 2014), so competition likely occurred, thereby potentially limiting Val uptake and possibly explaining the differences between our study and the prior work with swine explants. The apparent uptake affinity for Val was 10-39%

lower than the uptake affinity for Leu and Ile despite that these AA both use the same transporters. The affinity for Leu by System L transporters has been observed to be greater than for Val, consistent with the observed uptake rate constants herein (Hagenfeldt et al., 1980).

Increasing supplies of Val independent of the other AA would be expected to reduce uptake of other EAA through competitive inhibition, particularly Ile and Leu, which both possess strong anabolic signalling properties (Wolfson and Sabatini, 2017). In lactating sows, increasing dietary Val supplies reduced net mammary lysine uptake indicative of such inhibition (Guan et al., 2002). The lack of positive milk protein responses to branched-chain AA supplementation in dairy cows (Korhonen et al., 2002, Appuhamy et al., 2011b, Kassube et al., 2017) may have occurred because of competition by Val for cellular uptake.

The calculated  $k_m$  of Ile was nearly equal to typical in-vivo concentrations; hence transport may approach saturation at the upper range of in vivo concentrations. Increasing supplies of Leu and Ile resulted in curvilinear responses in casein synthesis and phosphorylation of mTOR related proteins in vitro (Arriola Apelo et al., 2014c, Arriola Apelo et al., 2014d). The declining marginal responses to increasing supplies of Leu and Ile may be due to competition for transport resulting in constant or even declining intracellular AA concentrations of other EAA. In humans, increased intake of EAA mixtures containing low Leu or high Leu proportions resulted in increased arterial Leu supply to the leg (2-3 fold higher vs low Leu), but no change in intracellular Leu concentrations, indicating that membrane transport might be rate limiting (Glynn et al., 2010). Increasing extracellular supplies of Ile decreased mRNA expression of SLCA5 in mammary epithelial cells, which should reduce transport capacity for Val (Zhou et al., 2018). Expression of transporters are integrated with anabolic AA use (Taylor, 2014, Li et al., 2015) and extracellular AA concentrations (Chen et al., 2018), hence, excess supplies of some

AA may downregulate expression of transporters that mediate transport of other AA. Low affinity by the AA transporters as well as less transporters on the membrane driven by the increase in total AA concentration extracellular from 0.36 to 6.24 mM would further precipitate the decline in uptake affinity and might be what occurred with Val.

The saturation of Arg uptake as total AA concentrations increased suggests that it may be a potential limitation for milk protein synthesis as extracellular AA concentrations increase. Arginine transport is sodium independent and is mediated by two different systems: one is specific to cationic AA (System y<sup>+</sup>) and thought to handle the majority of Arg uptake (80%), and the other handles cationic and neutral AA (System y<sup>+</sup>L) (Sharma and Kansal, 2000, Shennan and Boyd, 2014). Increasing dietary protein supplies in lactating sows decreased expression of CAT-2B, a System y<sup>+</sup> transporter, demonstrating adaptive expression (Laspiur et al., 2009). Increasing supplies of Lys also competitively inhibited uptake via System y<sup>+</sup> (Baumrucker, 1984). For the other transport system (system y<sup>+</sup>L), Leu has been shown to be a major competitive inhibitor (Sharma and Kansal, 2000). Because our treatments were applied for a 24 h period prior to measurement of transport, it is possible that the responses were due to a combination of adaptive regulation, i.e. decreased transporter expression, and competitive inhibition from the increased supplies of AA across treatment. Increasing Arg supplies in vitro have been shown to increase casein synthesis and phosphorylation of mTOR related proteins (Ma et al., 2018) and some evidence supports this in-vivo (Tian et al., 2017) and via a meta-analysis (Hanigan et al., 2018). Further research on the effects of high and low protein diets on Arg uptake is needed given its potential role on milk protein synthesis.

Proline is taken up primarily by sodium driven, System A transporters and is also the second most abundant AA in milk protein (Lapierre et al., 2012b, Shennan and Boyd, 2014).

Alanine, Gly, and Ser are all transported by system A, and have been observed to inhibit uptake of Pro by 95 and 87%, respectively (Gay and Hilf, 1980, Zebisch and Brandsch, 2013). Such transport inhibition by Gly and Ser even occurs during amino acid starvation (Gay and Hilf, 1980). The increasing concentrations of Gly and Ser might have competitively inhibited Pro uptake leading to the decline in Pro uptake from Trt2 to Trt4. One of the system A transporters, SNAT2 is adaptively regulated with low concentrations of AA leading to greater expression (Tovar et al., 2000, Gaccioli et al., 2006, Shennan and Boyd, 2014). In mammary tissue explants, amino acid free media increased SNAT2 RNA expression by >25 fold versus a complete media (Lopez et al., 2006). Thus, another possibility is that increased concentrations of AA decreased expression of System A transporters, thereby decreasing Pro uptake. In contrast to Arg and Val, inadequate Pro is taken up by the udder. The milk Pro output to Pro uptake ratio was observed to range from 0.27 to 0.36 (Doepel et al., 2016) (Chapter 6). The needed Pro is synthesized primarily from Arg (Basch et al., 1997) but interestingly the model parameter for Pro synthesis solved to a value of 0 in the current work. Intracellular Pro concentrations were quite high with the ratio to extracellular concentrations ranging from 9 (Trt4) to 28 (Trt1) which perhaps suppressed synthesis.

Amino acid uptake of Ala, Asp, Met, Ser, and Thr appeared to be in the linear range with derived  $k_m$  2-fold or more than typical in-vivo plasma concentrations. Methionine is transported by a wide range of transport systems that are sodium dependent and independent (Shennan and Boyd, 2014). This apparent redundancy perhaps reflects its relative importance to the cell. It has been shown to limit milk protein synthesis under a range of dietary conditions. Perhaps Met being a limiting AA has resulted in the evolution to have various mechanisms for entering the cell and an apparent constant proportional capture of extracellular Met by cells. Methionine

mechanistically activates mTORC1 pathway through 1-carbon pathway and interaction with GATOR1 (Gu et al., 2017) and is always the start codon for protein translation in eukaryotes.

Another EAA that appears to be taken up linearly is Thr. Threonine is not taken up in excess to milk protein needs and dietary shortages of Thr elicit substantial changes in blood flow to maintain supplies (Doepel et al., 2016). Just like Met, Thr possesses the ability to enter the cell via sodium dependent or independent transporters, which might explain the lack of apparent saturation of uptake when Thr supplies are increased. Of the seven EAA we examined in this study, the two EAA with the least uptake saturation compared to in-vivo concentrations lack transporter specificity, i.e. Met and Thr.

Alanine and Ser are also taken up by sodium independent and dependent transporters (Shennan and Boyd, 2014), thereby presenting flexibility for entry into the cell. Aspartic acid is thought to be taken up primarily by System L transport, however, conversion of Asn to Asp in the media could also occur as Asn is transported by sodium dependent transport. Hence, evidence herein supports that AA capable of using multiple transporters possess greater transporter capacity for entry into the cell than AA that can use only a single class of transporters.

### **5.5.2. Amino Acid Pools**

Mass and concentrations of extracellular AA are subject to net flux across the cell membranes just as are intracellular concentrations. Most extracellular AA concentrations changed less over the course of the incubation period as media concentrations were increased. This was driven by greater net AA uptake by cells to maintain homeostasis of intracellular free AA when incubated in lower concentrations of AA, and a smaller pool of extracellular AA to support cellular use. Cells possess the ability to migrate additional transporters to the cell

membrane, change expression of transporters, or post translationally modify transport activity to maintain intracellular concentrations (Lopez et al., 2006, Taylor, 2014). The decline in most EAA reflects the inability of cells to synthesise these AA, and thus use results in a net loss to the extracellular pool. The capture or release of AA by the lysosome varies greatly by AA and storage of most EAA are governed by mTOR and autophagy regulation (Abu-Remaileh et al., 2017). In contrast, storage of most NEAA in the lysosome depend on the V-ATPase pump and are not sensitive to mTOR regulation. Cell storage and demand appears to vary by AA, particularly between EAA and NEAA which would likely dictate observed intracellular free AA concentrations.

The rapid turnover of the extracellular pool for some AA illustrates the substantial bi-directional transport activity occurring. Previous research with tissue slices or cells used isotope movement to quantify net uptake (Pocius and Baumrucker, 1980, Baumrucker, 1984, Hurley et al., 2000, Jackson et al., 2000, Shennan et al., 2002). Efflux was not accounted for in these studies. It was apparent in our results that as extracellular concentrations increased with treatment, efflux for many AA was equal to or exceeded uptake of the AA. The prior studies often calculated uptake for kinetic calculations based on intracellular accumulation of the isotope over a short time period (10-30 min). However, even over such a short time period, the high rates of AA efflux from the cells would have resulted in significant intracellular label being returned to the media leading to underestimates of transport rates. If our results are extrapolated, linear accumulation of AA intracellularly likely wasn't occurring as much as inferred in these previous studies since only the isotopically labelled AA was measured, not the  $^{12}\text{C}$  form of the

AA that was likely being effluxed at a high rate (Pocius and Baumrucker, 1980, Baumrucker, 1984, Hurley et al., 2000, Jackson et al., 2000, Shennan et al., 2002).

The rapid exchange of AA across the cellular membrane resulted in extracellular turnover rates greater than 50%/h for Thr, Ala, Gly, Pro, Ser, and Tyr (Table 8, Supplemental Material, Table 9). Threonine, Ala, and Ser are transported by sodium independent and dependent mechanisms and likely are important for driving the influx of System L AA, hence the rapid turnover, particularly for Ala and Ser. These results draw into question the implied importance of optimal AA ratios relative to milk protein. Transport clearly changes the profile of AA appearing intracellularly and if there is regulatory control of efflux or at the minima mass action, infers strong capability for transport to take varied daily intake of AA and profiles and manipulate it to match intracellularly needs precisely to the tune of what is needed for RNA translation. The reduction in efflux, in some cases to zero (Met) in Trt1, as intracellular AA supplies decline provides a mechanism to increase net uptake independent of extracellular supplies. Efflux is thought to be governed by mass action provided there are adequate transporters on the membrane.

Given the complexity of AA transport and the regulation of some transport activity in response to AA supply and profile, it seems unlikely that a single, unique profile of AA would be adequate to define milk protein responses. The actions of NEAA on the transport of EAA alone would seem to nullify such efforts. If the EAA profile and ratios to key AA such as Met and Lys all depend on a constant supply and profile of NEAA, then clearly they are not tenable given the complete inattention to predictions of NEAA supplies and concentrations. In support of that conclusion, balancing lactating cow diets to precisely achieve a three to one ratio of Lys to Met showed no benefit in milk yield across 14 studies in diets with low CP (Sinclair et al., 2014) and

does not appear to explain animal responses with high confidence (Hanigan et al., 2000). Hence, identification of the most limiting AA by comparing absorbed supplies to milk protein yield is inappropriate as transport modifies the profile which has previously been simulated (Maas et al., 1998).

Increased cell numbers as AA supply increased indicates greater cell proliferation which has previously been observed (Dai et al., 2018) and is reflective of an enhanced anabolic state. Increased cell numbers were reflected in increased total cell weight over the 25-h treatment period, but isolated cell and protein weights were decreased which might suggest decreased cell diameter and volume. Amino acid deprivation, particularly, Leu, induces autophagy via mTORC1 and downstream PAQR3 which greatly reduces cell diameter (Wang et al., 2017). Reduced cell diameter would lead to increased protein concentrations and weight per cell. If such changes are of concern, changes in cell volume and perhaps protein mass might be avoided if the treatment incubation period was shortened, i.e. 1 h. However, such a short period would not allow the effects of altered gene expression to be manifested, and thus a portion of the potential transport responses would be overlooked. The effects of AA deprivation have been demonstrated to increase transcription of transporters (i.e. integrated stress response pathway and ATF4 increased expression) to enhance affinity for channelling AA into the cell (Lopez et al., 2006, Baird and Wek, 2012, Taylor, 2014). Capturing such gene expression changes typically requires at least 4 h with longer times required for proteins that turn over at a slow rate (Cheng et al., 2016). Hence our choice of a 24 h treatment period. However, the downside is that this appears to have resulted in reduced cell numbers and volume for Trt1 which makes interpretation of the results slightly more difficult. Accounting for compartment volume and concentration changes, as was undertaken, accommodates the effects of such changes on transport kinetic

measurements, however, those variables were subject to high measurement error. This likely contributed to the greater error in AA flux estimates for Trt1.

Increasing extracellular AA supplies have previously translated to corresponding increases intracellularly (Pocius and Baumrucker, 1980, Appuhamy et al., 2012, Arriola Apelo et al., 2014c). Clark et al. (1980) previously observed decreased intracellular concentrations of Pro, Ser, Ala, Gly, and Glu in isolated bovine cells subjected to increasing concentrations of media AA. The strong quadratic net uptake response to extracellular AA supply for these NEAA implies potential transport inhibition by other AA, decreased activity of transporters, greater metabolic use, or simply greater efflux. Of the AA that did not exhibit a quadratic increase (Arg, Met, Ser), transport can be facilitated by multiple transporters that are both sodium independent and sodium dependent. The diminishing response in casein synthesis that was observed with increasing Ile, Leu, Met, and Thr concentrations (above 298, 221, 102, and 70  $\mu\text{M}$ ) (Arriola Apelo et al., 2014d) might occur because intracellular supplies of these AA were not increasing at the higher concentrations, which appears to be the case for Ile and Leu in particular. The diminishing response to infused casein (Martineau et al., 2016) likely is partly mediated by lack of a linear increase in intracellular EAA concentrations if our results are extrapolated. The concentrative uptake of EAA and NEAA was obvious as the average ratio between cellular concentrations and media concentrations was 4.2 for NEAA in Trt2 comparing to 19.8 for EAA in Trt2. Cells are known to accumulate certain NEAA (Ala, Gly, and Gln) to function as exchange currency to drive uptake of EAA (Broer and Broer, 2017). The concentration gradients of AA associated with System L transporters (driven by efflux) are thought to be small, which is what we observed (Christensen, 1990). That intracellular demand, particularly milk protein demand would be quite different in-vivo between treatments likely, may

hinder inference in our results since metabolic demand is thought to play a significant role in regulating uptake (Souba and Pacitti, 1992, Cant et al., 2018).

The change in protein-bound AA concentrations appears to be isolated to NEAA with Phe and Thr as the only EAA varying with treatment. Perhaps, as release from lysosomes varies by AA (Abu-Remaileh et al., 2017), the composition of proteins in the fast turnover protein pool are enriched for NEAA supporting the observation of lack of significance in protein bound EAA turn over. The difference could also be due to technique. Acid hydrolysis at a single time point results in bias in AA composition. Ser and Thr are more susceptible to destruction in the harsh acid hydrolysis conditions leading to underestimates of their recovery (Darragh et al., 1996). In contrast, release of BCAA is slow and not generally complete during a 20 h acid hydrolysis, and thus they are also biased. Our results indicate this loss during acid hydrolysis obviously occurred for Ser, Gly, and possibly to some extent Thr. To accommodate loss of this pool, the fast protein pool was varied between 1 and 75% for these AA instead of 1 to 10% to identify optimal model fit. Acid hydrolysis loss of these AA should not have changed the isotope ratio of the pool, only the mass. By using larger percentage of protein bound pool, we achieved good representation of the experimental fast turnover pool size despite a smaller overall pool size caused by acid hydrolysis. Hence, while the AA concentrations reported for several AA in Table 4 are most likely incorrect, establishment of a second pool for the fast turnover portion could still be identified.

The discrepancies between targeted and measured AA concentrations of the treatment media (Supplemental Materials, Table 1 and 2) arise from the uncertainty regarding algae AA concentrations and the challenge of simultaneously achieving desired AA concentration and atom percent enrichment as previously discussed (Chapter 4). The much higher concentrations

of Asp and Glu observed was expected as the  $^{13}\text{C}$  and  $^{15}\text{N}$  enriched algae contain high concentrations of these AA due to complete conversion of Asn and Gln to Asp and Glu during acid hydrolysis. The larger differences in Gly, Ser, Thr, and, to some extent, Val may reflect variation in measured algae concentrations or experimental measurement error. Asparagine, Cys, Gln, and Trp were not measured or isotopically enriched, however these AA were present in the treatment media. Histidine was not enriched in the  $^{15}\text{N}$  treatment media nor in the external tracer for determination of concentration. For Lys, analytical assay variation also led to highly variable results, therefore it will not be discussed. The observed concentrations and corresponding isotope ratios were identified in all twelve pools for 7 EAA and 7 NEAA, enabling modelling of their respective uptake and fluxes.

### **5.5.3. Parameters, Fluxes, and Turnover**

Mass action kinetics of udder AA uptake infer a fixed uptake rate constant regardless of extracellular AA concentrations which for most AA was not observed since the rate constants were affected by treatment. Increased transporter activity occurs by two primary mechanism: increased affinity for the AA usually by a conformational change ( $k_m$  effect) or increased number of transporter proteins by change in protein expression, protein stability, or movement to the membrane ( $V_{\max}$  effect). Mass action uptake rate constants derived in the 12-pool model were generally the greatest for Trt2 and declined for Trt1, Trt3, and Tr4. These results suggest nonlinear transport with respect to concentrations. This was supported by the generally improved fits for a monomolecular model to the flux and concentration data as compared to linear model fits (Table 9). The decrease in affinity for uptake between Trt1 and Trt2 for most AA except Val, Asp, Glu, and Tyr suggests that despite the lowest AA concentrations no further increases in transporter activity were possible. This might infer maximization of transporter expression or conformational changes.

Model fit statistics in nearly all parameters and pools were worse for Trt1, so model error in derivation in uptake rate constant may have also occurred. Nevertheless, our results for Trt2 to Trt4 suggest increased transporter activity which is expected given the ability of cell to increase expression of transporters, change conformation, and migrate them to the membrane (Gaccioli et al., 2006, Lopez et al., 2006, Taylor, 2014, Broer and Broer, 2017). This suggests as AA concentrations exceed in-vivo concentrations, diminished affinity by epithelial cells, thereby leaving increased proportion of AA available for splanchnic catabolism. The decreased efficiency in converting absorbed protein into milk protein as increasing amounts of casein are infused has been previously observed (Whitelaw et al., 1986). Perhaps, it is related to decreased cell uptake of these increased AA supplies.

Changes in efflux rate constant across treatment were less evident but increased efflux affinity was generally observed as AA supplies were increased. Efflux regulation is thought to exhibit mass action kinetics (Christensen, 1990). However, efflux is also a function of influx regulation. If more transporters were recruited to the membrane when extracellular supplies were changed, or increased transporter expression occurred, then an increase in apparent rate of efflux should occur. Maximal efflux activity appeared to occur within Trt2 and Trt3 for system L associated AA which also corresponded with maximal transporter activity for these AA. In contrast, sodium independent AA system A did not follow this trend. Some exhibited maximal efflux activity in Trt4 (Ala, Ser) whereas others had small to no changes across Trt (Gly, Pro).

The fast turnover protein synthesis rate constant was highest for Trt1 compared to the other treatments with most of the EAA (Arg, Ile, Leu, Met, and Val). Autophagy is upregulated when cells are depleted of AA. Autophagy represents a highly conserved mechanism for maintaining intracellular free AA pools (Mizushima, 2011). The high turnover rate with Trt1 for

most EAA likely infers protein turnover has increased and degradation of protein might be occurring. In contrast, Pro was the only NEAA to exhibit a significant increase in fast protein turnover in Trt1. Since NEAA can be synthesized by cells, perhaps the need to enhance turnover and degradation of protein to meet NEAA is less evident than for EAA. The fast protein flux as percent of AA influx was 18% and 159% for EAA and NEAA on average across all AA and treatments. It should be pointed out that the fast protein pool as a proportion of the bound protein varied between 0.5% to 75% for Leu and Ser. Nevertheless, it is evident that protein synthesis to accommodate turnover is sizable and reflects a significant ATP cost to maintain as has been previously shown (Hanigan et al., 2009). It is also interesting to consider that when protein supplies are reduced in a dairy cow, increased turnover may occur to maintain AA supplies for milk protein which would increase ATP demand in the udder.

## **5.6. Conclusion**

Uptake, release, catabolic, anabolic, and protein synthesis fluxes for 14 AA were derived from an experiment with four concentrations of extracellular AA, and used to derive kinetic parameters for AA uptake, release, oxidation, transamination, and protein synthesis in a fast turnover pool. Uptake of EAA did not appear to be subject to regulation; however, the composition of the AA was fixed which may have abrogated the need for regulation. Several NEAA were poorly described by linear or monomolecular models suggesting they were subject to significant regulation. Uptake of Arg, Pro, and Val were close to saturated at the upper end of the in-vivo range having  $k_m$  of 76, 68, and 122  $\mu M$  respectively which are generally below mean in vivo plasma concentrations for each. Alanine, Asp, Met, Ser, and Thr exhibited  $k_m$  that were more than two-fold greater than typical in-vivo concentrations in dairy cows, indicating they exhibit mass action responses in vivo. Increasing extracellular concentrations largely translated to increased intracellular concentrations, however, for most AA a quadratic effect was observed

except for Arg, Gly, Met, and Ser. Cellular efflux, as a proportion of AA uptake, ranged from 0 to 295% demonstrating the major impact of efflux in managing intracellular AA profiles when faced with varying extracellular concentrations or an imbalanced supply. Turnover rates for the extracellular AA pools illustrated the rapid exchange of AA with some pools turning over in 35 min (Ser). Intracellular pool turnover was generally greater with some as short as 2 min (Arg). Thus attempts to fit a static model to data from short duration exposure to isotope will underestimate transport rates. Uptake affinity declined with increased extracellular concentrations demonstrating apparent adaptive uptake and that transport efficiency into the cell will decline when protein supplies are increased. Exploration of the potential of absorbed AA to affect uptake efficiency in-vivo and the potential inhibitory effects of oversupply of Arg and Val are warranted. The complexity of AA transport was evident with varying results by AA and that adaption to changing amino-nitrogen supplies does occur with AA transport. However, in general, assumption of proportional uptake of AA to varying supplies close to in-vivo concentrations appears to be appropriate for most AA. Mass-action kinetics when modelling AA uptake appears adequate.

## 5.7. References

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**Table 5. 1.** Treatment amino acid concentrations ( $\mu M$ ) in the extracellular media

$\mu M$	Treatment AA Concentrations <sup>1</sup>			
	Trt1	Trt2	Trt3	Trt4
Ala	42	268	499	728
Arg	13	80	150	218
Asn	1.2	7.4	14	20
Asp	0.4	2.3	4.2	6.1
Cys	0.4	2.3	4.3	6.3
Glu	8.7	55	102	149
Gln	59	374	695	1014
Gly	57	358	667	972
His	8.2	52	97	141
Ile	17	108	200	292
Leu	26	165	308	449
Lys	9.0	57	106	154
Met	3.7	24	44	64
Phe	8.2	52	96	140
Pro	16	104	194	283
Ser	15	93	173	251
Thr	17	110	205	298
Trp	12	79	147	214
Tyr	9.2	59	109	159
Val	39	250	466	679
Total	360	2300	4280	6240

<sup>1</sup>Treatment AA profiles mimicked typical lactating dairy cows plasma and concentrations were varied being 16% of in-vivo in Trt1 (0.36 mM), 100% of in-vivo Trt2 (2.30 mM), 186% of in-vivo Trt3 (4.28 mM), and 271% of in-vivo Tr4 (6.24 mM).

**Table 5. 2.** Effect of treatment and time on extracellular free amino acid concentrations

uM <sup>2</sup>	Treatment <sup>1</sup>				SEM	P-value			
	Trt1	Trt2	Trt3	Trt4		Treatment	Time	Linear <sup>3</sup>	Quadratic <sup>3</sup>
Ala	52 <sup>d</sup>	286 <sup>c</sup>	498 <sup>b</sup>	650 <sup>a</sup>	5.6	<0.001	0.002	<0.001	<0.001
Arg	10 <sup>d</sup>	66 <sup>c</sup>	124 <sup>b</sup>	180 <sup>a</sup>	2.5	<0.001	<0.001	<0.001	0.98
Asp	4 <sup>d</sup>	21 <sup>c</sup>	40 <sup>b</sup>	51 <sup>a</sup>	1.8	<0.001	<0.001	<0.001	0.06
Glu	12 <sup>d</sup>	102 <sup>c</sup>	163 <sup>b</sup>	201 <sup>a</sup>	2.2	<0.001	<0.001	<0.001	<0.001
Gly	159 <sup>d</sup>	349 <sup>c</sup>	497 <sup>b</sup>	604 <sup>a</sup>	4.6	<0.001	<0.001	<0.001	<0.001
Ile	18 <sup>d</sup>	114 <sup>c</sup>	198 <sup>b</sup>	264 <sup>a</sup>	2.2	<0.001	<0.001	<0.001	<0.001
Leu	40 <sup>d</sup>	181 <sup>c</sup>	313 <sup>b</sup>	415 <sup>a</sup>	3.4	<0.001	<0.001	<0.001	<0.001
Met	2 <sup>d</sup>	19 <sup>c</sup>	36 <sup>b</sup>	49 <sup>a</sup>	0.6	<0.001	<0.001	<0.001	0.01
Phe	18 <sup>d</sup>	51 <sup>c</sup>	78 <sup>b</sup>	92 <sup>a</sup>	1.6	<0.001	<0.001	<0.001	<0.001
Pro	21 <sup>d</sup>	119 <sup>c</sup>	192 <sup>b</sup>	246 <sup>a</sup>	2.2	<0.001	0.006	<0.001	<0.001
Ser	31 <sup>d</sup>	136 <sup>c</sup>	220 <sup>b</sup>	274 <sup>a</sup>	5.0	<0.001	0.85	<0.001	<0.001
Thr	45 <sup>d</sup>	161 <sup>c</sup>	253 <sup>b</sup>	319 <sup>a</sup>	2.5	<0.001	0.002	<0.001	<0.001
Tyr	7 <sup>d</sup>	38 <sup>c</sup>	66 <sup>b</sup>	87 <sup>a</sup>	0.8	<0.001	0.001	<0.001	<0.001
Val	49 <sup>d</sup>	256 <sup>c</sup>	445 <sup>b</sup>	586 <sup>a</sup>	4.6	<0.001	0.002	<0.001	<0.001

<sup>a-d</sup>Means within a row with different superscripts differ ( $P<0.01$ ).

<sup>1</sup>Treatment: Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM).

<sup>2</sup>Concentration includes <sup>12</sup>C, <sup>13</sup>C, and <sup>15</sup>N mass of each AA.

<sup>3</sup>Linear and quadratic contrasts of treatment AA concentrations effects.

**Table 5. 3.** Effect of treatment and time on intracellular free amino acid concentrations

$\mu M^2$	Treatment <sup>1</sup>				SEM	P-value			
	Trt1	Trt2	Trt3	Trt4		Treatment	Time	Linear <sup>3</sup>	Quadratic <sup>3</sup>
Ala	772 <sup>b</sup>	3051 <sup>a</sup>	3350 <sup>a</sup>	3416 <sup>a</sup>	221	<0.001	0.84	<0.001	<0.001
Arg	5 <sup>c</sup>	46 <sup>b</sup>	87 <sup>a</sup>	102 <sup>a</sup>	19	<0.001	0.51	<0.001	0.45
Asp	231 <sup>b</sup>	702 <sup>a</sup>	501 <sup>b</sup>	547 <sup>b</sup>	66	<0.001	<0.001	0.009	0.001
Glu	4265 <sup>b</sup>	5183 <sup>a</sup>	4670 <sup>ab</sup>	4649 <sup>ab</sup>	419	0.55	0.12	0.73	0.25
Gly	3457 <sup>a</sup>	3200 <sup>ab</sup>	2842 <sup>b</sup>	2751 <sup>b</sup>	240	0.11	0.22	0.02	0.72
Ile	69 <sup>c</sup>	387 <sup>b</sup>	457 <sup>a</sup>	464 <sup>a</sup>	32	<0.001	0.36	<0.001	<0.001
Leu	147 <sup>c</sup>	575 <sup>b</sup>	666 <sup>a</sup>	674 <sup>a</sup>	45	<0.001	0.59	<0.001	<0.001
Met	14 <sup>d</sup>	81 <sup>c</sup>	141 <sup>b</sup>	175 <sup>a</sup>	15	<0.001	0.07	<0.001	0.22
Phe	215 <sup>b</sup>	362 <sup>a</sup>	327 <sup>a</sup>	309 <sup>a</sup>	28	0.004	0.67	0.05	0.004
Pro	584 <sup>b</sup>	2448 <sup>a</sup>	2372 <sup>a</sup>	2286 <sup>a</sup>	181	<0.001	0.09	<0.001	<0.001
Ser	533 <sup>c</sup>	896 <sup>b</sup>	904 <sup>b</sup>	1158 <sup>a</sup>	81	<0.001	0.03	<0.001	0.49
Thr	440 <sup>c</sup>	1104 <sup>b</sup>	1220 <sup>ab</sup>	1319 <sup>a</sup>	87	<0.001	0.88	<0.001	0.002
Tyr	141 <sup>b</sup>	306 <sup>a</sup>	311 <sup>a</sup>	313 <sup>a</sup>	26	<0.001	0.09	<0.001	0.002
Val	188 <sup>b</sup>	967 <sup>a</sup>	1056 <sup>a</sup>	981 <sup>a</sup>	72	<0.001	0.15	<0.001	<0.001

<sup>a-d</sup>Means within a row with different superscripts differ ( $P<0.01$ ).

<sup>1</sup>Treatment: Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM).

<sup>2</sup>Concentration includes <sup>12</sup>C, <sup>13</sup>C, and <sup>15</sup>N mass of each AA.

<sup>3</sup>Linear and quadratic contrasts of treatment AA concentrations effects.

**Table 5. 4.** Effect of treatment on intracellular protein bound amino acid concentrations

mM <sup>2</sup>	Treatment <sup>1</sup>				SEM	P-value
	Trt#1	Trt#2	Trt#3	Trt#4		Trt
Ala	38 <sup>a</sup>	27 <sup>b</sup>	24 <sup>c</sup>	24 <sup>c</sup>	0.8	<0.001
Arg	20	20	18	19	1.5	0.48
Asp	92 <sup>a</sup>	74 <sup>b</sup>	69 <sup>c</sup>	67 <sup>c</sup>	1.7	<0.001
Glu	153 <sup>a</sup>	144 <sup>b</sup>	141 <sup>b</sup>	135 <sup>c</sup>	3.0	0.001
Gly	6 <sup>a</sup>	5 <sup>b</sup>	4 <sup>c</sup>	4 <sup>c</sup>	0.2	<0.001
Ile	39	38	38	36	0.9	0.33
Leu	82	80	82	77	3.1	0.70
Met	16	16	15	15	0.7	0.59
Phe	37 <sup>a</sup>	37 <sup>a</sup>	36 <sup>ab</sup>	34 <sup>b</sup>	0.7	0.02
Pro	31	29	29	28	1.0	0.24
Ser	11 <sup>a</sup>	8 <sup>b</sup>	7 <sup>b</sup>	7 <sup>b</sup>	0.4	<0.001
Thr	21 <sup>a</sup>	15 <sup>b</sup>	13 <sup>c</sup>	13 <sup>c</sup>	0.5	<0.001
Tyr	10 <sup>a</sup>	6 <sup>b</sup>	6 <sup>b</sup>	6 <sup>b</sup>	0.2	<0.001
Val	44	44	43	42	1.1	0.47

<sup>1</sup>Treatment: Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM).

<sup>2</sup>Concentration includes <sup>12</sup>C, <sup>13</sup>C, and <sup>15</sup>N mass of each AA.

**Table 5. 5.** Essential amino acid parameter estimates (min<sup>-1</sup>) derived by fitting the model to the observed treatment data<sup>1</sup>

Item <sup>2</sup>	Treatment, min <sup>-1</sup>											
	Trt #1			Trt #2			Trt #3			Trt #4		
	Estimate	95% CI <sup>3</sup>	CV	Estimate	95% CI <sup>3</sup>	CV	Estimate	95% CI <sup>3</sup>	CV	Estimate	95% CI <sup>3</sup>	CV
<b>Arg</b>												
kxAAAnAA	0.009 <sup>ab</sup>	0.002-0.015	37.5	0.012 <sup>b</sup>	0.010-0.015	10.8	0.011 <sup>b</sup>	0.010-0.013	7.6	0.006 <sup>a</sup>	0.005-0.007	12.2
knAAxAA	0.070 <sup>ab</sup>	0-0.266	142.8	0.095 <sup>ab</sup>	0.031-0.159	34.2	0.118 <sup>b</sup>	0.089-0.146	12.4	0.034 <sup>a</sup>	0-0.070	55.9
knAAOx	0.148 <sup>ab</sup>	0.049-0.246	34.1	0.151 <sup>b</sup>	0.125-0.177	8.7	0.143 <sup>b</sup>	0.121-0.165	7.8	0.100 <sup>a</sup>	0.082-0.119	9.4
knAAAnKA				0.029	0.013-0.045	27.8	0.023	0.012-0.035	25.8	0.005	0-0.013	87.7
knAAAtfAA	0.290 <sup>c</sup>	0.248-0.333	7.5	0.038 <sup>b</sup>	0.032-0.043	7.8	0.014 <sup>a</sup>	0.012-0.017	9.2	0.014 <sup>a</sup>	0.012-0.016	6.9
<b>Ile</b>												
kxAAAnAA	0.012 <sup>ab</sup>	0.009-0.015	11.6	0.016 <sup>c</sup>	0.015-0.017	4.2	0.011 <sup>b</sup>	0.010-0.013	6.7	0.007 <sup>a</sup>	0.005-0.009	13.1
knAAxAA	0.012 <sup>a</sup>	0.001-0.023	47.8	0.064 <sup>b</sup>	0.063-0.064	0.5	0.073 <sup>c</sup>	0.072-0.073	0.5	0.063 <sup>b</sup>	0.063-0.064	0.4
knAAOx	0.049 <sup>b</sup>	0.040-0.058	9.4	0.036 <sup>ab</sup>	0.030-0.042	8.7	0.030 <sup>a</sup>	0.023-0.036	11.1	0.024 <sup>a</sup>	0.017-0.031	15.1
knAAAnKA				0.008 <sup>a</sup>	0.007-0.009	8.0	0.010 <sup>b</sup>	0.010-0.011	3.9	0.008 <sup>a</sup>	0.008-0.009	4.1
knAAAtfAA	0.021 <sup>b</sup>	0.019-0.023	4.8	0.007 <sup>a</sup>	0.004-0.011	21.3	0.007 <sup>a</sup>	0.003-0.010	25.6	0.006 <sup>a</sup>	0.001-0.011	39.8
<b>Leu</b>												
kxAAAnAA	0.012 <sup>ab</sup>	0.008-0.015	13.6	0.018 <sup>bc</sup>	0.016-0.020	6.1	0.015 <sup>b</sup>	0.014-0.016	3.4	0.010 <sup>a</sup>	0.010-0.011	3.7
knAAxAA	0.018 <sup>a</sup>	0.002-0.034	45.9	0.087 <sup>b</sup>	0.076-0.097	6.1	0.113 <sup>c</sup>	0.106-0.121	3.3	0.101 <sup>bc</sup>	0.093-0.110	4.2
knAAOx	0.037 <sup>bc</sup>	0.031-0.043	8.6	0.033 <sup>b</sup>	0.029-0.036	4.9	0.027 <sup>ab</sup>	0.024-0.030	5.3	0.022 <sup>a</sup>	0.018-0.026	9.0
knAAAnKA				0.008	0.007-0.010	8.7	0.010	0.008-0.011	5.8	0.008	0.007-0.009	7.9
knAAAtfAA	0.024 <sup>bc</sup>	0.021-0.026	5.1	0.019 <sup>b</sup>	0.015-0.023	9.8	0.015 <sup>ab</sup>	0.013-0.017	7.1	0.013 <sup>a</sup>	0.012-0.014	4.7
<b>Met</b>												
kxAAAnAA	0.008 <sup>a</sup>	0.007-0.009	7.1	0.018 <sup>c</sup>	0.016-0.020	6.3	0.014 <sup>bc</sup>	0.012-0.016	5.8	0.011 <sup>b</sup>	0.010-0.012	6.0
knAAxAA				0.018 <sup>a</sup>	0.013-0.022	13.4	0.040 <sup>b</sup>	0.033-0.047	9.0	0.041 <sup>b</sup>	0.034-0.048	8.6
knAAOx	0.040 <sup>c</sup>	0.023-0.058	22.0	0.013 <sup>b</sup>	0.009-0.017	13.9	0.003 <sup>a</sup>	0.001-0.006	41.5			
knAAAnKA	0.012	0-0.024	55.8	0.008	0.005-0.011	18.1	0.003	0.002-0.005	25.5	0.003	0.002-0.005	24.7
knAAAtfAA	0.034 <sup>c</sup>	0.024-0.043	14.7	0.014 <sup>b</sup>	0.013-0.016	5.9	0.009 <sup>a</sup>	0.008-0.010	5.1	0.007 <sup>a</sup>	0.007-0.008	4.5
<b>Phe</b>												
kxAAAnAA	0.007 <sup>a</sup>	0.002-0.013	40.4	0.028 <sup>b</sup>	0.021-0.034	11.8	0.028 <sup>b</sup>	0.023-0.034	10.2	0.014 <sup>a</sup>	0.011-0.016	8.9
knAAxAA	0.025 <sup>a</sup>	0-0.054	59.4	0.121 <sup>b</sup>	0.104-0.138	7.3	0.152 <sup>b</sup>	0.132-0.173	7.0	0.158 <sup>b</sup>	0.136-0.179	6.9

knAAnKA	0.001 <sup>a</sup>	0.001-0.002	25.8	0.004 <sup>b</sup>	0.003-0.005	13.6	0.002 <sup>ab</sup>	0.002-0.003	12.4	0.002 <sup>a</sup>	0.001-0.002	15.0
knAAtfAA	0.009 <sup>a</sup>	0.009-0.010	3.4	0.010 <sup>a</sup>	0.009-0.011	7.0	0.011 <sup>ab</sup>	0.010-0.012	5.4	0.014 <sup>b</sup>	0.012-0.016	7.2
Thr												
kxAAnAA	0.004 <sup>a</sup>	0.003-0.005	13.0	0.030 <sup>c</sup>	0.024-0.035	8.9	0.030 <sup>c</sup>	0.025-0.034	7.5	0.020 <sup>b</sup>	0.018-0.022	5.3
knAAxAA	0.006 <sup>a</sup>	0.003-0.008	22.9	0.087 <sup>b</sup>	0.070-0.104	10.1	0.119 <sup>b</sup>	0.101-0.137	7.7	0.093 <sup>b</sup>	0.083-0.103	5.4
knAAOx				0.011 <sup>b</sup>	0.008-0.013	10.5	0.005 <sup>a</sup>	0.003-0.006	18.5			
knAAnKA	0.001	0.000-0.002	45.8	0.000	0.000-0.001	34.0						
knAAtfAA	0.002 <sup>b</sup>	0.002-0.002	10.3	0.001 <sup>a</sup>	0.001-0.001	13.8	0.001 <sup>a</sup>	0.000-0.001	18.5	0.001 <sup>a</sup>	0.001-0.001	15.6
Val												
kxAAnAA	0.010 <sup>bc</sup>	0.005-0.016	26.7	0.010 <sup>c</sup>	0.010-0.011	4.3	0.006 <sup>b</sup>	0.006-0.007	4.0	0.004 <sup>a</sup>	0.003-0.004	5.3
knAAxAA	0.024 <sup>ab</sup>	0-0.054	63.4	0.045 <sup>b</sup>	0.039-0.050	6.1	0.042 <sup>ab</sup>	0.038-0.045	4.8	0.033 <sup>a</sup>	0.029-0.038	6.9
knAAOx	0.033 <sup>c</sup>	0.027-0.039	9.5	0.023 <sup>b</sup>	0.020-0.026	6.4	0.017 <sup>ab</sup>	0.015-0.020	7.3	0.013 <sup>a</sup>	0.010-0.017	12.2
knAAtfAA	0.009 <sup>b</sup>	0.007-0.010	7.9	0.004 <sup>a</sup>	0.004-0.005	6.2	0.004 <sup>a</sup>	0.004-0.004	5.8	0.004 <sup>a</sup>	0.003-0.004	5.1

<sup>a-d</sup>Means within a row with different superscripts differ ( $P<0.01$ ).

<sup>1</sup>Treatment: Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM).

<sup>2</sup>Rate constants: knAAOx=oxidation, knAAtfAA=fast protein turnover, knAAAtsAA=slow protein turnover, knAAxAA=efflux, kxAAnAA=uptake.

<sup>3</sup>95% CI derived from Markov Chain Monte Carlo simulation (n=5000 runs).

**Table 5. 6.** Essential amino acid flux predictions derived from the parameterized model and the treatment data<sup>1</sup>

Item	Amino Acid Fluxes, $\mu\text{M}/\text{min}^2$																	
	Net Uptake			Uptake			Exit			Turnover			Transamination			Oxidation		
	Mean	5%	95%	Mean	5%	95%	Mean	5%	95%	Mean	5%	95%	Mean	5%	95%	Mean	5%	95%
<b>Arg</b>																		
Trt1	0.4 <sup>a</sup>	0.3	0.4	0.5 <sup>a</sup>	0.2	0.8	0.1 <sup>a</sup>	0.0	0.4	0.6 <sup>ab</sup>	0.5	0.6				0.3 <sup>a</sup>	0.2	0.3
Trt2	3.0 <sup>b</sup>	2.8	3.2	4.7 <sup>b</sup>	3.8	5.6	1.7 <sup>b</sup>	0.9	2.6	0.7 <sup>b</sup>	0.6	0.7	0.5	0.3	0.7	2.7 <sup>b</sup>	2.5	2.9
Trt3	4.3 <sup>c</sup>	3.9	4.6	7.6 <sup>c</sup>	6.7	8.6	3.4 <sup>c</sup>	2.7	4.1	0.4 <sup>a</sup>	0.3	0.5	0.7	0.3	1.0	4.1 <sup>c</sup>	3.7	4.5
Trt4	4.5 <sup>c</sup>	3.9	5.1	5.8 <sup>bc</sup>	4.6	7.1	1.4 <sup>ab</sup>	0.1	2.7	0.6 <sup>ab</sup>	0.5	0.6	0.2	-0.1	0.5	4.1 <sup>c</sup>	3.4	4.7
<b>Ile</b>																		
Trt1	1.0 <sup>a</sup>	0.9	1.1	1.2 <sup>a</sup>	1.0	1.4	0.2 <sup>a</sup>	0.0	0.4	0.4 <sup>a</sup>	0.3	0.4				0.8 <sup>a</sup>	0.7	0.9
Trt2	3.6 <sup>c</sup>	3.5	3.8	10.3 <sup>b</sup>	9.6	11.1	6.7 <sup>b</sup>	6.1	7.3	0.8 <sup>b</sup>	0.7	0.8	0.8 <sup>a</sup>	0.7	1.0	3.8 <sup>b</sup>	3.6	4.0
Trt3	3.6 <sup>c</sup>	3.4	3.9	13.4 <sup>c</sup>	12.5	14.3	9.8 <sup>c</sup>	9.0	10.5	0.9 <sup>b</sup>	0.8	1.0	1.4 <sup>b</sup>	1.2	1.6	4.0 <sup>b</sup>	3.7	4.3
Trt4	2.8 <sup>b</sup>	2.4	3.3	11.8 <sup>bc</sup>	10.8	12.8	9.0 <sup>c</sup>	8.3	9.7	0.8 <sup>b</sup>	0.8	0.9	1.2 <sup>ab</sup>	0.9	1.4	3.4 <sup>b</sup>	2.9	3.9
<b>Leu</b>																		
Trt1	1.7 <sup>a</sup>	1.6	1.9	2.4 <sup>a</sup>	1.8	3.0	0.6 <sup>a</sup>	0.1	1.2	0.9 <sup>a</sup>	0.8	0.9				1.3 <sup>a</sup>	1.1	1.5
Trt2	5.2 <sup>b</sup>	4.9	5.4	18.8 <sup>b</sup>	16.9	20.7	13.6 <sup>b</sup>	11.8	15.4	3.0 <sup>b</sup>	2.5	3.5	1.3 <sup>a</sup>	1.1	1.5	5.1 <sup>b</sup>	4.8	5.4
Trt3	5.3 <sup>b</sup>	4.9	5.7	28.0 <sup>c</sup>	26.3	29.6	22.6 <sup>c</sup>	21.1	24.2	3.0 <sup>b</sup>	2.6	3.3	1.9 <sup>b</sup>	1.7	2.1	5.4 <sup>b</sup>	5.0	5.8
Trt4	4.4 <sup>b</sup>	3.8	5.1	26.1 <sup>c</sup>	24.4	27.8	21.7 <sup>c</sup>	20.1	23.3	2.7 <sup>b</sup>	2.5	3.0	1.7 <sup>ab</sup>	1.4	1.9	4.8 <sup>b</sup>	4.1	5.4
<b>Met</b>																		
Trt1	0.3 <sup>a</sup>	0.2	0.3	0.3 <sup>a</sup>	0.2	0.3				0.1 <sup>a</sup>	0.1	0.2	0.0	0.0	0.1	0.2 <sup>a</sup>	0.1	0.2
Trt2	0.8 <sup>c</sup>	0.8	0.9	1.4 <sup>b</sup>	1.2	1.5	0.5 <sup>a</sup>	0.4	0.6	0.4 <sup>b</sup>	0.4	0.4	0.2	0.1	0.3	0.4 <sup>b</sup>	0.3	0.4
Trt3	0.6 <sup>b</sup>	0.5	0.7	2.6 <sup>c</sup>	2.3	2.8	2.0 <sup>b</sup>	1.7	2.3	0.4 <sup>b</sup>	0.4	0.5	0.2	0.1	0.3	0.1 <sup>a</sup>	0.0	0.3
Trt4	0.4 <sup>ab</sup>	0.3	0.5	2.7 <sup>c</sup>	2.3	3.0	2.3 <sup>b</sup>	1.9	2.6	0.4 <sup>b</sup>	0.4	0.4	0.2	0.1	0.3			
<b>Phe</b>																		
Trt1	0.0 <sup>b</sup>	-0.1	0.0	1.1 <sup>a</sup>	0.2	2.0	1.2 <sup>a</sup>	0.3	2.1	0.4 <sup>a</sup>	0.4	0.5	0.0 <sup>a</sup>	0.0	0.1			
Trt2	-0.5 <sup>a</sup>	-0.6	-0.3	8.0 <sup>b</sup>	6.6	9.5	8.5 <sup>b</sup>	7.1	9.9	0.7 <sup>b</sup>	0.7	0.8	0.3 <sup>b</sup>	0.2	0.3			

Trt3	-0.2 <sup>ab</sup>	-0.4	-0.1	10.5 <sup>b</sup>	8.7	12.2	10.7 <sup>b</sup>	9.0	12.4	0.8 <sup>b</sup>	0.7	0.8	0.2 <sup>ab</sup>	0.1	0.2			
Trt4	-0.5 <sup>a</sup>	-0.6	-0.4	7.9 <sup>b</sup>	6.6	9.1	8.4 <sup>b</sup>	7.2	9.5	0.7 <sup>b</sup>	0.7	0.8	0.1 <sup>a</sup>	0.1	0.1			
Thr																		
Trt1	0.4 <sup>b</sup>	0.3	0.5	1.0 <sup>a</sup>	0.8	1.3	0.6 <sup>a</sup>	0.4	0.9	0.2	0.2	0.3	0.1	0.0	0.2			
Trt2	2.2 <sup>d</sup>	1.9	2.4	27.7 <sup>b</sup>	23.3	32.2	25.6 <sup>b</sup>	21.1	30.0	0.2	0.2	0.3	0.1	0.0	0.2	3.1 <sup>b</sup>	2.7	3.6
Trt3	1.0 <sup>c</sup>	0.6	1.4	44.9 <sup>c</sup>	38.7	51.1	43.9 <sup>c</sup>	37.8	50.0	0.3	0.2	0.4				1.7 <sup>a</sup>	1.2	2.2
Trt4	-0.9 <sup>a</sup>	-1.1	-0.6	39.3 <sup>c</sup>	35.5	43.2	40.2 <sup>c</sup>	36.4	44.1	0.3	0.2	0.4					0.0	0.0
Val																		
Trt1	1.6 <sup>a</sup>	1.4	1.8	2.8 <sup>a</sup>	1.4	4.1	1.1 <sup>a</sup>	-0.2	2.5	0.4 <sup>a</sup>	0.3	0.5				1.6 <sup>a</sup>	1.3	1.8
Trt2	4.3 <sup>bc</sup>	4.0	4.6	15.6 <sup>c</sup>	14.4	16.8	11.3 <sup>b</sup>	10.3	12.3	1.0 <sup>b</sup>	0.9	1.1				5.8 <sup>c</sup>	5.4	6.2
Trt3	3.5 <sup>b</sup>	3.0	4.0	16.1 <sup>c</sup>	14.9	17.2	12.5 <sup>b</sup>	11.6	13.5	1.2 <sup>b</sup>	1.1	1.3				5.2 <sup>bc</sup>	4.7	5.8
Trt4	2.4 <sup>ab</sup>	1.7	3.2	12.8 <sup>b</sup>	11.6	14.0	10.3 <sup>b</sup>	9.3	11.4	1.2 <sup>b</sup>	1.1	1.3				4.1 <sup>b</sup>	3.3	5.0

<sup>1</sup>Treatment: Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM).

<sup>2</sup>Average flux over 60 minutes using derived rate constants and experimental data

<sup>3</sup>5% and 95% confidence interval standard error represents the standard deviation of 1000 simulated model runs conducted using randomly drawn parameters from Markov Chain Monte Carlo derived parameter posteriors.

**Table 5. 7.** Evaluations of predictions of essential amino acid model isotope ratios after the model was fit by treatment to the observed data<sup>1</sup>

Item	Statistic <sup>2</sup>	Isotope Ratios <sup>3</sup>					
		E <sub>nAA</sub> <sup>13C</sup>	E <sub>xAA</sub> <sup>13C</sup>	E <sub>nAA</sub> <sup>15N</sup>	E <sub>xAA</sub> <sup>15N</sup>	E <sub>tAA</sub> <sup>13C</sup>	E <sub>tAA</sub> <sup>15N</sup>
Arg							
Trt1	CCC	0.48	0.48	0.23	0.14	0.80	-0.13
	RMSE	22.2	9.1	26.3	34.5	34.5	4.1
Trt2	CCC	0.67	0.78	0.85	0.38	0.89	-0.06
	RMSE	16.6	2.7	31.2	26.7	15.7	5.9
Trt3	CCC	0.25	0.68	0.55	0.86	0.92	0.01
	RMSE	29.0	1.8	92.9	10.3	9.5	6.6
Trt4	CCC	0.87	0.14	0.22	0.03	0.97	-0.01
	RMSE	23.7	2.5	95.9	96.1	5.9	6.8
Ile							
Trt1	CCC	0.35	-0.11	0.84	0.23	0.98	0.00
	RMSE	14.8	18.5	6.9	8.2	12.8	4.9
Trt2	CCC	0.98	0.96	0.96	0.99	0.98	0.03
	RMSE	10.7	3.0	11.1	2.7	13.7	4.9
Trt3	CCC	0.99	0.97	0.98	0.99	0.98	0.04
	RMSE	8.4	2.0	8.3	2.5	15.5	3.8
Trt#4	CCC	0.98	0.96	0.96	0.99	0.99	0.03
	RMSE	12.2	1.7	12.4	2.6	12.5	3.3
Leu							
Trt1	CCC	0.16	-0.20	0.92	0.33	0.86	0.00
	RMSE	13.4	15.5	6.9	13.4	39.5	23.3
Trt2	CCC	0.98	0.97	0.98	0.98	0.94	0.00
	RMSE	9.7	2.9	8.3	4.0	29.1	15.9
Trt3	CCC	1.00	0.97	0.99	0.99	0.98	0.01
	RMSE	5.3	2.3	5.6	2.7	15.8	12.0
Trt4	CCC	0.99	0.98	0.96	0.99	0.98	0.02
	RMSE	9.6	1.6	11.9	2.4	14.1	12.6
Met							
Trt1	CCC	0.47	0.00	0.57	0.00	0.94	-0.08
	RMSE	33.1	19.6	22.6	38.5	9.1	0.9
Trt2	CCC	0.13	0.77	0.86	0.76	0.99	0.04
	RMSE	29.7	6.2	22.4	12.1	6.7	1.7
Trt3	CCC	0.95	0.86	0.92	0.82	0.98	0.01
	RMSE	17.2	6.1	10.9	13.2	7.3	2.4
Trt4	CCC	0.98	0.85	0.84	0.83	0.99	-0.02

	RMSE	13.6	5.7	16.7	13.6	4.7	3.1
Phe							
Trt1	CCC	0.48	0.86	0.59	0.44	0.99	0.00
	RMSE	44.0	50.0	4.6	7.1	8.4	1.2
Trt2	CCC	0.95	0.96	0.72	0.89	0.94	0.01
	RMSE	21.2	29.8	13.8	18.3	26.5	1.9
Trt3	CCC	0.95	0.96	0.90	0.92	0.95	0.00
	RMSE	21.5	24.7	8.2	18.2	25.6	4.2
Trt4	CCC	0.97	0.96	0.52	0.94	0.97	0.00
	RMSE	18.9	23.7	25.8	15.1	20.1	4.4
Thr							
Trt1	CCC	0.43	0.37	0.27	0.46	0.88	0.00
	RMSE	17.3	14.1	5.2	8.8	0.9	0.6
Trt2	CCC	0.82	0.97	0.77	0.80	0.84	0.00
	RMSE	30.4	3.4	2.8	1.2	1.3	1.1
Trt3	CCC	0.94	0.98	0.97	0.89	0.78	0.00
	RMSE	17.1	2.7	1.6	1.7	1.8	2.0
Trt4	CCC	0.98	0.99	0.93	0.96	0.79	-0.01
	RMSE	10.4	1.7	2.7	1.0	1.9	2.1
Val							
Trt1	CCC	0.47	-0.09	0.83	0.52	0.92	0.00
	RMSE	16.0	21.7	8.5	5.5	11.2	6.7
Trt2	CCC	0.96	0.89	0.96	0.98	0.94	0.03
	RMSE	17.4	2.2	10.8	3.0	10.4	5.5
Trt3	CCC	0.99	0.88	0.96	0.98	0.97	0.05
	RMSE	6.0	1.5	11.5	2.7	6.4	4.2
Trt4	CCC	0.98	0.70	0.94	0.95	0.97	0.05
	RMSE	9.9	1.8	13.9	3.2	6.5	3.6

<sup>1</sup>Treatment: Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM).

<sup>2</sup>Test statistic: RMSE=root mean square prediction error, CCC=concordance correlation coefficient.

<sup>3</sup>Isotope ratios:  $E_{nAA}^{13C}$ =<sup>13</sup>C/<sup>12</sup>C area ratio of free intracellular AA,  $E_{xAA}^{13C}$ =<sup>13</sup>C/<sup>12</sup>C area ratio of free media AA,  $E_{nAA}^{15N}$ =<sup>15</sup>N/<sup>12</sup>C area ratio of free intracellular AA,  $E_{xAA}^{15N}$ =<sup>15</sup>N/<sup>12</sup>C area ratio of free media AA,  $E_{tAA}^{13C}$ =<sup>13</sup>C/<sup>12</sup>C area ratio of protein bound AA, and  $E_{tAA}^{15N}$ =<sup>15</sup>N/<sup>12</sup>C area ratio of protein bound AA.

**Table 5. 8.** Turnover of media, intracellular free, and intracellular protein bound essential amino acid pools in response to treatment<sup>1</sup>

	Treatment Pool Mass Turnover, % / hour			
	Trt1	Trt2	Trt3	Trt4
Arg				
Extracellular Free AA	14.2	27.1	29.8	8.4
Intracellular Free AA	3080	1774	1685	937
Fast Protein Bound AA	21.4	18.7	10.2	15.7
Ile				
Extracellular Free AA	12.2	62.6	50.3	33.8
Intracellular Free AA	471	619	627	525
Fast Protein Bound AA	27.9	44.1	48.2	43.8
Leu				
Extracellular Free AA	18.7	79.2	73.8	51.5
Intracellular Free AA	463	810	915	796
Fast Protein Bound AA	69.0	179.2	168.2	159.8
Met				
Extracellular Free AA		40.5	64.7	55.3
Intracellular Free AA	499	360	351	325
Fast Protein Bound AA	19.6	46.8	55.6	51.5
Phe				
Extracellular Free AA	45.5	176.9	174.4	88.5
Intracellular Free AA	171	712	920	921
Fast Protein Bound AA	9.3	7.2	5.9	5.1
Thr				
Extracellular Free AA	14.3	163.9	173.6	122.4
Intracellular Free AA	62.9	568	733	547
Fast Protein Bound AA	8.8	9.0	12.1	14.1
Val				
Extracellular Free AA	25.7	45.3	28.3	17.2
Intracellular Free AA	383	394	343	271
Fast Protein Bound AA	29.1	52.2	58.2	56.1

<sup>1</sup>Treatment: Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM)

**Table 5. 9.** Evaluation of amino acid uptake ( $\mu\text{mol}/\text{mmol}$  of bound AA protein/ $\text{min}^{-1}$ ) given changing extracellular amino acid concentrations

AA	AA uptake kinetic models <sup>1</sup>								
	Model	RMSE <sup>2</sup>	CCC <sup>3</sup>	BIC <sup>4</sup>	P-value <sup>5</sup>	Slope <sup>6</sup>	V <sub>max</sub> <sup>7</sup>	k <sub>m</sub> , uM <sup>8</sup>	In-vivo, uM <sup>9</sup>
Ala	Linear	11.6	0.98	23.6	-	0.058*	-	-	-
	MM	6.6	0.99	19.2	<0.001	-	103*	1130	268
Arg	Linear	34.6	0.77	15.1	-	0.019	-	-	-
	MM	25.9	0.87	12.7	<0.001	-	5.6	76	80
Asp	Linear	3.7	0.99	-26.6	<0.001	0.001*	-	-	-
	MM	4.7	0.99	-24.7	-	-	0.3*	49*	2
Glu	Linear	25.9	0.23	-12.3	-	-	-	-	-
	MM	26.8	0.13	-12.1	-	-	-	-	-
Gly	Linear	18.2	0.03	32.9	-	-	-	-	-
	MM	16.3	0.33	32.1	-	-	-	-	-
Ile	Linear	25.2	0.85	13.1	-	0.013*	-	-	-
	MM	14.0	0.95	8.5	<0.001	-	5.9*	114	107
Leu	Linear	20.7	0.91	11.2	-	0.009*	-	-	-
	MM	12.1	0.97	6.9	<0.001	-	6.9*	294	165
Met	Linear	12.6	0.97	1.4	-	0.051*	-	-	-
	MM	10.4	0.98	-0.2	<0.001	-	8.4	122	24
Phe	Linear	38.3	0.54	14.6	-	0.030	-	-	-
	MM	34.3	0.61	13.7	<0.001	-	5.9	74	52
Pro	Linear	37.9	0.52	18.6	-	0.013	-	-	-
	MM	28.5	0.74	16.3	<0.001	-	6.5*	68	104
Ser	Linear	13.8	0.96	27.7	-	0.165*	-	-	-
	MM	8.8	0.98	24.1	<0.001	-	93*	259	93
Thr	Linear	22.0	0.93	29.1	-	0.136*	-	-	-
	MM	21.0	0.93	28.8	<0.001	-	150	891	110
Tyr	Linear	14.7	0.97	15.6	-	0.126*	-	-	-
	MM	9.7	0.99	12.2	<0.001	-	24*	103	59
Val	Linear	32.9	0.97	16.2	-	0.004	-	-	-
	MM	21.3	0.84	12.7	<0.001	-	5.1*	122	250

<sup>1</sup>Amino acid uptake was standardized to the protein bound AA pool of the respective amino acid and fit to a linear model or Michaelis-Menten (MM) function with the dependent variable being extracellular amino acid concentration.

<sup>2</sup>RMSE: Root mean square prediction error percent of mean.

<sup>3</sup>CCC: Concordance correlation coefficient.

<sup>4</sup>BIC: Bayesian information criterion.

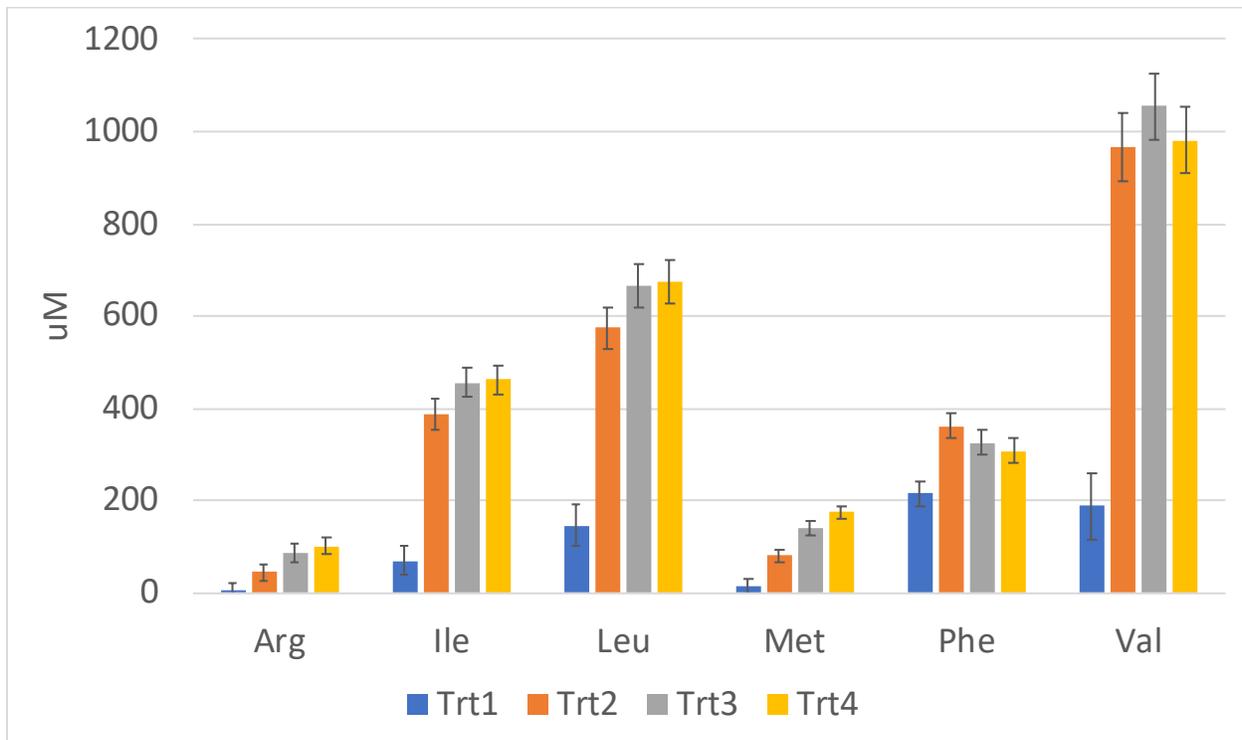
<sup>5</sup>P-value of log-likelihood ratio test comparison between models, model with corresponding P-value indicates better fit.

<sup>6</sup>Slope coefficient for linear model, \* indicates significance,  $P < 0.20$ .

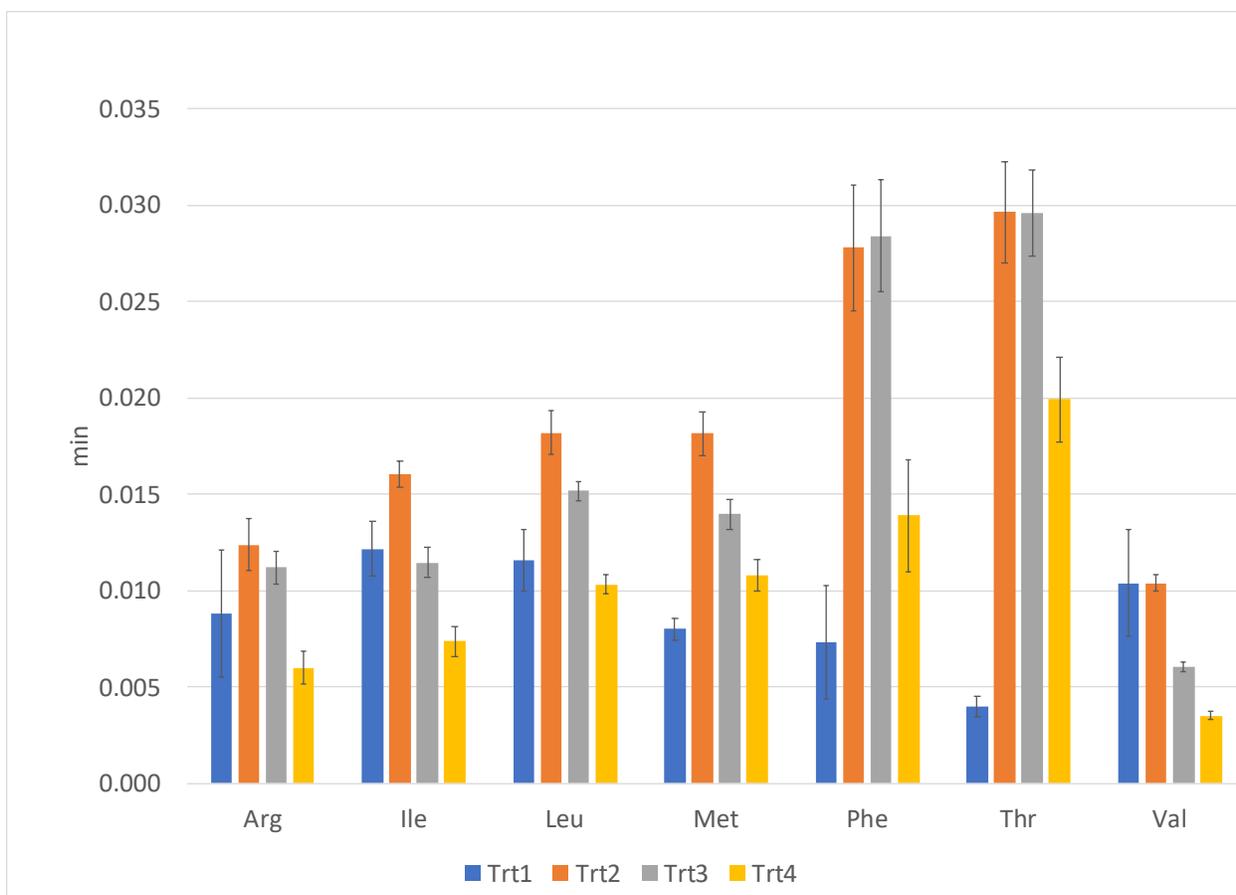
<sup>7</sup>V<sub>max</sub> derived from Michaelis-Menten function, units are  $\mu\text{mol}/\text{mmol}$  of AA protein/ $\text{min}^{-1}$ , \* indicates significance,  $P < 0.20$ .

<sup>8</sup>k<sub>m</sub> derived from Michaelis-Menten function, \* indicates significance,  $P < 0.20$ .

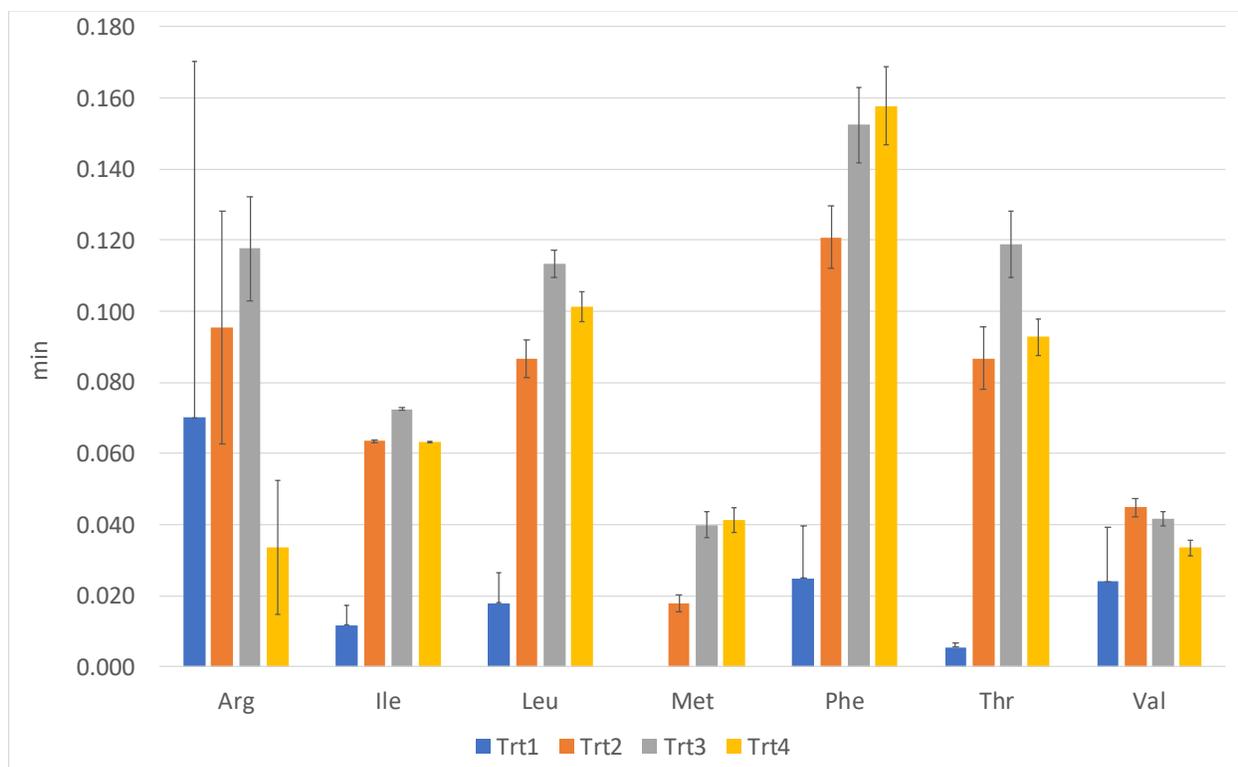
<sup>9</sup>Represents typical blood plasma AA concentration in lactating dairy cows (Swanepoel et al., 2016).



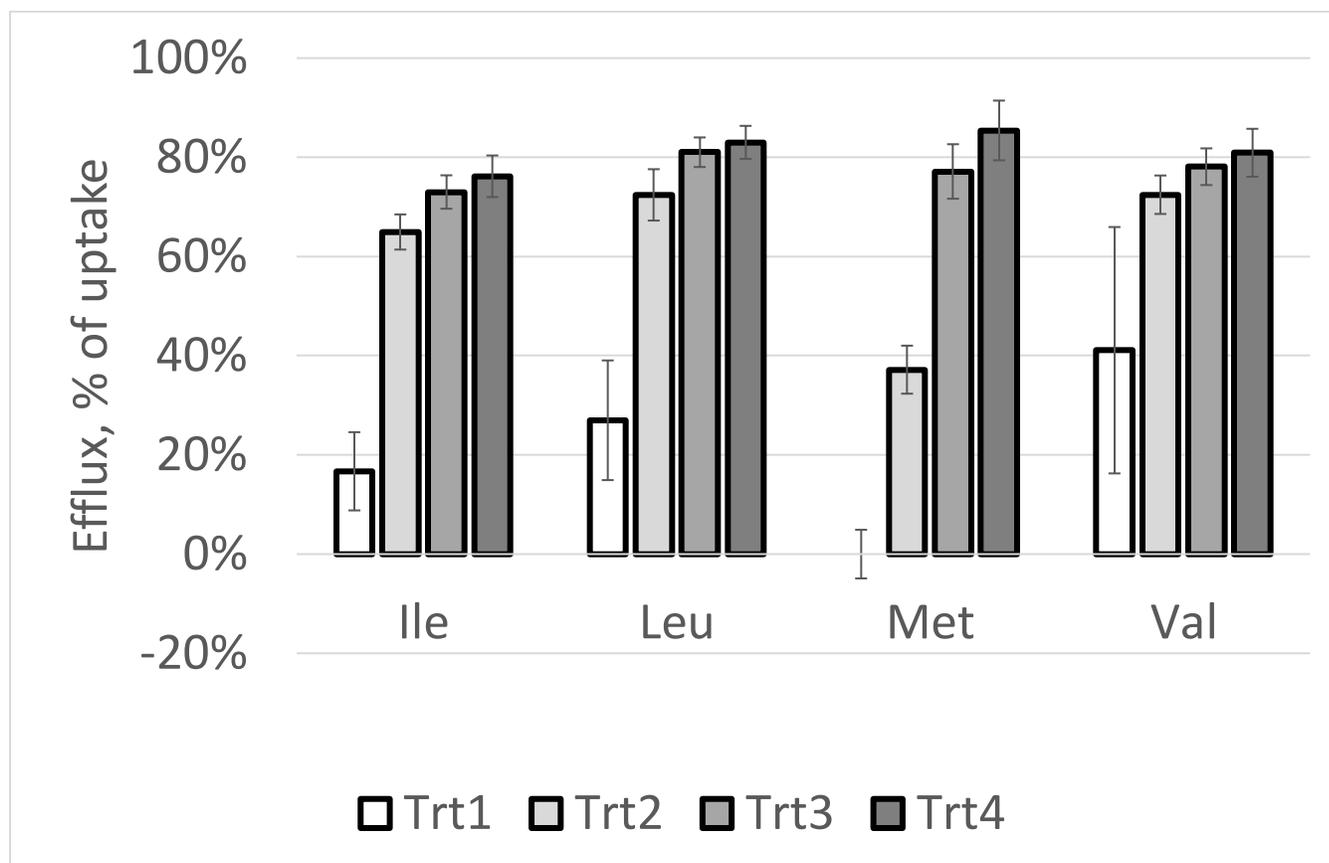
**Figure 5. 1.** Intracellular free essential amino acid concentrations over the course of the experiment by treatment. Treatments were the following; Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM).



**Figure 5. 2.** Amino acid uptake rate constants by treatment. Unit is  $\text{min}^{-1}$ . Treatments were; Trt1=16% of in-vivo (0.36  $\text{mM}$ ), Trt2 = 100% of in-vivo (2.30  $\text{mM}$ ), Trt3= 186% of in-vivo (4.28  $\text{mM}$ ), and Trt4= 271% of in-vivo Tr4 (6.24  $\text{mM}$ ).



**Figure 5. 3.** Amino acid efflux rate constants by treatment. Unit is  $\text{min}^{-1}$ . Treatments were the following; Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM).



**Figure 5. 4.** Amino acid efflux as a percent of uptake. Treatments were the following; Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM).

## 5.8. Supplemental Material

**Table 5.S 1.** Observed treatment amino concentrations of <sup>15</sup>N enriched media

uM <sup>2</sup>	Treatment AA Concentrations <sup>1</sup>				SEM	P-value
	Trt1	Trt2	Trt3	Trt4		
Ala	61	326	546	776	9.6	<0.001
Arg	9.9	66	126	186	2.0	<0.001
Asp	0.6	91	176	274	4.9	<0.001
Glu	15.7	158	295	451	8.0	<0.001
Gly	160	376	503	634	3.3	<0.001
Ile	22	125	217	318	4.9	<0.001
Leu	40	186	323	474	8.5	<0.001
Met	1.9	23	42	64	1.0	<0.001
Phe	8.4	54	97	144	2.2	<0.001
Pro	33	125	210	297	2.9	<0.001
Ser	26	194	319	513	26	<0.001
Thr	53	195	315	443	5.7	<0.001
Tyr	4.7	57	101	145	1.7	<0.001
Val	54	286	507	759	14	<0.001

<sup>1</sup>Treatment AA profiles mimicked typical lactating dairy cows and concentrations were varied being 16% of in-vivo in Trt1 (0.36 mM), 100% of in-vivo Trt2 (2.30 mM), 186% of in-vivo Trt3 (4.28 mM), and 271% of in-vivo Tr4 (6.24 mM).

<sup>2</sup>AA concentrations are the summation of measured <sup>12</sup>C, <sup>13</sup>C, and <sup>15</sup>N masses of each AA.

**Table 5.S 2.** Observed treatment amino concentrations of <sup>13</sup>C enriched media

uM <sup>2</sup>	Treatment AA Concentrations <sup>1</sup>				SEM	P-value
	Trt1	Trt2	Trt3	Trt4		
Ala	61	309	503	675	13	<0.001
Arg	13	81	135	193	5.6	<0.001
Asp	2.5	21	39	45	3.2	<0.001
Glu	6.1	96	139	181	5.2	<0.001
Gly	196	322	419	513	3.7	<0.001
Ile	24	129	209	287	5.6	<0.001
Leu	45	201	325	446	8.7	<0.001
Met	1.3	21	36	52	1.2	<0.001
Phe	45	120	180	241	4.1	<0.001
Pro	32	123	190	256	4.0	<0.001
Ser	34	142	229	277	13	<0.001
Thr	47	169	252	328	5.2	<0.001
Tyr	4.3	38	65	92	1.8	<0.001
Val	57	275	453	621	11	<0.001

<sup>1</sup>Treatment AA profiles mimicked typical lactating dairy cows and concentrations were varied being 16% of in-vivo in Trt1 (0.36 mM), 100% of in-vivo Trt2 (2.30 mM), 186% of in-vivo Trt3 (4.28 mM), and 271% of in-vivo Tr4 (6.24 mM).

<sup>2</sup>AA concentrations are the summation of measured <sup>12</sup>C, <sup>13</sup>C, and <sup>15</sup>N masses of each AA.

**Table 5.S 3.** Effects of treatment on volume, cell, and protein associated measurements

Item	Treatment <sup>1</sup>					SEM	Treatment	P-value		
	Trt1	Trt2	Trt3	Trt4	Time			Linear <sup>2</sup>	Quadratic <sup>2</sup>	
Starting plate weight, g	19.4	19.4	19.4	19.4	0.06	0.97	0.61	0.74	0.90	
Plate weight before lysis, g	20.0	20.2	20.2	20.3	0.14	0.49	0.66	0.17	0.87	
Cell weight from starting mass, g	0.64	0.78	0.82	0.93	0.14	0.51	0.73	0.14	0.90	
Cell weight from ending mass, g	0.55	0.65	0.77	0.82	0.17	0.66	0.60	0.20	0.89	
Harvested cell weight, g	0.29	0.32	0.34	0.39	0.03	0.09	0.40	0.01	0.83	
Protein weight, g	0.074	0.086	0.093	0.094	0.002	<0.001	0.19	<0.001	0.02	
Cells (1000) / mL	1371	1200	1508	1675	126	0.09	-	0.03	0.17	
Cells (1000) / total	2148	1871	2438	2710	183	0.04	-	0.01	0.13	
Cell, ng	0.73	0.84	0.67	0.60	0.06	0.07	-	0.03	0.13	
Protein, µg/mL	3602	4056	4232	3498	738	0.77	-	0.96	0.37	

<sup>1</sup>Treatment AA profiles mimicked typical lactating dairy cows and concentrations were varied being 16% of in-vivo in Trt1 (0.36 mM), 100% of in-vivo Trt2 (2.30 mM), 186% of in-vivo Trt3 (4.28 mM), and 271% of in-vivo Tr4 (6.24 mM).

<sup>2</sup>Linear and quadratic contrasts of treatment AA concentrations effects.

**Table 5.S 4.** Effect of treatment on intracellular free amino acid mass

umol <sup>2</sup>	Treatment <sup>1</sup>				SEM	P-value			
	Trt1	Trt2	Trt3	Trt4		Trt	Time	Linear <sup>3</sup>	Quadratic <sup>3</sup>
Ala	183	846	1054	1180	18	<0.001	<0.001	<0.001	<0.001
Arg	1	11	28	34	5	<0.001	0.44	<0.001	0.71
Asp	54	179	155	185	9	<0.001	<0.001	<0.001	<0.001
Glu	1009	1426	1443	1581	37	<0.001	0.18	<0.001	<0.001
Gly	835	892	899	964	16	<0.001	0.08	<0.001	0.78
Ile	17	107	142	157	3	<0.001	<0.001	<0.001	<0.001
Leu	36	159	208	230	4	<0.001	<0.001	<0.001	<0.001
Met	3	20	44	59	2	<0.001	<0.001	<0.001	0.58
Phe	53	100	101	103	2	<0.001	<0.001	<0.001	<0.001
Pro	142	675	727	780	14	<0.001	<0.001	<0.001	<0.001
Ser	126	248	283	394	11	<0.001	<0.001	<0.001	0.60
Thr	106	307	381	452	7	<0.001	<0.001	<0.001	<0.001
Tyr	35	86	95	104	2	<0.001	<0.001	<0.001	<0.001
Val	46	270	326	332	6	<0.001	<0.001	<0.001	<0.001

<sup>1</sup>Treatment: Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM).

<sup>2</sup>Mass includes <sup>12</sup>C, <sup>13</sup>C, and <sup>15</sup>N mass of each AA.

<sup>3</sup>Linear and quadratic contrasts of treatment AA concentrations effects.

**Table 5.S 5.** Effect of treatment on intracellular protein bound amino acid mass

millimole <sup>2</sup>	Treatment <sup>1</sup>				SEM	<i>P</i> -value
	Trt1	Trt2	Trt3	Trt4		Trt
Ala	2.6	2.1	2.1	2.0	0.04	<0.001
Arg	1.4	1.6	1.5	1.7	0.13	0.20
Asp	6.0	5.8	5.8	5.8	0.10	0.11
Glu	10.5	11.4	12.0	11.7	0.21	<0.001
Gly	0.4	0.4	0.4	0.4	0.01	0.01
Ile	2.6	3.0	3.2	3.2	0.08	<0.001
Leu	5.5	6.4	7.0	6.7	0.23	<0.001
Met	1.1	1.2	1.3	1.3	0.05	0.01
Phe	2.5	2.9	3.1	3.0	0.06	<0.001
Pro	2.1	2.3	2.4	2.4	0.06	<0.001
Ser	0.7	0.6	0.6	0.6	0.02	<0.001
Thr	1.4	1.2	1.1	1.1	0.02	<0.001
Tyr	0.7	0.5	0.5	0.5	0.01	<0.001
Val	3.0	3.5	3.7	3.7	0.09	<0.001

<sup>1</sup>Treatment: Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM).

<sup>2</sup>Mass includes <sup>12</sup>C, <sup>13</sup>C, and <sup>15</sup>N mass of each AA.

**Table 5.S 6.** Nonessential amino acid parameter estimates ( $\text{min}^{-1}$ ) derived by fitting the model to the observed treatment data<sup>1</sup>

Item <sup>2</sup>	Treatment, $\text{min}^{-1}$											
	Trt #1			Trt #2			Trt #3			Trt #4		
	Estimate	95% CI	CV	Estimate	95% CI	CV	Estimate	95% CI	CV	Estimate	95% CI	CV
<b>Ala</b>												
knAAnKA	0.012 <sup>a</sup>	0.008-0.016	18.4	0.044 <sup>b</sup>	0.040-0.049	5.1	0.046 <sup>b</sup>	0.043-0.049	3.37	0.043 <sup>b</sup>	0.039-0.046	4.6
knAAOx	0.070 <sup>c</sup>	0.056-0.083	9.8	0.029 <sup>b</sup>	0.019-0.040	18.2	0.021 <sup>ab</sup>	0.014-0.027	15.3	0.012 <sup>a</sup>	0.007-0.016	19.6
knAAAtfAA	0.003	0-0.006	62.5	0.001	0-0.002	83.7	0.001	0.000-0.001	50	0.001	0.000-0.001	28.7
knAAxAA	0.023 <sup>a</sup>	0.018-0.027	10.6	0.053 <sup>b</sup>	0.045-0.061	7.7	0.067 <sup>bc</sup>	0.061-0.074	4.88	0.070 <sup>c</sup>	0.063-0.078	5.7
ksynthesis	7.86	6.33-9.40	9.9	8.90	4.75-13.06	23.8	9.01	5.98-12.04	17.2	7.21	5.01-9.42	15.6
kxAAnAA	0.026 <sup>ab</sup>	0.023-0.029	6.7	0.032 <sup>b</sup>	0.029-0.035	4.4	0.025 <sup>b</sup>	0.024-0.027	3.17	0.021 <sup>a</sup>	0.019-0.023	4.8
<b>Asp</b>												
knAAOx	0.015	0.000-0.029	50.0	0.005	0-0.015	115.1	0.009	0-0.021	66.4	0.010	0.001-0.018	43.3
knAAAtfAA	0.007	0.005-0.008	11.7	0.005	0.004-0.006	8.0	0.006	0.003-0.009	25.9	0.005	0.004-0.006	12.9
knAAxAA	0.001	0.001-0.001	7.1	0.002	0.001-0.003	30.7	0.002	0.002-0.003	17.8	0.002	0.002-0.003	11.1
ksynthesis	0.80 <sup>a</sup>	0.15-1.46	41.4	1.70 <sup>ab</sup>	0.94-2.47	23.0	2.39 <sup>b</sup>	1.49-3.30	19.4	2.55 <sup>b</sup>	2.06-3.03	9.8
kxAAnAA	0.005 <sup>b</sup>	0.003-0.008	20.7	0.002 <sup>ab</sup>	0.001-0.004	27.3	0.002 <sup>ab</sup>	0.001-0.003	20.8	0.002 <sup>a</sup>	0.001-0.002	15.2
<b>Glu</b>												
knAAOx				0.015 <sup>a</sup>	0.013-0.018	8.0	0.019 <sup>ab</sup>	0.017-0.021	5.01	0.024 <sup>b</sup>	0.022-0.027	5.1
knAAAtfAA	0.003 <sup>a</sup>	0.002-0.003	9.3	0.006 <sup>b</sup>	0.006-0.007	4.2	0.019 <sup>c</sup>	0.019-0.020	1.86	0.008 <sup>b</sup>	0.007-0.009	3.8
knAAxAA	0.000 <sup>a</sup>	0.000-0.001	15.2	0.003 <sup>b</sup>	0.002-0.003	10.0	0.002 <sup>ab</sup>	0.001-0.003	25.3	0.002 <sup>ab</sup>	0.001-0.004	26.7
ksynthesis	3.23 <sup>a</sup>	2.56-3.90	10.5	19.60 <sup>b</sup>	17.33-21.88	5.9	23.54 <sup>bc</sup>	21.69-25.39	4.01	24.86 <sup>c</sup>	22.59-27.14	4.7
kxAAnAA	0.010 <sup>b</sup>	0.009-0.011	4.8	0.004 <sup>a</sup>	0.003-0.006	14.7	0.002 <sup>a</sup>	0.001-0.003	28.2	0.003 <sup>a</sup>	0.002-0.004	13.4
<b>Gly</b>												
knAAOx	0.014 <sup>a</sup>	0.009-0.018	16.6	0.027 <sup>b</sup>	0.023-0.032	8.9	0.017 <sup>a</sup>	0.014-0.020	10.1	0.009 <sup>a</sup>	0.005-0.012	22.3
knAAAtfAA	0.000	0.000-0.000	40.6	0.0002	0.000-0.000	17.8	0.000	0.000-0.000	31.7	0.000	0.000-0.000	22.2
knAAxAA	0.031 <sup>bc</sup>	0.026-0.036	8.1	0.032 <sup>c</sup>	0.029-0.036	5.6	0.024 <sup>b</sup>	0.022-0.026	4.05	0.020 <sup>a</sup>	0.019-0.021	3.0
ksynthesis	12.20 <sup>bc</sup>	9.76-14.64	10.2	13.34 <sup>c</sup>	11.32-15.35	7.7	9.30 <sup>b</sup>	7.89-10.72	7.75	6.11 <sup>a</sup>	4.57-7.66	12.9
kxAAnAA	0.016 <sup>c</sup>	0.013-0.019	9.8	0.011 <sup>c</sup>	0.010-0.013	5.6	0.007 <sup>b</sup>	0.006-0.008	4.52	0.004 <sup>a</sup>	0.004-0.005	5.1
<b>Pro</b>												
knAAnKA	0.003	0.001-0.006	38.3	0.004	0.002-0.006	27.0						

knAAOx	0.005 <sup>ab</sup>	0.003-0.007	19.0	0.007 <sup>b</sup>	0.006-0.009	11.1	0.004 <sup>b</sup>	0.003-0.006	13.4	0.002 <sup>a</sup>	0.001-0.003	25.3
knAAAtfAA	0.005 <sup>b</sup>	0.004-0.005	6.1	0.002 <sup>a</sup>	0.002-0.002	4.6	0.002 <sup>a</sup>	0.002-0.002	3.59	0.002 <sup>a</sup>	0.002-0.002	3.0
knAAxAA	0.010 <sup>a</sup>	0.009-0.012	9.2	0.021 <sup>c</sup>	0.019-0.023	4.8	0.020 <sup>c</sup>	0.018-0.021	3.49	0.015 <sup>b</sup>	0.014-0.016	3.3
kxAAnAA	0.011	0.010-0.012	5.6	0.018 <sup>c</sup>	0.017-0.019	3.4	0.010 <sup>b</sup>	0.010-0.011	2.57	0.006 <sup>a</sup>	0.006-0.007	2.3
Ser												
knAAOx	0.045 <sup>ab</sup>	0-0.102	64.0	0.042 <sup>b</sup>	0.033-0.050	10.1	0.032 <sup>ab</sup>	0.021-0.044	18.6	0.021 <sup>a</sup>	0.014-0.028	17.4
knAAAtfAA	0.003	0-0.010	122.5	0.001	0.000-0.001	44.7				0.000	0.000-0.001	59.6
knAAAtsAA				0.000	0.000-0.001	46.1						
knAAxAA	0.037 <sup>a</sup>	0.015-0.058	29.5	0.069 <sup>ab</sup>	0.050-0.088	13.9	0.067 <sup>ab</sup>	0.048-0.087	14.7	0.075 <sup>b</sup>	0.064-0.086	7.7
ksynthesis	5.73	0.63-10.84	45.5	7.01	5.85-8.17	8.5	6.66	4.97-8.38	12.9	7.28	5.88-8.69	9.8
kxAAnAA	0.026	0.012-0.041	27.6	0.027	0.020-0.034	12.4	0.017	0.013-0.022	13.8	0.017	0.014-0.020	7.6
Tyr												
knAAOx				0.015 <sup>b</sup>	0.012-0.019	11.2	0.011 <sup>b</sup>	0.009-0.014	12	0.005 <sup>a</sup>	0.002-0.008	27.0
knAAAtfAA				0.001	0.000-0.003	42.2				0.001	0.000-0.003	65.8
knAAxAA	0.024 <sup>a</sup>	0.016-0.032	17.3	0.042 <sup>a</sup>	0.030-0.053	14.1	0.063 <sup>b</sup>	0.055-0.072	6.99	0.064 <sup>b</sup>	0.055-0.074	7.7
ksynthesis	0.09 <sup>a</sup>	0.045-0.132	25.2	0.26 <sup>b</sup>	0.176-0.339	16.1	0.20 <sup>b</sup>	0.148-0.259	13.8			
kxAAnAA	0.013	0.008-0.018	20.5	0.013	0.010-0.016	13.2	0.012	0.011-0.014	6.64	0.009	0.008-0.011	8.6

<sup>1</sup>Treatment: Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM).

<sup>2</sup>Rate constants: knAAOx=oxidation, knAAAtfAA=fast protein turnover, knAAAtsAA=slow protein turnover, knAAxAA=efflux, ksynthesis=fractional synthesis, kxAAnAA=uptake.

<sup>3</sup>95% CI derived from Markov Chain Monte Carlo simulation (n=5000 runs).

**Table 5.S 7.** Nonessential amino acid flux predictions derived from the parameterized model and the treatment data<sup>1</sup>

Item	Amino Acid Fluxes, $\mu\text{M}/\text{min}^2$																				
	Net Uptake			Uptake			Exit			Turnover			Transamination			Oxidation			Synthesis		
	Mean	5%	95%	Mean	5%	95%	Mean	5%	95%	Mean	5%	95%	Mean	5%	95%	Mean	5%	95%	Mean	5%	95%
<b>Ala</b>																					
Trt1	2.4 <sup>b</sup>	2.2	2.6	6.8 <sup>a</sup>	6.0	7.6	4.4 <sup>a</sup>	3.5	5.2	0.5	0.0	1.2	2.3 <sup>a</sup>	1.4	3.2	13.4 <sup>a</sup>	11.2	15.6	12.0	9.8	14.1
Trt2	6.4 <sup>d</sup>	5.9	7.0	51.2 <sup>b</sup>	47.1	55.3	44.7 <sup>b</sup>	40.6	48.9	0.6	0.0	1.4	37.2 <sup>b</sup>	33.4	41.1	24.5 <sup>b</sup>	17.3	31.6	17.3	9.7	24.9
Trt3	4.0 <sup>c</sup>	3.2	4.8	74.3 <sup>c</sup>	70.0	78.6	70.3 <sup>c</sup>	66.0	74.7	0.7	0.1	1.2	47.7 <sup>c</sup>	44.1	51.2	21.6 <sup>b</sup>	16.0	27.3	18.0	12.3	23.6
Trt4	-0.2 <sup>a</sup>	-1.9	1.4	82.9 <sup>c</sup>	75.4	90.5	83.2 <sup>d</sup>	75.9	90.5	0.7	0.4	1.1	50.3 <sup>c</sup>	45.8	54.9	13.9 <sup>ab</sup>	9.2	18.7	14.8	10.6	19.0
<b>Asp</b>																					
Trt1	0.4	0.3	0.5	0.5 <sup>a</sup>	0.4	0.6	0.0 <sup>a</sup>	0.0	0.0	0.3 <sup>a</sup>	0.2	0.4				0.7	0.1	1.3	0.8 <sup>a</sup>	0.2	1.4
Trt2	0.5	0.2	0.7	0.8 <sup>ab</sup>	0.5	1.1	0.3 <sup>b</sup>	0.3	0.4	0.8 <sup>b</sup>	0.7	1.0				0.8	-0.1	1.7	1.7 <sup>ab</sup>	1.0	2.4
Trt3	0.6	0.3	1.0	1.0 <sup>b</sup>	0.7	1.4	0.4 <sup>b</sup>	0.3	0.5	1.1 <sup>b</sup>	0.6	1.5				1.6	0.1	3.1	2.4 <sup>b</sup>	1.5	3.3
Trt4	0.7	0.4	1.0	1.2 <sup>b</sup>	0.9	1.5	0.5 <sup>b</sup>	0.4	0.5	1.0 <sup>b</sup>	0.7	1.2				2.0	0.9	3.0	2.5 <sup>b</sup>	2.1	3.0
<b>Glu</b>																					
Trt1	0.8 <sup>b</sup>	0.7	0.9	1.2	1.1	1.3	0.4 <sup>a</sup>	0.3	0.5	2.7 <sup>a</sup>	2.2	3.2							3.3 <sup>a</sup>	2.6	4.0
Trt2	-1.7 <sup>a</sup>	-2.2	-1.3	1.7	1.2	2.2	3.5 <sup>b</sup>	2.9	4.0	8.7 <sup>b</sup>	7.9	9.5				21.2 <sup>a</sup>	18.6	23.9	20.1 <sup>b</sup>	17.9	22.2
Trt3	-1.8 <sup>a</sup>	-2.8	-0.9	0.9	0.3	1.6	2.7 <sup>b</sup>	1.4	4.1	25.6 <sup>d</sup>	24.6	26.5				25.7 <sup>a</sup>	23.2	28.2	24.2 <sup>c</sup>	22.4	26.1
Trt4	-1.2 <sup>a</sup>	-2.5	0.1	2.0	1.3	2.7	3.2 <sup>b</sup>	1.5	4.9	10.5 <sup>c</sup>	9.6	11.3				32.2 <sup>b</sup>	29.0	35.4	25.6 <sup>c</sup>	23.3	28.0
<b>Gly</b>																					
Trt1	-8.4 <sup>a</sup>	-9.3	-7.5	16.8 <sup>ab</sup>	13.7	19.8	25.2 <sup>bc</sup>	21.7	28.6	0.1	0.0	0.3				11.0 <sup>ab</sup>	7.6	14.5	18.6 <sup>bc</sup>	15.1	22.1
Trt2	-3.1 <sup>b</sup>	-3.6	-2.5	24.4 <sup>bc</sup>	21.7	27.1	27.5 <sup>c</sup>	24.7	30.2	0.2	0.1	0.2				23.5 <sup>c</sup>	19.6	27.3	24.2 <sup>c</sup>	20.6	27.9
Trt3	-0.9 <sup>c</sup>	-1.5	-0.3	21.4 <sup>b</sup>	19.7	23.2	22.3 <sup>b</sup>	20.8	23.9	0.1	0.1	0.2				15.6 <sup>b</sup>	12.8	18.4	16.9 <sup>b</sup>	14.5	19.3
Trt4	-2.7 <sup>b</sup>	-3.7	-1.7	16.8 <sup>a</sup>	15.3	18.3	19.5 <sup>a</sup>	18.5	20.5	0.2	0.1	0.2				8.5 <sup>a</sup>	5.1	11.9	10.9 <sup>a</sup>	8.3	13.5
<b>Pro</b>																					
Trt1	0.4 <sup>c</sup>	0.3	0.5	1.9 <sup>a</sup>	1.7	2.1	1.5 <sup>a</sup>	1.2	1.7	0.7 <sup>a</sup>	0.6	0.8	0.5 <sup>a</sup>	0.1	0.8	0.7 <sup>a</sup>	0.5	0.9			
Trt2	-0.1 <sup>b</sup>	-0.4	0.3	12.8 <sup>c</sup>	12.0	13.6	12.8 <sup>bc</sup>	11.9	13.7	1.1 <sup>b</sup>	1.0	1.2	2.3 <sup>b</sup>	1.1	3.4	4.3 <sup>c</sup>	3.6	5.1			
Trt3	-1.0 <sup>a</sup>	-1.3	-0.6	11.9 <sup>c</sup>	11.3	12.5	12.9 <sup>c</sup>	12.2	13.5	1.2 <sup>b</sup>	1.1	1.3				2.9 <sup>b</sup>	2.3	3.5			
Trt4	-1.5 <sup>a</sup>	-1.9	-1.0	9.8 <sup>b</sup>	9.4	10.2	11.3 <sup>b</sup>	10.7	11.9	1.5 <sup>c</sup>	1.4	1.6				1.6 <sup>ab</sup>	0.9	2.3			
<b>Ser</b>																					
Trt1	-0.4 <sup>b</sup>	-0.6	-0.2	5.3 <sup>a</sup>	2.6	8.0	5.7 <sup>a</sup>	2.9	8.5	0.4	-0.5	1.4				7.0	-0.4	14.5	9.1	1.7	16.5

Trt2	0.9 <sup>c</sup>	0.6	1.3	21.5 <sup>b</sup>	16.9	26.2	20.6 <sup>b</sup>	16.0	25.2	0.2	0.0	0.4	12.4	10.4	14.5	12.9	10.9	15.0
Trt3	0.5 <sup>bc</sup>	-0.3	1.3	22.9 <sup>b</sup>	16.9	28.9	22.4 <sup>bc</sup>	16.4	28.4				10.8	7.5	14.1	12.4	9.4	15.4
Trt4	-3.1 <sup>a</sup>	-4.3	-2.0	28.8 <sup>b</sup>	24.7	33.0	31.9 <sup>c</sup>	27.8	36.1	0.2	0.0	0.4	8.9	6.0	11.7	13.6	11.3	16.0
Tyr																		
Trt1	-0.3 <sup>a</sup>	-0.4	-0.3	0.4 <sup>a</sup>	0.2	0.6	0.8 <sup>a</sup>	0.5	1.0							0.1 <sup>a</sup>	0.1	0.2
Trt2	-0.1 <sup>b</sup>	-0.2	0.0	3.0 <sup>b</sup>	2.2	3.8	3.1 <sup>b</sup>	2.3	3.9	0.1	0.0	0.2	1.2 <sup>b</sup>	1.0	1.4	0.6 <sup>b</sup>	0.4	0.8
Trt3	-0.2 <sup>ab</sup>	-0.3	-0.1	4.9 <sup>c</sup>	4.3	5.5	5.2 <sup>c</sup>	4.6	5.8				0.9 <sup>b</sup>	0.8	1.1	0.5 <sup>b</sup>	0.4	0.7
Trt4	-0.4 <sup>ab</sup>	-0.6	-0.2	4.9 <sup>c</sup>	4.1	5.8	5.3 <sup>c</sup>	4.6	6.1	0.1	0.0	0.2	0.4 <sup>a</sup>	0.2	0.6			

<sup>1</sup>Treatment: Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM).

<sup>2</sup>Average flux over 60 minutes using derived rate constants and experimental data.

<sup>3</sup>5% and 95% confidence interval standard error represents the standard deviation of 1000 simulated model runs conducted using randomly drawn parameters from Markov Chain Monte Carlo derived parameter posteriors.

**Table 5.S 8.** Fit statistics for predictions of observed essential amino acid isotope ratios by amino acid and treatment<sup>1</sup>

Item	Statistic <sup>2</sup>	Isotope Ratios <sup>3</sup>					
		EnAAi	ExAAi	EnAAii	ExAAii	EtAAi	EtAAii
Ala							
Trt1	CCC	0.62	0.81	0.62	0.47	0.17	0.00
	RMSE	15.8	16.7	11.9	9.3	3.1	4.6
Trt2	CCC	0.91	0.99	0.98	0.96	0.13	0.03
	RMSE	11.6	4.9	6.3	5.9	3.0	3.8
Trt3	CCC	0.93	0.99	1.00	0.96	0.31	0.05
	RMSE	10.8	4.0	3.2	5.9	2.1	3.8
Trt4	CCC	0.93	0.99	0.96	0.98	0.51	0.04
	RMSE	11.1	4.1	9.7	4.8	1.8	3.5
Asp							
Trt1	CCC	0.55	0.23	0.81	0.08	0.77	0.01
	RMSE	50.1	46.9	28.2	8.8	2.1	1.0
Trt2	CCC	0.25	0.83	0.71	0.49	0.94	0.05
	RMSE	39.2	34.0	37.0	19.5	1.6	1.5
Trt3	CCC	0.33	0.89	0.82	0.21	0.81	0.18
	RMSE	32.9	23.5	31.5	13.8	2.9	2.0
Trt4	CCC	-0.03	0.87	0.83	0.35	0.86	-0.04
	RMSE	34.0	28.4	25.1	17.9	2.7	2.2
Glu							
Trt1	CCC	0.88	0.48	0.77	0.05	0.97	-0.01
	RMSE	21.9	62.7	5.5	6.6	2.5	1.5
Trt2	CCC	0.44	0.93	0.88	0.67	0.96	0.20
	RMSE	52.7	25.3	14.1	12.5	3.6	1.4
Trt3	CCC	0.18	0.92	0.89	0.68	0.86	0.32
	RMSE	75.2	26.9	13.9	9.9	7.0	1.8
Trt4	CCC	0.41	0.87	0.88	0.63	0.91	0.00
	RMSE	65.4	31.8	15.2	10.2	6.5	1.9
Gly							
Trt1	CCC	0.83	0.94	0.67	0.94	0.27	-0.02
	RMSE	5.1	8.2	4.0	5.5	1.0	1.2
Trt2	CCC	0.90	0.96	0.90	0.94	0.66	0.06
	RMSE	8.4	3.9	4.3	7.8	0.9	1.4
Trt3	CCC	0.96	0.96	0.95	0.96	0.38	0.01
	RMSE	6.5	2.3	3.5	4.4	1.6	2.3
Trt4	CCC	0.97	0.98	0.97	0.98	0.58	-0.01
	RMSE	5.1	1.0	2.7	2.3	1.2	2.0

Pro							
Trt1	CCC	0.88	0.66	0.84	0.57	0.97	0.00
	RMSE	28.1	14.1	5.3	14.0	10.0	9.3
Trt2	CCC	0.97	0.95	0.96	0.98	0.96	0.01
	RMSE	20.9	6.6	4.9	8.5	10.3	9.6
Trt3	CCC	0.99	0.97	0.98	0.98	0.99	0.02
	RMSE	11.5	3.3	3.6	7.1	6.2	5.8
Trt4	CCC	0.99	0.96	0.94	0.98	0.98	0.05
	RMSE	14.6	2.7	6.3	6.5	6.6	4.2
Ser							
Trt1	CCC	0.59	0.90	0.13	0.83	0.09	-0.01
	RMSE	15.2	10.9	5.6	5.2	3.3	1.0
Trt2	CCC	0.40	0.90	0.79	0.97	0.16	0.02
	RMSE	22.4	8.1	7.3	5.1	4.2	1.6
Trt3	CCC	0.52	0.72	0.83	0.89	NA	NA
	RMSE	24.0	8.5	8.5	8.7	3.9	2.2
Trt4	CCC	0.85	0.77	0.91	0.96	0.14	-0.06
	RMSE	13.3	9.7	6.3	5.2	3.1	1.9
Tyr							
Trt1	CCC	0.75	0.71	0.05	0.63		
	RMSE	10.62	21.08	6.22	3.05	8.20	8.29
Trt2	CCC	0.84	0.80	0.91	0.94	0.18	0.02
	RMSE	6.92	3.68	10.45	7.67	2.36	2.57
Trt3	CCC	0.87	0.84	0.99	0.96		
	RMSE	4.20	1.56	5.29	5.63	2.71	3.09
Trt4	CCC	0.77	0.49	0.97	0.98	0.15	0.02
	RMSE	4.65	1.76	10.02	4.03	2.41	2.79

<sup>1</sup>Treatment: Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM).

<sup>2</sup>Test statistics: RMSPE=root mean square prediction error, CCC=concordance correlation coefficient.

<sup>3</sup>Isotope ratios: EnAAi=<sup>13</sup>C/<sup>12</sup>C area ratio of free intracellular AA, ExAAi=<sup>13</sup>C/<sup>12</sup>C area ratio of free media AA, EnAAii=<sup>15</sup>N/<sup>12</sup>C area ratio of free intracellular AA, ExAAii=<sup>15</sup>N/<sup>12</sup>C area ratio of free media AA, EtAAi=<sup>13</sup>C/<sup>12</sup>C area ratio of protein bound AA, and EtAAii=<sup>15</sup>N/<sup>12</sup>C area ratio of protein bound AA.

**Table 5.S 9.** Media, intracellular free, and intracellular protein bound nonessential amino acid pool turnover per hour by treatment and amino acid<sup>1</sup>

	Treatment Pool Turnover, % / hour			
	Trt1	Trt2	Trt3	Trt4
<b>Ala</b>				
Extracellular Free AA	100.7	166.7	143.8	123.9
Intracellular Free AA	600.6	495.2	534.1	499.9
Fast Protein Bound AA	40.7	47.3	52.3	57.6
<b>Asp</b>				
Extracellular Free AA	2.7	5.9	4.3	4.0
Intracellular Free AA	218.4	135.9	179.8	152.7
Fast Protein Bound AA	6.2	12.1	16.3	16.7
<b>Glu</b>				
Extracellular Free AA	18.0	53.3	31.5	27.7
Intracellular Free AA	42.9	138.4	238.5	181.4
Fast Protein Bound AA	54.3	137.9	422.7	162.9
<b>Gly</b>				
Extracellular Free AA	146.6	77.2	44.5	30.6
Intracellular Free AA	263.4	342.9	252.5	169.0
Fast Protein Bound AA	3.6	3.8	3.4	4.1
<b>Pro</b>				
Extracellular Free AA	50.7	106.6	65.6	43.2
Intracellular Free AA	106.1	138.9	119.7	91.4
Fast Protein Bound AA	18.2	20.7	21.9	26.2
<b>Ser</b>				
Extracellular Free AA	170.7	154.8	101.2	113.1
Intracellular Free AA	570.1	696.7	636.2	599.4
Fast Protein Bound AA	129.3	2.8		16.2
<b>Tyr</b>				
Extracellular Free AA	130.9	80.4	76.4	59.5
Intracellular Free AA	111.4	297.7	401.9	367.1
Fast Protein Bound AA		36.4		29.2

<sup>1</sup>Treatment: Trt1=16% of in-vivo (0.36 mM), Trt2 = 100% of in-vivo (2.30 mM), Trt3= 186% of in-vivo (4.28 mM), and Trt4= 271% of in-vivo Tr4 (6.24 mM)

## **CHAPTER 6: Effects of jugular infused methionine, lysine, and histidine as a group or leucine and isoleucine as a group on lactating dairy cows**

### **6.1. Abstract**

Essential AA (EAA), particularly leucine, isoleucine, methionine, and histidine possess signaling properties for promoting cellular anabolic metabolism whereas methionine, lysine, and histidine are considered to be substrate limiting AA. The objective of this study was to evaluate production responses to supplementation of two AA groups in a 2 x 2 factorial design. Eight cows (99 +/- 18 DIM) were assigned to four jugular infusion treatments consisting of saline (CON), methionine plus lysine plus histidine (MKH), isoleucine plus leucine (IL), or MKH plus IL in a replicated 4 x 4 Latin square design. Periods were 18 d in length comprising 8 d of rest followed by 10 d of jugular treatment infusion. Daily infusion amounts were 21 g of methionine, 38 g of lysine, 20 g of histidine, 50 g of leucine and 22 g of isoleucine. Cows were ad libitum fed a common diet consisting of 15.2 % crude protein and 1.61 Mcal/kg NE<sub>L</sub> on a dry matter basis that met rumen degradable protein requirements but was 15% deficient in metabolizable protein. Milk and energy-corrected milk yields increased by 2.3 kg/d and 1.9 kg/d respectively with infused IL, and no change was observed for MKH. Milk protein concentration increased by 0.13 percentage units for MKH whereas milk protein yield increased for both MKH and IL by 84 g/d and 64 g/d, respectively. The milk protein yield increase for MKH+IL was 145 g/d versus CON. Gross feed efficiency tended to increase with the IL infusion and N efficiency tended to increase with the MKH infusion. Arterial EAA concentrations less Met, Lys, and His declined by 7.2 % in response to MKH infusion. Arterial EAA less Ile and Leu also declined in response to IL infusion by 6.2%. Net total AA (TAA) and EAA uptake by the udder tended to increase in response to MKH infusion whereas, mammary blood flow increased in response to IL infusion, but TAA and EAA net uptake were unaffected. Apparent udder affinity increased for TAA and

EAA less Met, Lys, and His in response to MKH infusion whereas affinity for EAA less Ile and Leu increased for IL infusion. Venous Met and Leu concentrations increased by 192% and 35% from the MKH and IL infusions compared to CON, respectively, which indicates that intracellular concentration of these EAA changed substantially. Increases in milk protein yield were observed from two groups of amino acids independently and additively which contradicts the single limiting amino acid theory that a single nutrient will limit milk protein yield.

**Key words:** milk protein, methionine, lysine, histidine, leucine, isoleucine

## 6.2. Introduction

Improving our ability to manipulate milk protein yield to increase profitability and N efficiency, the latter being quite low at 25% (Hristov et al., 2004), is critical for human food supply security and industry sustainability. Milk protein yield is determined by AA availability and enzyme catalytic potential with the former considered to be the major regulator of milk protein synthesis.

Mitchell and Block (1946a) formulated a conceptual framework using the assumption that the most limiting AA will regulate synthesis and addition of other AA will have no effect. The most limiting EAA for milk protein production are considered to be Met, Lys, and possibly His. Milk protein responses to Met are well documented and fairly consistent; responses to Lys and His are somewhat more variable but have often been observed (Schwab et al., 1976, Noftsker and St-Pierre, 2003, St-Pierre and Sylvester, 2005, Appuhamy et al., 2011b, Lee et al., 2012, Zanton et al., 2014, Giallongo et al., 2015, Giallongo et al., 2016, Giallongo et al., 2017). However, the conceptual framework of Mitchell and Block (1946a) can yield variable and unpredictable responses (Hanigan et al., 2000). For example, in diets less than 15% CP, supplementation of Met and Lys resulted in no overall benefit in fat-corrected milk yield and

only a small benefit in milk protein yield across 16 experiments (Sinclair et al., 2014). Some studies actually observe negative responses to Met and Lys (Guinard and Rulquin, 1995, Robinson et al., 2000). More understanding of the mechanisms mediating Met, Lys, and His effects on milk protein yield, and interactions with other factors are needed to better predict responses to supplies of these EAA.

Net AA uptake by the udder as a proportion of arterial daily influx is quite low, having been shown to be 22% (Hanigan et al., 2004). This leaves a significant proportion of arterial AA available for splanchnic tissue catabolism. Given such a low udder capture of AA, a logical strategy is to increase intracellular demand via increased protein synthesis rates which would increase the pull of AA from arterial supplies and decrease AA available for splanchnic catabolism. Changes in phosphorylation of translation factors related to protein synthesis rate and milk protein yield have been observed in-vitro and in a few in-vivo trials (Rius et al., 2010a, Toerien et al., 2010, Appuhamy et al., 2012, Arriola Apelo et al., 2014d, Liu et al., 2017). Mechanistically, Leu appears to be the most potent of the EAA through binding to Sestrin2 in the cytoplasm which disrupts the GATOR2-Sestrin2 bond (Saxton et al., 2016b). Methionine and Arg mechanistically have also been linked to mTOR regulation (Sabatini, 2017).

Evidence that supplementing Leu or the BCAA increases milk protein yield via increased phosphorylation of translation factors is not well documented, particularly in longer term studies (>5 d) (Arriola Apelo et al., 2014a, Doelman et al., 2015b, Nichols et al., 2017). Infusion of BCAA has previously not increased milk protein yield in a number of studies (Hopkins et al., 1994, Mackle et al., 1999, Korhonen et al., 2002, Appuhamy et al., 2011b, Kassube et al., 2017, Curtis et al., 2018). Several reasons may exist for the lack of a response; the BCAA were not limiting, inappropriate infusion amounts, other EAA were more limiting thus blocking Leu

signaling, AA transport competition, or stimulation of increased non-mammary use of EAA. Experiments using removal of BCAA or Leu from a EAA infusate as a model have shown negative effects on milk protein yield (Rulquin and Pisulewski, 2006, Doelman et al., 2015b, Tian et al., 2017) while others have shown no response (Weekes et al., 2006). Infusion of only Leu and Ile, both of which possess strong mTOR signaling properties versus Val not possessing a known mechanism to our knowledge, might yield different results than the previously mentioned studies. Additionally, utilizing high producing dairy cows and infusion amounts similar to replacing EAA supplies in typical industry diets and that are sizeable to elicit a change in blood plasma concentrations might also yield different results. Regarding AA transport, Val cellular uptake competes with Leu and Ile by using System L transporters (Shennan and Boyd, 2014). Though speculative, addition of Val to treatment BCAA infusates may counteract benefits of Leu and Ile. This hypothesis on Val is also supported by a recent meta-analysis that found a negative milk protein response to increasing Val dietary supplies (Hanigan et al., 2018).

Our hypotheses were that supplementation of Met, Lys, and His as a group and Ile and Leu as a group would independently increase milk protein yield and that these effects would be additive. The objectives of this study were to determine milk and components response to these two groups of EAA in healthy dairy cows (>50 kg milk/d) and observe changes in metabolism that may mediate changes in milk protein yield.

## **6.3. Materials and Methods**

### **6.3.1. Experimental Design, Animals, and Feeding Management**

All animal procedures were approved by the Virginia Tech Animal Care and Use Committee. Eight Holstein cows in second lactation were blocked by DIM (2 blocks) and

randomly assigned to 1 of 4 treatment sequences within a 2 x 2 factorial arrangement in 2 orthogonally replicated 4 x 4 Latin squares. For one of the blocks, cows were biopsied for muscle and mammary tissue on d 18 (n= 4 cows) whereas the cows in the other block were infused with isotopic labelled algae (n=4 cows). Treatments were jugular infusion of saline (**CON**), methionine, lysine, and histidine (**MKH**), isoleucine and leucine (**IL**), and the combination of MKH and IL (**MKH+IL**). Period length was 18 d with an initial adaptation period of 28 d. The entire experiment was completed in 100 d. At the beginning of the experiment (i.e. prior to the adaptation period), animals averaged 71 DIM (SD = 18 d). Cows were housed in a 12-stall pen for the adaptation period and the first 8 d of each period. On d 8 of each period, cows were moved to individual metabolism stalls and housed until d 18 of each period. Cows were fed a TMR *ab libitum* twice daily in the metabolism stalls with a target minimum refusal of 5% and milked twice daily at (0100 and 1230 h). Starting at 1200 h on d 15, cows were fed every 2 h for 36 h at 100% of the observed previous 7-d DMI average to standardize feed intake for blood flow and isotope infusion measurements. Milk, feed intake, and body weight were recorded daily. The corn silage was from plants containing the brown midrib 3 gene and was sourced from a single silage bag. Corn silage DM was assayed 5 times per period in duplicate by drying for 24 h at 100°C. Dietary adjustments were made when DM changed by more than 2 units. Refusals and TMR DM were assayed 3 times per period by drying for 24 h at 100°C. Corn silage, alfalfa hay, corn grain, wet brewers grain, grain mix, and TMR were sampled 3 times per period, composited by period, and stored at -20°C. Ingredients were assayed for DM using a forced-air oven at 55°C for 48 h and then sent to Cumberland Valley Analytical Services (Waynesboro, PA) for analysis of CP, ADF, NDFom, lignin, IV-NDF 30 h, IV-NDF 120 h, IV-NDF 240 h, ADICP, NDICP, soluble CP, ammonia, starch, IV-Starch 7

h, EE, fatty acids, ash, minerals, and fermentation (NIR2 package) (Supplemental Materials, Table 1).

Cows were fed a common diet for the entire study, including the adaptation period that was formulated to meet requirements for energy, rumen degradable protein, minerals, and vitamins but be deficient in metabolizable protein for a 680 kg Holstein cow producing 47 kg milk containing 3.60% fat and 3.10% protein (Table 1, Table 2). The amount of jugular infused amino acids for the MKH (21 g/d of Met, 20 g/d of His, and 38 g/d of Lys) and IL (22 g/d of Ile and 50 g/d of Leu) treatments was estimated based on meta-analytical work in progress, predictions from the NDS Professional ration formulation software (version 6.55; RUM&N, NDS Professional, Reggio Nell'Emilia, Emilia-Romagna, Italy), the amounts of each AA contained in a typical diet containing heat-treated soybean meal, blood meal, and rumen-protected methionine, and the recommended supply relative to metabolizable energy supply (Supplemental Materials, Table S7) (VanAmburgh et al., 2015). The ratio of Lys to Met supplied from the diet plus infusate was targeted at 2.65 to 1 whereas the ratio of Leu to Ile was targeted to be similar to what is observed in true milk protein (1.67 to 1) (Lapierre et al., 2012b). Leucine and Ile are metabolized with similar efficiencies from absorbed protein supplies (Myers et al., 2018), hence, the targeted ratio. The amount of Leu and Ile infused was limited by the solubility of Leu in 3 L of saline.

### **6.3.2. Jugular Infusions and Catheterizations**

Pharmaceutical-grade L-methionine, L-histidine, L-lysine-HCL, L-isoleucine, and L-leucine were obtained from ACP Chemicals (Toronto, Ontario). Infusates were prepared approximately 1-3 d prior to jugular infusion by dissolving 21 g of methionine, 20 g of histidine, and 47.5 g of lysine-HCL in 3 L of 0.9% saline for the MKH treatment, 22 g of isoleucine and 50 g of leucine in 3 L of 0.9% saline for the IL treatment, and 21 g of methionine, 20 g of histidine,

47.5 g of lysine-HCL, 22 g of isoleucine, and 50 g of leucine in 3 L of 0.9% saline for the MKH+IL treatment. The 0.9% saline was pharmaceutical-grade (MWI Animal Health, Boise, ID). Treatment solutions were sterile filtered through 0.22-um membrane filters (Sterivex, Millipore, Billerica, MA) and stored at 4°C until the day of infusion. Additionally, 2 g of <sup>13</sup>C labeled AA derived from algae (U-<sup>13</sup>C, 97-99% enriched, Cambridge Isotope Laboratories, Andover, MA) was dissolved in 1 L of 0.9% saline, sterile filtered through 0.22-um membrane filters, and infused for 5-h on d 16 of each period for one block of cows (n=4).

On d 8 of each period, cows were fitted with a jugular catheter (90 cm x 2.03 mm i.d. microrenathane, Braintree Scientific Inc., Braintree, MA) for jugular infusion of the treatment solutions. Catheter placement alternated sides by period. Catheters were checked every 4 h for efficacy and infection at the site of insertion and removed on d 18. Treatment and isotope solutions were jugular infused at 135 mL/h and 167 mL/h, respectively, using clinical infusion pumps (Abott Lifecare, San Antonio, TX; model RF-5000). Treatment and isotope solutions bags were gravimetrically weighed prior to and following infusion. On d 16 at 0800 h during period 2 to 4, cows were fitted with a subcutaneous mammary abdominal vein catheter (13 cm x 14 g, Jorvet, Loveland, CO) for venous sampling, and this catheter was removed on d 16 at 2400 h.

### **6.3.3. Sample Collection and Analysis**

Milk samples were collected twice daily on d 7 prior to treatment infusions and then from d 9 to d 18 during the treatment infusions at 0100 and 1230 h, stored at 4°C, and analyzed within 72 h for fat, true protein, lactose, SCC, and MUN concentrations with a CombiFoss FT + Fourier transform infrared analyzer (Foss, Hillerod, Denmark) by United DHIA (Radford, VA). On d 16, one block (n=4 cows) were frequently milked for measurement of isotope enrichment in milk protein at approximately 1500, 1700, and 1900 h or 2, 4, and 6 h relative to the start of the

infusion. To ensure complete milk removal, 5 cc of oxytocin was administered intramuscularly. Blood was sampled twice daily at approximately 0800 and 1800 h from the coccygeal vessel via venipuncture into potassium EDTA and sodium heparin vacutainers (Becton Dickinson, Rutherford, NJ) on d 7, 9, and 12. On d 16, blood was again sampled from the coccygeal vessel and the subcutaneous mammary abdominal vein by venipuncture in period 1 and by an indwelling catheters during period 2 to 4 at approximately 1300, 1500, 1700, 1900, 2100, and 2300 h for one block of cows (n=6 timepoints). For the 2<sup>nd</sup> block of cows, blood was sampled at the additional times of 1330, 1400, 1430, 1600, and 1800 (n=11 timepoints) for isotope enrichment of plasma AA. The coccygeal vessel sampling was considered arterial (Emery et al., 1965, Hanigan et al., 1991) and will be referenced as arterial blood herein despite that the blood sampling origin could be arterial or venous veins. Upon sampling, blood samples were placed on ice and then centrifuged (<6 h from first blood sample) at 1600 x g for 15 min. The resulting plasma was stored in polypropylene tubes at -20°C until further analysis. Tissue samples from the longissimus dorsi and the rear quarter of the udder were biopsied (average = 542 mg, SD = 139) (Toerien et al., 2010) on d 18 immediately following the 1230 milking on the tissue biopsy designated block of cows (n=4).

Approximately 0.50 mL of blood plasma were gravimetrically sampled and acid deproteinized using 10% sulfosalicylic acid and centrifuged at 16,000 g for 20 min at 4°C. The resulting supernatant (0.25 mL) was gravimetrically combined with a mixture of U-[<sup>13</sup>C,<sup>15</sup>N] AA derived from algae (Lot #16824, Product #CNLM-452) supplemented with [<sup>15</sup>N<sub>3</sub>, 99% enriched] His (Lot #15858, Product #NLM-1513), [<sup>15</sup>N, 98% enriched] Cys (Lot #14674, Product #NLM-2295), [<sup>15</sup>N, 99% enriched] Thr (Lot #20756, Product #NLM-742), [methyl-<sup>13</sup>C<sub>3</sub>, 99% enriched] Met (Lot #24031, Product #CLM-206), [Amide-<sup>15</sup>N, 98% enriched] Asn (Lot #12319, Product

#NLM-120), and [Amide-<sup>15</sup>N, 98% enriched] Gln (Lot#15592, Product #NLM-557) and subsequently desalted by ion chromatography (AG 50W-X8 resin, Bio-Rad Life Science). All isotopes were purchased from Cambridge Isotope Laboratories Inc, Tewksbury, MA. Desalted samples were freeze dried, solubilized in acetonitrile (J. T. Baker Inc., Phillipsburg, NJ) and converted to N-(tert-butyldimethyl) AA derivatives by incubation for 1 h at 70°C in N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (SELECTRA-SIL; UCT Inc., Bristol, PA). The derivatized AA were separated by gas chromatography (Trace GC Ultra; Thermo Scientific) and quantified by mass spectrometry (DSQII; Thermo Scientific) (Calder et al., 1999). Calibration curves for the labeled AA mixture were gravimetrically determined using an AA standard (AAS18; Sigma-Aldrich, St. Louis, MO) supplemented with L-Asn, L-Gln, L-Trp, and L-Cys (Sigma-Aldrich, St. Louis, MO).

#### **6.3.4. Statistical Analysis and Calculations**

Metabolizable EAA flow was estimated using NDS Professional ration formulation software (version 6.55; RUM&N, NDS Professional, Reggio Nell'Emilia, Emilia-Romagna, Italy) and NRC (2001) nutrition models with observed least square treatment means (Table 3 and Supplemental Materials, Table 2). Efficiency of MP and EAA for milk protein using NRC 2001 was calculated using model derived maintenance requirements whereas growth and pregnancy were not considered. Energy-corrected milk was calculated as follows:  $ECM = [(12.86 \times \text{kg of fat}) + (7.04 \times \text{kg of protein}) + (0.3246 \times \text{kg of milk})]$ . Milk AA output was calculated using true milk protein with a 3.37% correction for blood-derived proteins and the AA composition of milk protein (Lapierre et al., 2012b). Mammary plasma flow (MPF) was estimated using the Fick principle with Phe and Tyr as the internal markers (Cant et al., 1993). Milk Phe and Tyr yield ( $\mu\text{mol/h}$ ) were determined from milk protein yield using the average 12-hr period (1300 to 0100)

from d 15 to d 17 of each period. Blood arterial and venous Phe and Tyr concentrations were sampled 6 (biopsy block of cows, n=4) or 11 times (isotope block of cows, n=4) on d 16 from 0130 to 1130 and analyzed individually which should improve precision versus pooling samples (Lapierre et al., 2012b). Net uptake of AA by the mammary gland (umol/h) was calculated using arterial-venous difference multiplied by MPF. Clearance of AA by the udder was calculated as net uptake / venous concentration (Cant et al., 2016).

One cow during period 3 was diagnosed with clinical mastitis and data for that cow in period 3 and 4 were discarded. Another cow was diagnosed with clinical mastitis during period 2 and data for only that period was discarded. Data was analyzed with a mixed model using the “lmer” package in R (version 3.4.3; R Core Team, 2017) and SAS version 9.4 (SAS Institute, 2010) when considering repeated measures. Milk production, milk composition, and DMI of treatments were analyzed by day whereas arterial AA, net AA uptake, AA clearance, and ratio of AA uptake to milk protein output were analyzed by experimental period. The following model was utilized for analyzing data summarized by period:

$$Y_{ijklm} = \mu + MKH_i + IL_j + MKH * IL_{ij} + Block_k + Period_L + Cow(Block)_{m(k)} + e_{ijklm}$$

where  $Y_{ijklm}$  = the dependent variable,  $\mu$  = population mean of Y,  $MKH_i$  = fixed effect of methionine, lysine, and histidine infusion (df=1),  $IL_j$  = fixed effect of isoleucine and leucine infusion (df=1),  $MKH * IL_{ij}$  = interaction of  $MKH_i$  and  $IL_j$  (df=1),  $Block_k$  = random effect of block (df=1),  $Period_L$  = random effect of period (df=3), and  $Cow(Block)_{m(k)}$  = random effect of cow nested within block (df=6). Main effects and interactions were declared significant at  $P \leq 0.05$  and trends at  $P \leq 0.10$  and denominator degrees of freedom for all tests were adjusted using the Kenward-Rogers option. Residual error was evaluated for homogeneity of error and for outliers and if studentized residuals exceeded 3, the sample was removed for statistical

analysis. Post-hoc mean-separation testing was conducted only on significant main effects using the “lsmeansLT” package with degrees of freedom adjusted using the Kenward-Rogers option. When the interaction was significant, only the interaction effects were considered during mean-separation tests.

For the data analyzed by day, the following model was constructed with the MIXED procedure of SAS version 9.4 (SAS Institute, 2010) that included the previously described model with the addition of repeated fixed effect day (9 df) and all 2-way and 3-way interactions with the other fixed effects. The covariance structure was auto-regressive (1) and was selected from the available covariance structures using the lowest Bayesian information criterion. When the treatment x day interaction was significant ( $P \leq 0.10$ ), the SLICE option was used to determine on which days of the period ( $P \leq 0.10$ ) treatment means would be separated using Fisher’s protected LSD ( $P \leq 0.10$ ). No treatment x day, two-way or three-way interactions were found to be significant ( $P > 0.20$ ) and thus are not reported or discussed. All results reported in the paper are least-square means of the treatment effects whereas the main-effect means are listed in the supplemental material.

#### **6.4. Results and Discussion**

The objective of this study was to test the hypothesis that multiple EAA can independently increase milk protein yield in lactating dairy cows which would be contradictory to current nutrition model software and theory (NRC, 2001). The MKH treatment was designed to represent industry and literature recommendations for EAA supplementation, and the resulting treatment achieved ratios and supply balances that would be considered optimal for maximizing milk protein yield (NRC, 2001; CNCPS v6.55) (Table 3, Supplemental Materials, Table 2). The IL treatment objective was to evaluate hypothesis that signaling properties of these specific EAA could increase milk protein yield. Some factorial requirement models (CNCPS v6.55) also

suggest that these EAA were deficient (-24 g/d Ile and -87 g/d Leu in the CON) and supplementation with the IL treatment reduced this deficiency (Table 3; Supplemental Materials, Table 2).

Evaluation of this hypothesis with subjects that represented high-producing and healthy lactating dairy cows fed typical industry diets was also an important criterion of our study. The resulting subjects were post-peak multiparous cows (all in 2<sup>nd</sup> lactation) with average DIM of 99 (+/- 18) following the adaptation period and milk yields greater than 45 kg/d. Cows in 2<sup>nd</sup> lactation were selected to minimize the issue of leaky external pudic vein valves that occurs with age which would allow non mammary blood to mix with subcutaneous abdominal venous blood drainage of mammary tissue (Bequette et al., 1999). The subjects were fed the treatment diet (Table 2) for 4 weeks prior to the first period to minimize confounding effects with protein mobilization of labile body stores. To avoid any uncertainty with respect to the bioavailability of ruminally protected AA, the supplemented AA were provided by jugular infusion (Vyas and Erdman, 2009). Additionally, jugular infusion eliminates uncertainty regarding digestion and gastrointestinal metabolism of AA that would result using post ruminal infusions given varying use by amino acid (Hanigan et al., 2004, Estes et al., 2018a).

#### **6.4.1. Experimental Diet and Nutrient Supply**

The dietary ingredient composition is listed in Table 1 and the nutrient composition of corn silage, alfalfa hay, wet brewers, corn grain, and the concentrate supplement are provided in the supplemental materials, Table 1. The diet contained on average 47.5% forage and 52.5% concentrate which mimics commercial high cow rations (Chase, 2016). The starch content was higher than expected at 31.1% DM, but given that the corn grain source was dry and potentially less fermentable, it did not appear to induce ruminal acidosis as evidenced by milk fat concentrations which averaged 3.5% (Table 4). Palmitic fatty acid diet supplementation was

done for energy purposes and to enhance nutrient partitioning to the mammary gland (de Souza et al., 2018) via insulin resistance (Rico et al., 2016). This might improve the probability of EAA treatments exerting effects on mammary tissue and less on extra-mammary tissues. Mixing errors were greater than expected for corn grain due to an employee issue which resulted in a 21.9% coefficient of variation on a diet DM basis (Table 1). The mixing variation affected all treatments similarly. This mixing variation could be considered problematic, but experiments with high producing cows that were subjected to extreme dietary variation have shown no major effects on cow performance (McBeth et al., 2013, Yoder et al., 2013).

The resulting diet was high in energy concentration, 1.60 NE<sub>L</sub> Mcal/kg (NRC, 2001) and 1.69 NE<sub>L</sub> Mcal/kg (CNCPS v6.55) but energy allowable milk was still in deficit ~ 2 kg of milk/d given observed milk yields. The discrepancy in energy allowable milk is likely for several reasons; 1) cows numerically lost body weight during each experimental period (not considered in model evaluation); 2) DMI likely was slightly inhibited in the metabolism stalls; 3) or the models have bias in describing nutrient digestion and metabolism. Formulation for a low CP diet by removing all high RUP feedstuffs (i.e. heat-treated soybeans, animal protein, etc.) as we did in this trial and replacing with rumen degradable feedstuffs may enhance organic matter digestion and microbial AA supply and empirical evidence supports nutrition model inaccuracies on the value of high RUP feed ingredients (Santos et al., 1998).

The experimental diet was nearly adequate in rumen degradable protein (NRC, 2001) and well above the rumen ammonia requirement (CNCPS v6.55) which achieved our objective to not hinder ruminal nutrient digestion or intake. Milk urea nitrogen concentrations were low (Table 4) but the observed values do not suggest major limitation on rumen ammonia concentrations (Kohn et al., 2002). The predicted MP deficit by the NRC (2001) and CNCPS (v6.55) models

was 14-15% or 10-11 kg of milk for all treatments, indicating significant under prediction by these commonly utilized industry nutrition models. The dramatic under prediction might reflect inaccurate supply estimates, particularly microbial protein, protein mobilization from body stores, maintenance requirement overestimation, or error in the marginal efficiency of metabolizable protein to milk protein. Microbial supply was possibly underestimated given removal of high RUP feed ingredients (Santos et al., 1998). Based on Met, the observed plasma arterial AA concentrations in the CON treatment (Table 5) were similar to that observed on commercial dairies feeding diets with 16.5% CP (24  $\mu$ M) (Swanepoel et al., 2016) and university trials (n=102 studies) (Patton et al., 2015) which might indicate that AA supply was not severely deficient as nutrition models would indicate. Overestimation of maintenance MP requirements will result in underestimation of milk yield, and thus this could contribute to the model prediction error. Model under prediction of protein allowable milk at similar levels to this study (~11 kg of milk) has previously been observed (Doelman et al., 2015a, Doelman et al., 2015b, Doepel et al., 2016) but not at the level of milk production in this study, which averaged 48.7 kg/d, approximately 20 kg/d higher than these studies. The marginal efficiency when varying MP supply close to model requirements is likely no more than 25-35% (Hanigan et al., 1998a, Arriola Apelo et al., 2014b), not 67% as indicated in most nutrition software, which in nearly all cases will result in nutrition models significantly under predicting milk yield when MP supply is less than requirement. However, the under prediction at high milk yield is unexpected given previous data and observation that the NRC (2001) over predicts milk yield at high levels of production (see Figure 16.3, NRC (2001))(Arriola Apelo et al., 2014b). This either means that the results of this study are an extreme outlier compared to the NRC 2001 dataset or that more

studies with higher producing dairy cows feeding diets deficient in MP are needed, the later which will have consequences on model development.

Metabolizable supply of Met and Lys were 28 % and 18 % below the suggested requirement (NRC, 2001) and 25% and 18% less than the optimal supply relative to metabolizable energy (CNCPS v6.55) for the CON treatment diet (Table 3, Supplemental Material, Table 2). The observed MKH treatment precisely met the Met requirement as suggested by NRC (2001) and CNCPS (v6.55) as was planned prior to the trial. Lysine supply met the requirement with respect to metabolizable energy as suggested by CNCPS (v6.55) but was in deficit according to the NRC (2001) by 2 % on a gram basis given the required MP and NRC optimal MP concentration of Lys. This treatment change in supply was confirmed with an 89% ( $P < 0.001$ ) and 34% ( $P < 0.001$ ) increase in arterial plasma Met and Lys concentrations in the MKH treatment cows (Table 5, Figure 1). Additionally, venous plasma Met and Lys concentrations increased by 192% ( $P < 0.001$ ) and 32% ( $P = 0.002$ ) from the MKH infusion (Supplemental Material, Table 5), which suggests that mammary epithelial cells and their transporters were exposed to dramatic differences, particularly Met, since venous concentrations closely represent extracellular concentrations (Hanigan et al., 1998c).

The suggested His requirement is similar to Met (Giallongo et al., 2017) or 1.1 times Met supply (Schwab et al., 2014). Our target was a ratio of 1.1 for His:Met supplies which was also predicated on the personal observation that typical high-producing dairy cow diets contain 5-7 % of dietary DM as animal protein blends or heat-treated soybean meal resulting in a supply difference similar to our targeted infusion of 20 g/d of His. The observed supply of His was 1.1 times Met supply according to NRC (2001) but was predicted to be in excess by 16% of CNCPS v6.55 requirements for the MKH treatment. For the CON treatment, the ratio of His to Met was

1.16 (NRC, 2001) and 9% below requirement (CNCPS v6.55). Arterial plasma His concentrations increased by 31% ( $P < 0.001$ ) in response to MKH treatment (Table 5, Figure 1) whereas venous concentrations increased 33% ( $P < 0.001$ ) (Supplemental material, Table 5), indicative of treatment effects on blood concentrations as expected.

Isoleucine and Leu requirements were not established by the NRC (2001) whereas the CNCPS does provide factorial requirements though limited emphasis is placed on following these requirements as there is limited experimental support for the requirements in lactating dairy cows. Isoleucine was supplied to target a similar ratio in MP as that of milk protein (Lapierre et al., 2012b) as Leu and Ile are found to be incorporated into milk protein from absorbed supply at similar efficiency (Myers et al., 2018). Isoleucine and Leu deficits were 16% and 31% of requirements according to CNCPS v6.55 in the CON treatment and the IL treatment partially reduced these deficits to 3% and 14% respectively. Plasma concentrations of Ile and Leu increased 11% ( $P = 0.02$ ) and 30% ( $P < 0.001$ ) (Table 5, Figure 2); venous concentrations increased by 35% ( $P=0.02$ ) and 30% ( $P=0.01$ ) in response to the IL treatment (Supplemental Material, Table 5). The 35% and 30% increase in extracellular Leu concentrations likely proportionally translated to a similar increase in intracellular concentrations (Arriola Apelo et al., 2014c), which should have activated protein translational machinery.

Overall, energy supply was slightly below requirement and resulted in cows numerically losing body weight whereas MP and AA were significantly deficient for CON, and MKH and IL partially corrected these deficiencies. Changes in arterial plasma AA concentration for the infused AA confirmed that treatments changes in supply did occur.

## **6.5. Intake**

Dry matter and dietary CP intake were not affected by treatment (Table 4). The jugular treatment infusions increased total CP intake for MKH ( $P = 0.01$ ) and IL ( $P=0.01$ ) with no

interaction of the main effects. Histidine supplementation has been shown previously to increase intake (Lee et al., 2012, Giallongo et al., 2017) whereas methionine supplementation, if excessive, can decrease intake (Satter et al., 1975, Robinson et al., 2000). Numerically, intake increased in response to MKH but counteracting effects of potentially excess Met may have negated any benefit from His supplementation. Another potential reason is that the trial was short-term (18 d periods) as His deficiency takes several weeks to occur due to use of plasma hemoglobin and muscle carnosine (Lapierre et al., 2008b, Lee et al., 2012). This may have buffered His diet deficiency in this trial. Infusions of Ile and Leu were not expected to change intake given previous findings (Korhonen et al., 2002, Rulquin and Pisulewski, 2006, Weekes et al., 2006, Appuhamy et al., 2011b). Infusion of Met, Lys, and the BCAA have previously decreased DMI (Kassube et al., 2017) but we observed no interaction effect of MKH and IL.

#### **6.5.1. Milk Components**

Milk protein yield increased by 5.5% and 4.3% from MKH ( $P<0.001$ ) and IL ( $P<0.01$ ) treatments (Table 4). There was no interaction between MKH and IL treatments indicating that milk protein yield can be stimulated independently and additively by EAA. The milk protein yield increase from provision of additional Met, Lys and His supplementation was expected and well-documented in literature (Lee et al., 2012, Zanton et al., 2014, Patton et al., 2015, Giallongo et al., 2017). Interestingly, milk protein yield responses predicted by only Met supply as a proportion of MP using derived equations from CNCPS v6.55 (VanAmburgh et al., 2015) and NRC (2001) were 68 and 45 g/d increases for the MKH treatment whereas the observed increase was 82 g/d. Milk protein yield increased at 3.9 grams per gram of MP-Met for the MKH treatment which is higher than what was found in a meta-analysis estimate of 2.23 grams of milk protein per gram of MP-Met (Zanton et al., 2014). However, the observed responses are a function of the supplies of all AA, hence, only predicting or correlating milk protein from Met

supplies is an incorrect approach, although often applied in meta-regression for derivation of optimal supply. The increase in milk protein yield by IL treatment independent of MKH treatment agrees with in-vitro findings (Appuhamy et al., 2012, Arriola Apelo et al., 2014d) but disagrees with previous in-vivo trials (Huhtanen et al., 2002, Korhonen et al., 2002, Appuhamy et al., 2011b, Arriola Apelo et al., 2014a). The observation also disagrees with the hypothesis that the maxima milk protein yield is capped solely by a single first-limiting EAA. Methionine, Lys, or His were limiting EAA given the observed positive response herein and previous literature observations, however, despite this limitation, milk protein yield also increased with Ile and Leu supplementation. Increased milk protein yields seem to have been at least partially facilitated by greater mammary plasma flow ( $P = 0.01$ ), and hence delivery of EAA to the mammary gland. In the previous studies that did not observe a response to BCAA supplementation, decreased concentration of other EAA in plasma were often observed when supplementing BCAA which is thought to implicate stimulation of muscle protein synthesis from the increased BCAA supplies (Appuhamy et al., 2011b, Curtis et al., 2018). Increased demand by extra-mammary tissues may counteract and prevent any increase in milk protein yield from increased supplies of BCAA.

The combination of MKH and IL yielded an additional 145 g/d of milk protein which was a 10% increase. The marginal MP efficiency of the response was between 43 and 77% across the 3 treatments, which is much higher than the marginal response of 10-25% typically observed from infused casein or EAA previously (Hanigan et al., 1998b, Hanigan et al., 2004, Weekes et al., 2006, Doelman et al., 2015a, Doepel et al., 2016). However, these studies often infuse all EAA or casein, which probably oversupplies certain EAA, leading to very low marginal efficiency and no improvements in overall N efficiency. The independent responses to

MKH and IL responses were additive, 82 g/d from MKH plus 64 g/d from IL sums to 146 g/d, which was nearly identical to the 145 g/d response to MKH plus IL infusion. Previous, in-vitro experiments have shown independent positive milk protein responses from Leu, Ile, and Met (Park et al., 1976, Arriola Apelo et al., 2014d) and modeling work has shown that an additive consideration of multiple individual EAA was more accurate than the single-limiting AA approach (Hanigan et al., 2000). Using the observed venous concentrations for Met, Leu, and Ile and Thr from this study with the regression model (Arriola Apelo et al., 2014d) resulted in predicted milk protein responses of 6.3% for MKH, 20.8% for IL, and 16.3% for the combination of MKH and IL. The observed milk protein responses were 5.5%, 4.3%, and 10% in our experiment for MKH, IL, and MKH+IL treatments respectively, suggesting that the in-vitro model was overly-sensitive to Leu and Ile supplies. The observed responses also indicate that achieving maximum milk protein yield in dairy cows may depend on more than Met, Lys, and His, hence, supplementation of EAA such as Ile and Leu in the form of synthetic AA or feed ingredients may further enhance response when Met, Lys, and His are supplemented.

Milk fat yield is usually not affected by supplementation of the classical limiting EAA, Met, Lys, and His (Lee et al., 2012). However, a recent meta-analysis demonstrated a positive 2 gram of milk fat per gram of infused of DL-Met (Zanton et al., 2014). We observed a numerical 0.95 gram milk fat/gram of infused DL-Met response. Supplementing Ile and Leu has previously not increased milk fat yield (Huhtanen et al., 2002, Korhonen et al., 2002). However, recent evidence has pointed towards mTORC1 regulation of SREBP1, a potent transcription factor for de-novo fat synthesis (Chen and Corl, 2016) as a mechanism potentially linking the 2 systems. In the current work milk fat yield increased numerically by 47 g/d ( $P=0.22$ ) in response to Ile and Leu indicating that milk fat synthesis may be increasing in response to mTORC1 activation.

Lactose yield was unaffected by the MKH infusion whereas IL infusion increased lactose yield ( $P=0.002$ ). The increased lactose yield could have been influenced from greater MPF, increased anabolic signals from mTORC1 activation, or increased partitioning of nutrients to mammary tissue.

### **6.5.2. Milk Yield**

Milk yield and ECM increased from supplementation of Ile and Leu by 2.3 kg/d ( $P=0.001$ ) and 1.9 kg/d ( $P=0.01$ ) respectively whereas no affect was observed for the MKH treatment or the interaction (Table 4, Figure 3). The increase in milk or ECM yields from supplementation of Ile or Leu has not been previously observed (Korhonen et al., 2002, Rulquin and Pisulewski, 2006, Appuhamy et al., 2011b, Arriola Apelo et al., 2014a, Doelman et al., 2015b, Kassube et al., 2017). However, a recent meta-analysis ( $n=258$  treatment means) indicated positive milk yield responses to Leu (Lean et al., 2018). Based on that meta-regression equation, the change in milk yield from CON to IL was predicted to be 1.5 kg/d which was close to the observed response results. Decreased activation of the mTORC1 pathway when Leu and Ile are deficient has previously been observed in-vitro and in some in-vivo studies ((Appuhamy et al., 2012, Arriola Apelo et al., 2014c, Doelman et al., 2015b, Liu et al., 2017) which might lead to decreased mammary tissue anabolic activity and milk protein yield. The increased protein yield that was observed in the IL treatment ( $P < 0.01$ ) may have increased  $\alpha$ -lactalbumin, thereby driving the lactose disaccharide formation of galactose and glucose via allosteric activation of galactosyl transferase. Increased lactose yield ( $P < 0.01$ ) propels greater milk yield ( $P = 0.001$ ), which was observed. Why increased milk protein yield ( $P < 0.001$ ) in the MKH treatment did not also increase lactose ( $P = 0.98$ ) and milk yields ( $P = 0.31$ ) might reflect the negative effect of numerically lower MPF (9.5% lower) and delivery of milk precursors to the udder as well as the less potent mTOR anabolic effect of Met, Lys, and His compared to Ile and

Leu. Another possible mechanism is increased insulin resistance associated with elevated circulating plasma Ile and Leu (Coughlan et al., 2013), thereby reducing nutrient demand by extra-mammary tissues and partitioning more nutrients toward mammary gland. Infusion of glucose results in a rise in plasma insulin and when coupled with BCAA infusion, this can depress milk protein yield due to increased extra-mammary tissue activity (Curtis et al., 2018). Insulin resistance associated with palmitic acid supplementation and a concomitant rise in plasma ceramides has been observed in lactating cows (Rico et al., 2016) and suggested as a mechanism for improved ECM. High circulating BCAA in dairy cows might also promote insulin resistance as observed in other species thereby increasing milk yields and persistency. Feeding more highly digestible RUP, which likely improved EAA supplies such as Leu and Ile increased milk yield by 5.4 kg/d and ECM by 1.9 kg over 10-wk and 9-wk feeding trials respectively (Noftsker and St-Pierre, 2003, Gutierrez-Botero et al., 2014b).

The lack of response in milk and ECM yields to the MKH treatment is consistent with some studies, particularly short-term studies and meta-analyses (Appuhamy et al., 2011b, Sinclair et al., 2014, Zanton et al., 2014) while inconsistent with others (Noftsker and St-Pierre, 2003, St-Pierre and Sylvester, 2005). One noticeable effect of the MKH treatment was the numerical decline in mammary plasma flow (MPF) ( $P=0.22$ ) whereas MPF was increased with IL treatment ( $P=0.01$ ) (Table 6). Mammary plasma flow is closely correlated to milk yield and excess Met has been shown to decrease MPF. Infusion of 0 to 32 g/d in low producing dairy cows (24 kg/d milk yields) decreased MPF quadratically (Guinard and Rulquin, 1995), with the decrease being 13.8% with 16 g/d Met infusion which is similar to the 9.5% decrease we observed from 21 g/d Met infusion. The numerical decreased MPF may have reduced metabolite supplies for milk metabolism or compromised the supply of other EAA.

Additionally, the mechanism for mTORC1 activation by Lys or His appears to be less potent if not nonexistent compared to Ile and Leu (Appuhamy et al., 2012). Methionine does activate mTORC1 via Sestrin2 binding, though with much lower affinity than leucine (Wolfson et al., 2016) or via SAMTOR via one-carbon metabolism (Gu et al., 2017). Hence, the lack of response to the MKH treatment is somewhat expected given lack of alternate mechanism and the numerical decreased MPF.

Given the complexity of mTOR regulation, perhaps it is not surprising the variation in cow responses to BCAA supplementation. The anabolic signaling of mTORC1 is regulated by a multitude of factors, i.e. oxygen, glucose, amino acids, energy, insulin (Sabatini, 2017), as well as inhibited by the integrated stress pathway via ATF4, i.e. amino acid and glucose deficiency, ER stress (unfolded proteins), and oxidative stress (Baird and Wek, 2012, Wolfson and Sabatini, 2017). Hence, variation in cow responses to the strategy of manipulating mTORC1 seems likely. Intracellular Leu and to a lesser extent, Ile, bind to Sestrin2 triggering GATOR2 to activate mTORC1 which then phosphorylates key initiation factors controlling rates of translation and protein synthesis (Saxton et al., 2016b, Wolfson et al., 2016). mTORC1 activation by Leu or Ile can be inhibited by Sestrin2 concentrations, and high levels of Sestrin2 expression can completely blunt Leu activation despite increasing Leu intracellular concentrations (Wolfson and Sabatini, 2017). Sestrin2 expression is regulated by the transcription factor ATF4 and activation of this transcription factor can arise from a variety of factors including long term AA starvation and endoplasmic reticulum stress via phosphorylation of eIF2a (Baird and Wek, 2012). Milk fat depression via trans-10, cis-12 CLA causes endoplasmic reticulum stress and inhibition of SREBP1 in mouse adipocytes, which has been shown to be related to phosphorylation of eIF2a and ATF4 increased expression (LaRosa et al., 2007). Hence, while speculative, severe milk fat

depression might block anabolic activation of mTORC1 via Sestrin2. In two previous studies (Appuhamy et al., 2011b, Arriola Apelo et al., 2014a), BCAA or individual Leu supplementation failed to increase milk protein yield, but in those studies the average treatment milk fat concentrations were 2.46% and 3.07%, both indicative of severe milk fat depression (Bauman et al., 2011). Additionally, milk fat depression (2.86% and 2.82%) as well as very low CP diets (11.2% DM, 12.4% DM) coupled with relatively low milk production given DIM (30.1 kg/d at 105 DIM, and 33.9 kg/d at 78 DIM) in 2 recent studies that evaluated mTORC1 signaling may explain the lack of mTORC1 signaling responses to the subtraction or provision of Leu (Doelman et al., 2015b, Nichols et al., 2017). In summary, other factors might block the effects of supplementing translation stimulatory EAA thereby imposing inference containing multivariate complexity.

### **6.5.3. Efficiency**

Gross feed efficiency (GFE) increased by 3.2% during infusion of Ile and Leu ( $P=0.07$ ). Greater partitioning of nutrients to mammary gland or enhanced anabolic activity of the udder (e.g. rBST supplementation) have been observed to increase feed efficiency. Nitrogen efficiency also increased slightly from Met, Lys, and His infusion ( $P=0.09$ ). Overall, the average N efficiency across treatments was 38.5%, which is substantially higher than the average efficiency of 25% for lactating dairy cows fed conventional diets (Hristov et al., 2004) but similar to a previous jugular infusion trial (Appuhamy et al., 2011b). The average efficiency of MP conversion to milk protein was 56.3%, much higher than what has been observed in some previous experiments (Rius et al., 2010b) but slightly less than the efficiency encoded in common nutrition models (i.e. NRC, 2001 and CNCPS v6.55). Milk urea nitrogen was unaffected by treatment and below typical concentrations, indicative of the high N efficiency, averaging 8.44 mg/dL. The lack of treatment effects suggests the increased N intake ( $P < 0.001$ )

from the infusions likely offset the gain in milk protein yield and reduced catabolism of other AA that weren't infused. No change in MUN concentrations despite an increase in milk protein yield has been found in previous studies (Appuhamy et al., 2011b, Lee et al., 2012, Giallongo et al., 2016). Importantly, MUN content did not increase as has been observed from previous EAA infusions studies (Nichols et al., 2016), which probably indicates that increased supply is only marginally (10-25%) being synthesized into milk protein with instead a large proportion being catabolized. Feeding a low CP diet with a high energy concentration likely contributed to the above average N efficiency.

#### **6.5.4. Arterial Plasma AA**

The arterial plasma concentrations of infused EAA all increased as expected (Table 5). This differs from some previous jugular infusion trials where Leu and Ile concentrations did not increase in response to Leu and Ile infusions (Appuhamy et al., 2011b, Kassube et al., 2017). Leu ( $P=0.03$ ), Phe ( $P<0.01$ ), Thr ( $P<0.001$ ), and Val ( $P=0.06$ ) all declined in arterial concentration in response to MKH, which was likely driven by increased demand by mammary tissue in support of greater milk protein yield. Previously, jugular infusions of Met and Lys increased milk protein yield but did not contribute to a decline in plasma concentrations of other EAA except for Ile (Appuhamy et al., 2011b, Kassube et al., 2017). However, dietary CP (16.0%) and MUN (12.5 mg/dl) were much higher for these prior studies than for this study, which may have buffered changes in the concentrations of those other EAA. Two of the EAA that decreased in concentration, Phe and Thr, had net uptake to milk protein output ratios that averaged 1.00 and 0.98 respectively, indicating that mammary catabolism of these EAA was not occurring. Threonine is generally taken up in excess of milk protein demand as it is considered a group 2 EAA (Lapierre et al., 2012b), thus the ratio near 1 for this work may indicate tight supplies of Thr. However, Thr venous concentration was still approximately 48.6  $\mu M$  for the

MKH treatment, which is 19.2% less than the CON ( $P = 0.04$ ) (Supplemental materials, Table 5).

Gly ( $P=0.02$ ), Ser ( $P=0.12$ ), and Tyr ( $P<0.01$ ) also had decreased arterial plasma concentrations. This observation is not usually observed with cows supplemented with Met, Lys, and His (Lee et al., 2012, Giallongo et al., 2016), however, the high level of protein yield coupled with a 14% deficit in MP might result in a tight supply of AA in general, hence, the drawdown of Gly, Ser, and Tyr. The Tyr venous concentration fell 81% in response to MKH infusion. Minimal synthesis of Tyr occurs in the mammary gland, observed to be 3 to 5% of Tyr derived from Phe in lactating goats (Bequette et al., 1999), so increased protein yield likely drove much greater extraction from the arterial blood.

Total AA, essential AA, and nonessential arterial plasma AA concentrations did not change in response to the AA infusions. Branched chain AA did decrease in response to MKH infusion ( $P=0.06$ ), reflecting increased demand for the milk protein since BCAA make up 50% of the EAA in milk protein. Branched chain AA did not change in response to IL treatment, likely because of offsetting decreases in Val ( $P=0.03$ ). Glucose infusion has often stimulated milk protein yield and has been observed to coincide with decreased BCAA concentrations (Raggio et al., 2006, Curtis et al., 2014, Nichols et al., 2016, Curtis et al., 2018). The decreases in concentrations presumably result at least partially from use for milk protein synthesis, but the glucose and resulting insulin signal may also stimulate extra-mammary use (Bequette et al., 2002). Supplementing methionine, lysine, or histidine usually does not cause a decrease in BCAA concentrations (Appuhamy et al., 2011b, Lee et al., 2012). However, again given the production level and MP deficit in our study, BCAA likely became short in supply. Reductions in the concentrations of the non-infused EAA and possibly some of the NEAA would act to

mitigate the stimulatory effects of the infused EAA, and this may explain the variability in responses to infused EAA. The concentration responses surface appears to be complicated given changes observed in multiple non-infused AA, but it must be mapped if we are to fully understand and predict responses to individual AA.

#### **6.5.5. Mammary Uptake of AA**

Mammary plasma flow increased by 24% ( $P=0.01$ ) in response to IL infusion whereas plasma flow numerically decreased by 9.5% ( $P=0.22$ ) for the MKH infusion (Table 6). Mammary plasma flow is highly correlated with milk yield (Prosser et al., 1996), hence, the 2.3 kg/d milk yield response from IL infusion correlates with the increased MPF. There are 2 potential reasons for the increased MPF: 1) greater anabolic activity associated with stimulation of mTORC1 that results in ADP buildup and release into the interstitium, which triggers nitric oxide synthesis from arginine (Cieslar et al., 2014), or 2) a shortage of one or more individual EAA (Cant et al., 2018) acting through an unknown mechanism. Using the regression equation of (Purdie et al., 2009), MPF was predicted to increase by 111 L/h for the IL treatment, and the observed increase was 180 L/h. Plasma flow stimulation has been observed in response to deficiencies in His, Lys, and Thr (Bequette et al., 2000, Doepel et al., 2016, Guo et al., 2017). This appears to be a mechanism to maintain an adequate supply of nutrients to the udder for maintenance of milk production even under deficient conditions. Clearly MKH and IL stimulated mammary activity which may have altered ADP concentrations. Plasma Thr concentrations also tended to decline ( $P=0.10$ ) which may have been the triggered for the local MPF response. Whether the change in MPF was caused by increased metabolism or shortage of an EAA remains to be elucidated. However, the plasticity of the mammary gland to increase milk protein yield without supplementation of the classic limiting EAA was demonstrated in this study.

Net uptake of several EAA increased in response to the treatment infusions and likely to support greater protein yield (Table 6). Amino acid substrates for greater yield must originate from either reduced catabolism or protein accretion in the mammary gland or greater uptake from the arterial blood. Despite the 9.5% numerical decline in MPF in response to MKH infusion, increased net uptake of Lys ( $P < 0.001$ ), Phe ( $P = 0.02$ ), Asn ( $P = 0.12$ ), Gln ( $P = 0.12$ ), Ser ( $P < 0.01$ ), and Tyr ( $P < 0.01$ ) did occur. Lysine is considered a group 2 EAA in that on average the net uptake to output in milk protein is 1.26 (Lapierre et al., 2012b). However as arterial supply increases, net uptake will increase, and the ratio of uptake to output will rise proportionally yielding even lower efficiency (Lapierre et al., 2012b). The reverse has been observed in goats in that as Lys supplies decrease, net uptake and the ratio of uptake to output will decrease proportionally (Guo et al., 2017). Venous concentration of Lys increased 32% ( $P < 0.01$ ) in response to MKH infusion which led to net udder uptake increasing by 32% ( $P < 0.001$ ). The Lys uptake to output ratio increased from 1.05 to 1.32 and the marginal transfer efficiency of the extra lysine into milk protein Lys was only 13%. The nitrogen in Lys can be used for synthesis of Gln, Glu, Asp, Asn, Ser, and Ala. It has been shown that Lys net uptake to milk protein output ratios approaching 1 result in reduced milk protein yield (Lapierre et al., 2009, Guo et al., 2017). Thus, some Lys catabolism is apparently obligatory, and one cannot assume that all EAA with uptake to output ratios greater than 1 are not deficient. Phe and Tyr uptake also increased from the MKH infusion, by 5.2% and 7.6%, which closely matched the milk protein response of 5.5%. Methionine uptake numerically increased by 12.7% ( $P = 0.22$ ) which is greater than the milk protein response. Infusion of DL-Met increases the proportion of D-Met in blood as the conversion to L-Met is slow (Lapierre et al., 2012a). As D-Met can be transported into cells, but not used other than conversion to L-Met, the lack of a significant

response in net uptake of Met may be due to masking of a net change in L-Met uptake. Histidine net uptake was unchanged ( $P=0.74$ ) as was the net uptake to output ratio ( $P=0.54$ ). Histidine is unique in that a significant portion of His supply can originate from peptides (carnosine, anserine) (Lapierre et al., 2008b). Plasma His could have been used by muscle to synthesize these peptides, and the peptides released to plasma for use by mammary tissue to synthesize milk protein (Lapierre et al., 2012b). Histidine catabolism by mammary tissue could have also been reduced. The latter should be reflected in uptake to output ratios, but His is subject to greater analytical variation (Lapierre et al., 2012b). The increase in net uptake of the other NEAA (Asn, Gln, and Ser) might reflect the increased demand to match the milk protein response or diminished catabolism of EAA for synthesis of NEAA due to a reduction in EAA availability. For example, Arg and BCAA net uptake did not change and these EAA serve as precursors for Gln, Ser, and Asn (Roets et al., 1979, Roets et al., 1983, Rubert-Aleman et al., 1999). The uptake to output ratio Leu ( $P=0.14$ ) and Val ( $P=0.10$ ) tended to decline, indicating less catabolism by mammary tissue. Overall, the aggregate of TAA ( $P=0.12$ ), EAA ( $P=0.08$ ), and NEAA ( $P=0.22$ ) all significantly, tended, or numerically increased in net uptake as one might expect given the increase in milk protein yield. Greater capture of arterial AA should reduce the amount of AA available for catabolism by the splanchnic tissue bed, and overall N efficiency did improve for the MKH treatment ( $P=0.09$ ).

The 24% increase in MPF with the IL infusion resulted in increased net uptake of Arg ( $P=0.02$ ), His ( $P=0.04$ ), Ile ( $P=0.04$ ), Leu ( $P<0.001$ ), and Phe ( $P<0.01$ ) (Table 6). Ile and Leu net uptake increased by 19% and 27% which compares closely to the 29.5 % and 34.6% increase in venous concentrations of Ile and Leu. Greater capture of these EAA likely increased intracellular concentrations which should facilitate greater activation of the mTORC1 pathway

and substrate for NEAA synthesis, the latter negated the need for greater uptake of Asn, Gln, and Ser as observed with the MKH infusion. Despite a 30  $\mu$ M drop in Val arterial concentration ( $P=0.03$ ), net uptake increased numerically ( $P=0.54$ ) which was driven by a higher Val clearance rate ( $P=0.09$ ) and MPF. The lack of response for Met and Tyr uptake is unknown as synthesis by the mammary gland is minimal to nonexistent for these AA; so how greater milk protein yield resulted without greater uptake is perplexing. In goats, as much as 18% of milk protein Met and 14% of milk protein Tyr may be derived from peptide uptake by the udder (Bequette et al., 1999). However, these values are overestimated as pointed out by (Hanigan et al., 1998c) due to very slow exchange with body protein pools but one cannot rule out contributions from peptides containing Met and Tyr.

#### **6.5.6. Mammary AA Clearance Rates**

Clearance of TAA increased ( $P=0.02$ ) with MKH infusion but did not change for IL infusion (Table 7). Assuming mammary demand is a determinant of transport activity, there may not have been a need for additional transport activity given the increase in TAA supply driven by increased MPF with the IL treatment. Histidine and Met clearance rates dropped by 31% ( $P=0.01$ ) and 64% ( $P<0.001$ ) respectively with the MKH infusion. These EAA generally are not taken up in excess when supply increases above mammary demand for milk protein (Lapierre et al., 2012b), so the dramatic decrease in clearance likely indicates these EAA were oversupplied with the MKH infusion. Clearance rates of Phe ( $P<0.001$ ), Thr ( $P=0.11$ ), Val ( $P=0.02$ ), Gln ( $P=0.12$ ), Gly ( $P=0.01$ ), and Ser ( $P=0.07$ ) all increased or tended to increase in response to MKH infusion which reflects greater affinity for extracting these AA from extracellular space and should reduce their availability for splanchnic catabolism. Clearance of Lys (which was also infused) did not change ( $P=0.95$ ) reflecting the variable catabolism in the cells. Clearance of

BCAA ( $P<0.01$ ) and EAA other than Met, Lys, and His ( $P=0.14$ ) increased in response to MKH infusion, which again reflects greater pull from mammary tissue for these AA.

Similar increases in clearance rates of non-infused AA were also observed with Arg ( $P=0.11$ ), His ( $P=0.02$ ), Val ( $P=0.09$ ), Ser ( $P=0.07$ ), and the EAA other than Ile and Leu ( $P=0.03$ ) with IL treatment infusion, again to support the greater milk protein yield (Table 7). Clearance rates of Ile and Leu did not change despite increased concentrations associated with infusion, which likely reflects the variable catabolism in cells of these AA.

#### **6.5.7. Mammary AA Uptake: Milk AA Output Ratio**

The capture of extracted TAA into milk protein averaged 90% across treatments, which precisely agrees with previous findings of (Cant et al., 1993) (Table 8). The capture of extracted His, Met, Phe, and Tyr averaged close to 100% whereas Arg, Ile, Leu, Lys, Thr, and Val were much higher. Histidine varied from 81 to 99% and this variation is expected with the analytical methods used for AA concentrations assessments. Of the infused EAA for MKH treatment, only Lys increased in the uptake to output ratio and this is usually observed (Lapierre et al., 2009). The uptake to output ratio declined for BCAA ( $P=0.07$ ) and the EAA less Met, Lys, and His which means less catabolism or transamination in the udder which might explain the decline in lactose concentration due to a reduction in cellular energy status. The ratio of one NEAA, Glu ( $P<0.01$ ), declined whereas the ratio for Ser increased ( $P=0.02$ ). Lysine is used to synthesize Glu (Lapierre et al., 2009) and the 31.7% increase in net Lys uptake flux likely reduced demand for extracellular Glu given a much larger Lys precursor pool to synthesize Glu. In contrast, Ser intracellular synthesis may have decreased due to reduced intracellular BCAA with the MKH treatment (Roets et al., 1979, Roets et al., 1983) thereby necessitating greater uptake ( $P<0.01$ ) to support milk synthesis. These changes are reflected in Ser higher observed ratio ( $P=0.07$ ). The Ser ratio was not affected by the IL treatment ( $P=0.62$ ) despite a similar increase in milk protein

yield, however, Leu and Ile net flux to the udder increased quite significantly, thereby providing substrate for Ser synthesis.

The ratio of Leu uptake to milk protein output increased by 21% ( $P < 0.01$ ) (Lapierre et al., 2012b) from the IL infusion (Table 8). This change is usually observed when increased amounts of Leu are present in arterial blood (Bequette et al., 1996). However, increased Ile supply with the IL treatment did not change the uptake to output ratio though it was numerically higher ( $P=0.14$ ). The ratio of His ( $P=0.10$ ) and Phe ( $P=0.07$ ) increased slightly with the IL treatment and were both close to unity. Greater MPF translated into greater net uptake of both of these EAA, which probably decreased need for delivery via peptides, thereby increasing the ratio closer to unity. Greater amounts of Gly relative to output were also observed ( $P=0.08$ ) with IL treatment which likely reflects reduced intracellular synthesis. This might have occurred given that the substrates for Gly synthesis are primarily Thr (Verbeke et al., 1972) and Val (Roets et al., 1979) and both of these declined in arterial concentration ( $P=0.10$  and  $P=0.03$ ) and were unchanged in net uptake, hence less availability for Gly synthesis. In general, changes in AA uptake to output ratios tended to be as expected in that changes in arterial supply translated to changes in flux and proportion catabolized by the udder. In some cases, these changes could be correlated to changes in certain NEAA. Again, the multidimensionality is clearly present in that a change in likely limiting AA supplies and corresponding milk protein yield affects the metabolism of many other AA.

#### **6.5.8. AA Efficiency to Milk Protein**

The observed efficiency of MP used for milk protein was much higher than the NRC (2001) would indicate, averaging 84.3% across treatments versus 67% encoded in the NRC (Table 9). The underprediction of milk yield by 10 to 11 kg/d by the NRC (2001) and CNCPS v6.55 models is likely caused by too low of efficiency (unlikely) or maintenance costs that are

estimated to be too high. If one assumes that maintenance needs reflect the AA profile of MP, then efficiencies of several EAA (His, Ile, Lys, Met, and Val) that are equal to or greater than 100% efficiency are not possible (Table 9). If one takes the approach of an overall efficiency (milk protein yield / MP supply) as recommended by (Lapierre et al., 2016), then the observed efficiencies for the aggregate and individual EAA seem much more appropriate. Indeed, the overall individual EAA efficiencies of the three treatments LSMs are very similar to what previously has been derived from model development literature datasets (VanAmburgh et al., 2015, Martineau et al., 2016). The total AA marginal efficiency of the treatments was much higher than what is commonly observed from infused EAA or casein (15-25%) (Hanigan et al., 1998a, Doelman et al., 2015a), ranging from 43 to 77%. The marginal efficiencies of MKH and IL treatments alone were less than overall efficiency, implying declining responses as supply increases, whereas the combination of MKH and IL increased marginal efficiency above the overall efficiency, implying that all AA as a sum were used more efficiently for milk protein. That the marginal efficiency did not improve for the MKH treatment which involved commonly described limiting EAA implies one or more of those EAA were oversupplied or that other EAA were co-limiting. The barrel-stave theory implies that marginal efficiency of MP must increase if these limiting EAA are supplemented.

Interestingly, the overall efficiencies of two of the commonly limiting EAA (Met and Lys) were much higher in the CON treatment at 0.90 and 0.77 than the 0.66 and 0.69 previously derived and encoded in CNCPS v6.55 (VanAmburgh et al., 2015). Efficiency of use for an individual EAA varies depending on supply level relative to requirement as well as other factors (Doepel et al., 2004). The marginal efficiency of Met, Lys, and His were extremely low, 9%, 13%, and 9% for incorporation into milk protein in the MKH treatment. This infers excessive

catabolism by the splanchnic tissue bed or demand for protein synthesis by extra-mammary tissues for Met and His whereas most of the Lys seemed to be taken up by the udder but catabolized. When Ile and Leu were added to Met, Lys, and His, the marginal efficiency doubled to 20%, 31%, and 20% respectively. For Ile and Leu marginal efficiencies, changes in response to IL and MKH+IL treatments largely followed Met, Lys, and His. If one followed a strict interpretation of the barrel and stave approach, the marginal efficiency of a limiting AA should not be less than its overall efficiency if the requirement is precisely met. In our case as an example, 91% of the extra methionine that was supplied in a diet balanced according to NRC (2001) and CNCPS v6.55 recommendations was catabolized or used by extra-mammary tissues and not milk protein yield. This infers the AA requirements are possibly wrong or that other factors limited milk protein yield such as other AA or energy. The derived fixed AA efficiency in CNCPS v6.55 was based on 8 studies (45 treatment means) consisting of dose response studies to Met and Lys with no consideration for other EAA. The Met and Lys efficiencies proposed by the optimal supplies relative to energy within CNCPS v6.55 are 57 and 67% respectively (Tylutki and VanAmburgh, 2015), which interestingly infer a lower efficiency than BCAA (Leu = 73%, Ile = 67%, and Val = 68%) which seems highly unlikely given the use of BCAA for udder catabolism and transamination (Lapierre et al., 2012b). The derived marginal efficiency for MP-Met to milk protein was 11.2% given the regression equation provided by (VanAmburgh et al., 2015) (assumed average supply of MP to be 2500 g/d in dataset) and this is the marginal efficiency below the prescribed requirement of 2.6% of MP. A similar calculation can be made for the NRC (2001) in that the marginal efficiency for Met is 9.4% (assumed average supply of MP to be 2500 g/d in dataset), which is nearly identical to the 9% observed Met marginal efficiency in our study. Our observations that infusion of other EAA (Ile and Leu)

doubled the marginal efficiency of Met, Lys, and His implies responses depend on more EAA than currently described in ration models. The low marginal efficiency of Met and His, the dramatic decrease in udder clearance of these EAA, and numerically reduced MPF may imply that these EAA were oversupplied and that model requirements are possibly incorrect.

Application of Oser's index (Oser, 1959) as described by (Chandler and Cawood, 2010) for diet AA formulation instead of barrel-stave approach (NRC, 2001) of considering the single most limiting EAA appears to be a better representation of recent studies (Arriola Apelo et al., 2014d, Liu et al., 2017) and this study in that supplies of multiple EAA appear to determine the level of response.

## **6.6. Conclusion**

Supplementing two groups of EAA; 1) Met, Lys, and His and 2) Ile and Leu, independently and additively increased milk protein yield in high producing dairy cows. These outcomes directly contradict the single-limiting AA theory for regulation of milk protein yield and instead, point towards regulation beyond simply substrate supply. Increased supplies of Ile and Leu also increased milk and ECM yields in dairy cows, which appeared to be facilitated by increased mammary plasma flow. Additionally, Met, Lys, and His supplementation improved nitrogen efficiency whereas Ile and Leu improved gross feed efficiency. Increased milk protein yield by these two groups of AA was assisted by changes in arterial AA concentrations, mammary plasma flow, and udder clearance rates of AA which yielded altered net uptake. There were also changes in the proportion of AA catabolized and used for milk protein which demonstrated plasticity in mammary protein metabolism. These results confirm our hypotheses based on in-vitro experimental data and observations that barrel-stave AA theory performs poorly in explaining in-vivo milk protein yields in that multiple EAA can independently affect milk protein yield. Nutritionists should be aware that the single EAA theory likely does not hold

and that other EAA beyond Met, Lys, and His are critical in optimizing dairy cattle performance when formulating lactating cow diets based on these results.

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**Table 6. 1.** Average ingredient composition of diet (% of DM)

Item	Mean	Standard Deviation
Corn Silage	38.11	2.40
Alfalfa Hay	9.37	1.17
Wet Brewers	9.26	0.81
Corn Grain	20.17	4.41
Soybean Meal	7.43	0.48
Citrus Pulp Dry	6.39	0.41
Soybean hulls, ground	4.04	0.26
Palmitic enriched fat supplement <sup>1</sup>	1.12	0.07
Sodium Bicarbonate <sup>2</sup>	0.943	0.061
Calcium Carbonate	0.600	0.039
Potassium Carbonate <sup>3</sup>	0.495	0.032
Molasses	0.314	0.020
Dicalcium phosphate	0.419	0.027
Urea	0.210	0.014
Sodium Bentonite <sup>4</sup>	0.191	0.012
Omnigen <sup>5</sup>	0.171	0.011
Salt	0.181	0.012
Magnesium Oxide	0.171	0.011
Potassium Chloride Red	0.162	0.010
Magnesium Potassium Sulfate	0.086	0.006
Flow matrix <sup>6</sup>	0.048	0.003
Tracer minerals and vitamins <sup>7</sup>	0.067	0.004

<sup>1</sup>Palmit 80, Global Agri-Trade Corp, Long Beach, CA

<sup>2</sup>Church & Dwight Co., Inc. Princeton, NJ

<sup>3</sup>DCAD PLUS, Church & Dwight Co., Inc. Princeton, NJ

<sup>4</sup>AB-20, Prince Agri Products, Inc., Quincy, IL

<sup>5</sup>Prince Agri Products, Inc., Quincy, IL

<sup>6</sup>PMI Nutritional Additives, Arden Hills, MN

<sup>7</sup>Contained 221 mg of Mn, 140 mg of Cu, 2 mg of Fe, 397 mg, and 15 mg of Co from Availa 4 (Zinpro Corp., Eden Prairie, MN), 16 mg of I (calcium iodate), 7.3 mg of Se (Sel-Plex 2000, Alltech Inc., Nicholasville, KY), 131 kIU of vitamin A, 30 kIU of vitamin D, and 550 IU of vitamin E.

**Table 6. 2.** Nutrient composition of diet (DM basis)

	Mean	Standard Deviation
CP	15.19	0.45
RDP <sup>1</sup>	9.98	-
RUP <sup>1</sup>	5.21	-
NDF	28.48	1.03
uNDF	9.74	-
peNDF	18.32	-
Starch	31.1	2.22
Sugar	4.87	-
Fat	4.89	0.08
PUFA	2.47	-
Ca	0.69	0.05
P	0.36	0.01
K	1.36	0.07
Mg	0.30	0.02
NEL, mcal/kg <sup>1</sup>	1.69	-
MP, % DM <sup>1</sup>	9.91	-

<sup>1</sup>Calculated (CNCPS v6.55) using observed DMI, feed composition, and period average BW.

**Table 6. 3.** Metabolizable amino acid supply as predicted by NRC (2001) given observed DMI, infused AA, milk and component yields, and BW expressed as percentage of metabolizable protein and supply relative to AA requirement

Item	Treatment <sup>1</sup>			
	CON	MKH	IL	MKH+IL
MP allowable milk, kg/d	37.0	38.0	39.6	39.6
MP, g/d	2599	2704	2689	2801
MP balance, g/d	-478	-468	-451	-513
RDP balance, g/d	-25	-23	-22	-23
RUP balance, g/d	-574	-656	-680	-797
<b>Histidine</b>				
Suggested requirement, g/d	68	70	69	73
Diet supply, g/d	57	57	57	58
Jugular infusion, g/d	0	20	0	20
Balance, g/d	-11	7	-12	5
Percent of MP	2.19	2.85	2.12	2.78
<b>Isoleucine</b>				
Diet supply, g/d	126	127	127	128
Jugular infusion, g/d	0	0	22	22
Percent of MP	4.85	4.70	5.54	5.36
<b>Leucine</b>				
Diet supply, g/d	231	233	233	236
Jugular infusion, g/d	0	0	50	50
Percent of MP	8.89	8.62	10.52	10.21
<b>Lysine</b>				
Suggested requirement, g/d	203	209	207	219
Diet Supply, g/d	166	168	168	169
Jugular infusion, g/d	0	38	0	38
Balance, g/d	-37	-3	-39	-12
Percent of MP	6.39	7.62	6.25	7.39
<b>Methionine</b>				
Suggested requirement, g/d	68	70	69	73
Diet supply, g/d	49	49	49	50
Jugular infusion, g/d	0	21	0	21
Balance, g/d	-19	0	-20	-2
Percent of MP	1.89	2.59	1.82	2.53

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

**Table 6. 4.** Effect of AA infusions on intake, milk production parameters, nutrient efficiency, and body weight (least-square treatment means)

Item	Treatment <sup>1</sup>				SEM	Effect ( <i>P</i> -value)		
	CON	MKH	IL	MKH + IL		MKH	IL	MKH*IL
<i>Intake</i>								
DMI, kg/d	25.3	25.6	25.5	25.8	0.41	0.45	0.31	0.90
Dietary CP, kg/d	3.85	3.87	3.88	3.92	0.06	0.45	0.31	0.90
Infused AA, g/d	0	78.9	71.9	150.8	0.04	<0.001	<0.001	0.53
Total CP, kg/d	3.85	3.96	3.95	4.06	0.06	0.01	0.01	0.99
<i>Milk production</i>								
Milk, kg/d	47.3	47.8	49.5	50.3	0.96	0.31	0.001	0.86
ECM, kg/d <sup>2</sup>	47.2	48.3	49.1	50.1	1.03	0.14	<0.01	0.93
Milk protein %	3.10	3.19	3.04	3.21	0.04	<0.001	0.45	0.09
Milk protein, g/d	1458	1517	1498	1603	32	<0.001	<0.01	0.28
Milk fat %	3.58	3.55	3.55	3.42	0.11	0.29	0.28	0.48
Milk fat, g/d	1675	1716	1744	1741	56	0.60	0.22	0.56
Lactose, %	5.04	4.99	5.05	4.97	0.03	<0.001	0.67	0.48
Lactose, g/d	2389	2388	2505	2508	53	0.98	<0.01	0.97
MUN (mg/dl)	8.17	8.68	8.50	8.40	0.53	0.55	0.94	0.39
<i>Efficiency</i>								
Protein efficiency <sup>3</sup>	38.1	38.1	38.3	39.6	0.01	0.09	0.19	0.18
GFE <sup>4</sup>	1.85	1.87	1.93	1.91	0.06	0.99	0.07	0.68
BW, kg/d	668	672	675	677	15.5	0.52	0.24	0.97
BW change, kg/d	-13.2	-13.5	-14.0	-8.46	9.94	0.69	0.76	0.66

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>ECM=energy-corrected milk calculated as (12.82 x kg of fat) + (7.13 x kg of protein) + (0.323 x kg of milk).

<sup>3</sup>Protein efficiency calculated as milk protein yield (kg/d) / total CP intake (kg/d).

<sup>4</sup>Gross feed efficiency calculated as ECM yield (kg/d) / DMI (kg/d).

**Table 6. 5.** Arterial amino acid plasma concentrations (least-square treatment means)<sup>1</sup>

Item	Treatments <sup>2</sup>				SEM	Effect ( <i>P</i> -value)		
	CON	MKH	IL	MKH + IL		MKH	IL	MKH*IL
Arg	80.8	87.6	81.8	83.7	4.6	0.20	0.66	0.45
His	64.7	84.8	63.4	84.0	3.0	<0.001	0.65	0.93
Ile	127.3	137.1	158.2	136.3	9.6	0.32	0.02	0.02
Leu	201.2	189.1	267.8	239.3	16.9	0.03	<0.001	0.33
Lys	85.2	121.4	87.6	109.6	5.9	<0.001	0.20	0.05
Met	23.6	45.9	23.6	43.4	2.2	<0.001	0.52	0.52
Phe	46.2	44.5	48.2	42.5	2.0	<0.01	0.98	0.11
Thr	99.0	89.0	95.4	83.2	4.9	<0.001	0.10	0.69
Val	285.6	269.5	263.9	231.8	22.2	0.06	0.03	0.51
Ala	265.9	270.8	270.7	271.4	20.5	0.76	0.78	0.82
Asn	22.9	23.0	23.3	21.4	2.2	0.53	0.68	0.48
Asp	13.8	14.3	13.0	13.5	1.4	0.59	0.43	0.98
Gln	146.0	143.3	152.0	146.1	11.1	0.23	0.23	0.65
Glu	109.9	104.8	106.3	108.4	9.5	0.68	0.99	0.31
Gly	291.8	252.0	280.3	257.0	31.7	0.02	0.79	0.49
Pro	108.6	109.3	109.0	110.3	5.6	0.77	0.83	0.92
Ser	90.4	84.9	91.7	84.3	6.5	0.12	0.93	0.797
Tyr	35.4	31.8	35.3	27.9	2.7	<0.01	0.22	0.24
TAA <sup>3</sup>	2103	2109	2167	2115	57.8	0.61	0.45	0.53
EAA <sup>4</sup>	1023	1078	1087	1060	55.8	0.67	0.50	0.21
BCAA <sup>5</sup>	623	596	688	608	48.8	0.06	0.18	0.34
-MKH <sup>6</sup>	849	817	912	817	50.8	0.05	0.32	0.30
-IL <sup>7</sup>	685	749	664	682	29.5	0.03	0.02	0.21
NEAA <sup>8</sup>	1089	1041	1080	1046	54.5	0.19	0.95	0.81

<sup>1</sup>Data represent average AA concentrations sampled from the coccygeal vessels for day 9, 12, and 16 of the experimental period.

<sup>2</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>3</sup>TAA = total amino acids (EAA + NEAA).

<sup>4</sup>EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val.

<sup>5</sup>BCAA = Ile, Leu, and Val.

<sup>6</sup>-MKH = Arg, Ile, Leu, Phe, Thr, and Val.

<sup>7</sup>-IL = Arg, His, Lys, Met, Phe, Thr, and Val.

<sup>8</sup>NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

**Table 6. 6.** Mammary plasma flow and net uptake of amino acids (least-square treatment means)<sup>1</sup>

Item	Treatment <sup>2</sup>				SEM	Effect ( <i>P</i> -value)		
	CON	MKH	IL	MKH + IL		MKH	IL	MKH*IL
Mammary plasma flow (MPF), L/h								
MPF, L/h	807	665	928	905	86.7	0.22	0.01	0.37
Net mammary uptake, mmol/h								
Arg	29.0	29.6	34.4	33.5	3.4	0.93	0.02	0.71
His	9.7	9.2	12.1	11.9	1.3	0.74	0.04	0.9
Ile	35.4	40.5	46.2	44.0	4.1	0.65	0.04	0.25
Leu	59.8	58.1	74.8	74.4	5.1	0.75	<0.001	0.85
Lys	38.7	50.4	38.6	53.0	3.5	<0.001	0.52	0.48
Met	12.1	13.8	11.6	12.7	1.6	0.22	0.51	0.78
Phe	18.9	19.5	19.8	21.2	0.9	0.02	<0.01	0.25
Thr	23.5	23.9	25.0	23.4	2.0	0.69	0.74	0.52
Val	52.6	46.0	52.4	51.0	5.6	0.31	0.54	0.50
Ala	32.3	31.4	32.5	38.2	10.4	0.72	0.62	0.62
Asn	7.0	8.8	8.5	9.3	1.0	0.12	0.23	0.59
Asp	4.4	4.3	3.4	3.5	1.1	0.95	0.38	0.91
Gln	27.3	30.4	29.6	33.9	3.3	0.12	0.22	0.8
Glu	37.2	28.7	40.6	38.6	5.1	0.21	0.12	0.43
Gly	10.6	14.7	20.8	24.7	5.9	0.43	0.06	0.98
Pro	17.2	15.5	16.9	19.5	2.9	0.85	0.44	0.37
Ser	18.2	22.2	18.9	24.8	3.0	<0.01	0.30	0.53
Tyr	18.2	19.7	18.8	20.1	0.7	<0.01	0.28	0.91
TAA <sup>3</sup>	576	722	565	624	73.4	0.12	0.40	0.5
EAA <sup>4</sup>	352	447	354	375	34.9	0.08	0.28	0.25
BCAA <sup>5</sup>	187	223	193	190	20.9	0.39	0.47	0.31
-MKH <sup>6</sup>	278	335	282	283	28.8	0.28	0.37	0.29
-IL <sup>7</sup>	231	296	221	244	23.8	0.05	0.15	0.31
NEAA <sup>8</sup>	224	274	212	249	42.0	0.22	0.59	0.85

<sup>1</sup>Data represents arterial and venous measurements (n=6 to 11 samples per cow) taken on d 16.

<sup>2</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>3</sup>TAA = total amino acids (EAA + NEAA).

<sup>4</sup>EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val.

<sup>5</sup>BCAA = Ile, Leu, and Val.

<sup>6</sup>-MKH = Arg, Ile, Leu, Phe, Thr, and Val.

<sup>7</sup>-IL = Arg, His, Lys, Met, Phe, Thr, and Val.

<sup>8</sup>NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

**Table 6. 7.** Mammary clearance rates (L/h) of amino acids (least-square treatment means)<sup>1</sup>

Item	Treatments <sup>2</sup>				SEM	Effect ( <i>P</i> -value)		
	CON	MKH	IL	MKH + IL		MKH	IL	MKH*IL
Arg	768	703	905	883	133	0.65	0.11	0.82
His	195	136	271	187	27.6	0.01	0.02	0.61
Ile	451	545	430	476	90.4	0.33	0.53	0.74
Leu	545	658	418	529	109	0.20	0.15	0.99
Lys	1219	1084	1193	1313	164	0.95	0.42	0.31
Met	1592	624	1868	635	321	<0.001	0.43	0.46
Phe	880	1155	818	1367	121	<0.001	0.30	0.06
Thr	394	495	425	528	66.0	0.11	0.60	0.99
Val	243	230	214	346	34.3	0.02	0.09	<0.01
Ala	180	150	168	221	60.5	0.73	0.40	0.23
Asn	727	902	718	956	189	0.16	0.88	0.84
Asp	740	807	605	678	269	0.76	0.56	0.99
Gln	273	345	280	328	57.5	0.12	0.91	0.75
Glu	706	554	639	639	137	0.48	0.93	0.48
Gly	49	69	39	118	28.4	0.01	0.26	0.10
Pro	219	195	238	287	55.2	0.79	0.26	0.44
Ser	347	506	503	707	144.0	0.07	0.07	0.81
Tyr	-989	-334	4349	185	1978	0.31	0.10	0.17
TAA <sup>3</sup>	308	334	287	404	36.2	0.02	0.37	0.11
EAA <sup>4</sup>	430	454	444	528	50.1	0.22	0.31	0.48
BCAA <sup>5</sup>	362	401	289	446	51.0	<0.01	0.64	0.06
-MKH <sup>6</sup>	401	446	413	509	53.9	0.14	0.42	0.57
-IL <sup>7</sup>	411	416	465	541	43.8	0.31	0.03	0.37
NEAA <sup>8</sup>	226	241	243	307	44.4	0.26	0.24	0.47

<sup>1</sup>Data represents arterial and venous measurements (n=6 to 11 samples per cow) taken on d 16.

<sup>2</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>3</sup>TAA = total amino acids (EAA + NEAA).

<sup>4</sup>EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val.

<sup>5</sup>BCAA = Ile, Leu, and Val.

<sup>6</sup>-MKH = Arg, Ile, Leu, Phe, Thr, and Val.

<sup>7</sup>-IL = Arg, His, Lys, Met, Phe, Thr, and Val.

<sup>8</sup>NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

**Table 6. 8.** Effect of AA infusions on mammary AA uptake:milk protein output (least-square treatment means)<sup>1</sup>

Item	Treatments <sup>2</sup>				SEM	Effect ( <i>P</i> -value)		
	CON	MKH	IL	MKH + IL		MKH	IL	MKH*IL
Arg	2.28	2.21	2.41	2.36	0.25	0.48	0.15	0.91
His	0.85	0.78	1.02	0.96	0.11	0.54	0.10	0.97
Ile	1.25	1.35	1.54	1.38	0.13	0.76	0.14	0.23
Leu	1.26	1.16	1.51	1.41	0.08	0.14	<0.01	0.98
Lys	1.08	1.32	1.03	1.31	0.08	<0.001	0.60	0.72
Met	0.97	1.07	0.93	0.96	0.12	0.49	0.39	0.71
Phe	1.00	0.98	1.01	1.01	0.02	0.30	0.07	0.57
Thr	1.00	0.98	1.02	0.90	0.07	0.26	0.66	0.46
Val	1.47	1.23	1.40	1.28	0.14	0.10	0.94	0.57
Ala	1.36	1.31	1.32	1.48	0.41	0.82	0.80	0.68
Asn	0.35	0.42	0.41	0.43	0.04	0.27	0.42	0.52
Asp	0.26	0.24	0.19	0.19	0.06	0.84	0.24	0.84
Gln	0.67	0.71	0.70	0.75	0.07	0.39	0.50	0.92
Glu	0.70	0.57	0.65	0.53	0.04	<0.01	0.13	0.83
Gly	0.66	0.91	1.23	1.44	0.35	0.44	0.08	0.94
Pro	0.31	0.27	0.29	0.32	0.05	0.90	0.74	0.39
Ser	0.47	0.55	0.48	0.58	0.08	0.02	0.62	0.75
Tyr	0.94	0.96	0.92	0.93	0.02	0.30	0.07	0.57
TAA <sup>3</sup>	0.88	0.87	0.92	0.92	0.05	0.89	0.36	0.96
EAA <sup>4</sup>	1.23	1.22	1.33	1.26	0.07	0.42	0.14	0.48
BCAA <sup>5</sup>	1.33	1.23	1.48	1.31	0.09	0.07	0.11	0.58
-MKH <sup>6</sup>	1.32	1.24	1.45	1.29	0.08	0.06	0.15	0.48
-IL <sup>7</sup>	1.21	1.22	1.24	1.23	0.07	0.98	0.62	0.86
NEAA <sup>8</sup>	0.61	0.59	0.59	0.65	0.07	0.73	0.73	0.59

<sup>1</sup>Data represents arterial and venous measurements (n=6 to 11 samples per cow) taken on d 16 and average milk AA yield from d 15 to d 17 between 1300 to 0100 h.

<sup>2</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>3</sup>TAA = total amino acids (EAA + NEAA).

<sup>4</sup>EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val.

<sup>5</sup>BCAA = Ile, Leu, and Val.

<sup>6</sup>-MKH = Arg, Ile, Leu, Phe, Thr, and Val.

<sup>7</sup>-IL = Arg, His, Lys, Met, Phe, Thr, and Val.

<sup>8</sup>NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

**Table 6. 9.** Effect of AA infusions on overall and marginal MP and individual EAA efficiency conversion to milk protein<sup>1</sup>

Item	Treatment <sup>2</sup>										
	CON		MKH			IL			MKH+IL		
	NRC (2001)	Overall <sup>3</sup>	NRC (2001)	Overall	Marginal	NRC (2001)	Overall	Marginal	NRC (2001)	Overall	Marginal
TAA	0.85	0.56	0.84	0.56	0.55	0.83	0.56	0.43	0.85	0.57	0.77
Arg	0.67	0.44	0.68	0.46	-	0.67	0.45	-	0.70	0.48	-
His	1.12	0.74	0.87	0.57	0.09	1.14	0.76	-	0.88	0.59	0.20
Ile	1.09	0.72	1.13	0.74	-	0.93	0.62	0.11	0.98	0.66	0.37
Leu	0.99	0.65	1.02	0.67	-	0.82	0.55	0.08	0.85	0.58	0.27
Lys	1.17	0.77	0.98	0.64	0.13	1.17	0.78	-	1.00	0.68	0.31
Met	1.37	0.90	1.00	0.66	0.09	1.39	0.93	-	1.01	0.68	0.20
Phe	0.88	0.58	0.91	0.60	-	0.89	0.59	-	0.92	0.63	-
Thr	0.82	0.54	0.85	0.56	-	0.82	0.55	-	0.85	0.58	-
Val	1.09	0.72	1.12	0.73	-	1.09	0.73	-	1.14	0.77	-

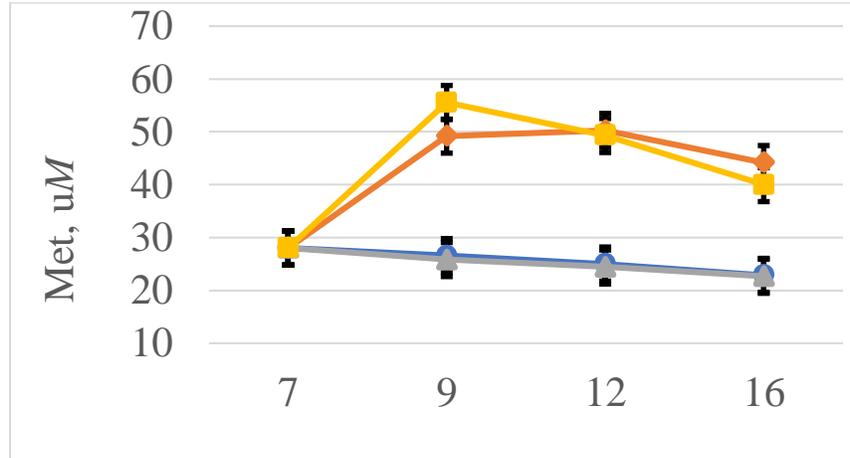
<sup>1</sup>Data represents least square means of intake, infusion, and milk production.

<sup>2</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

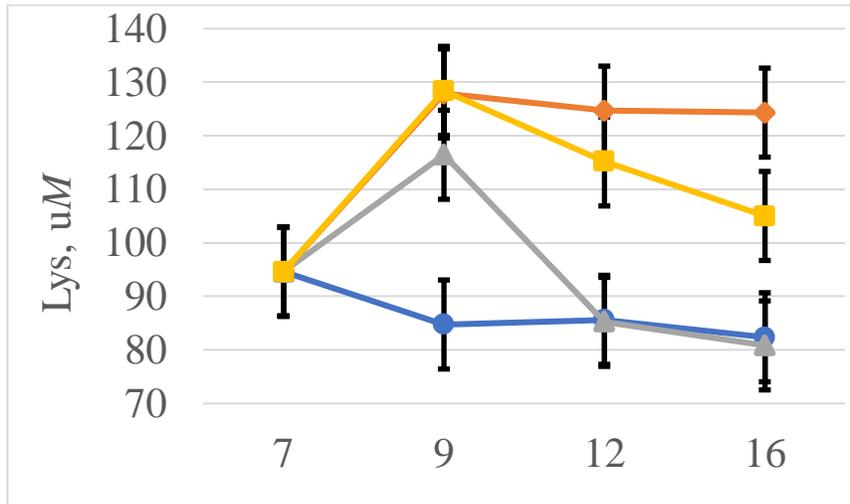
<sup>3</sup>Overall efficiency calculated by MP-AA supply / milk AA yield as described by (Lapierre et al., 2016)

# FIGURES

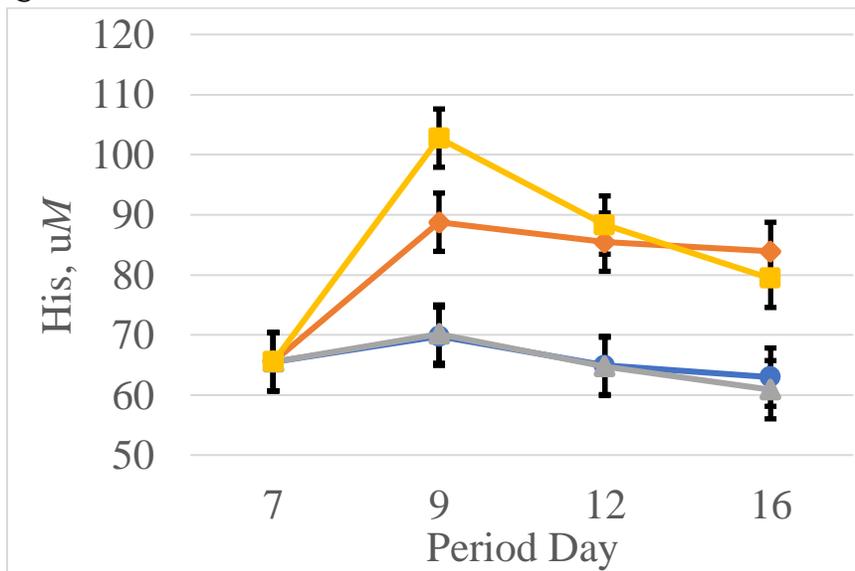
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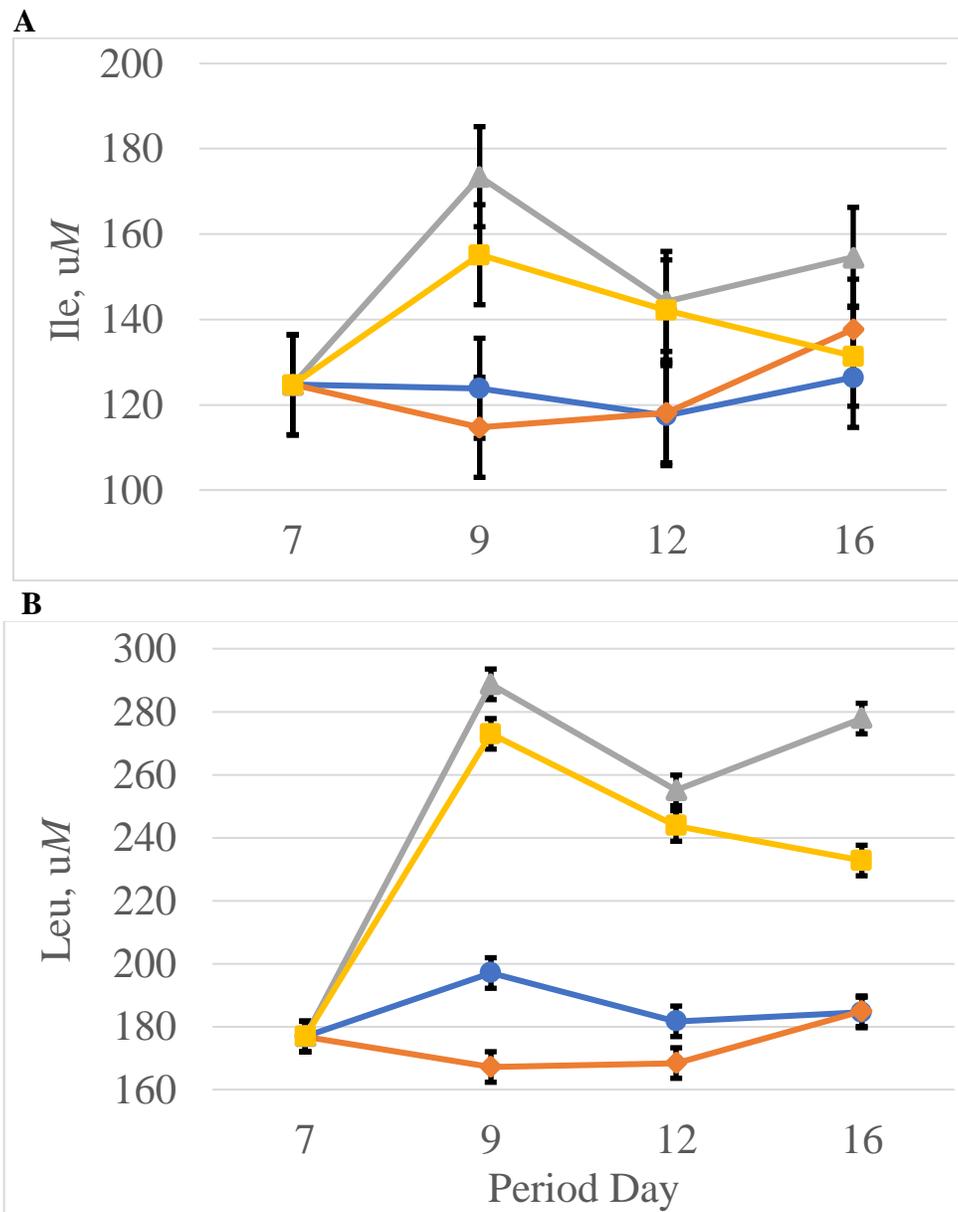
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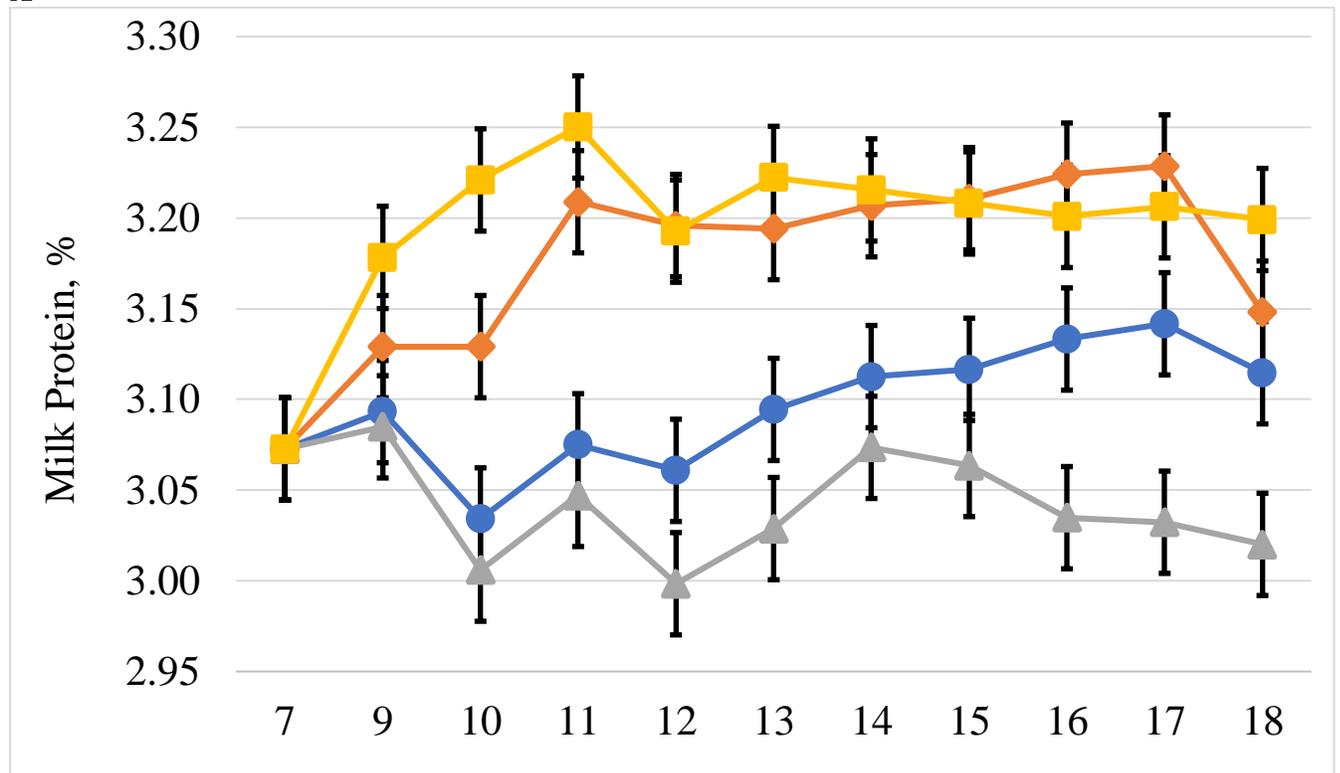
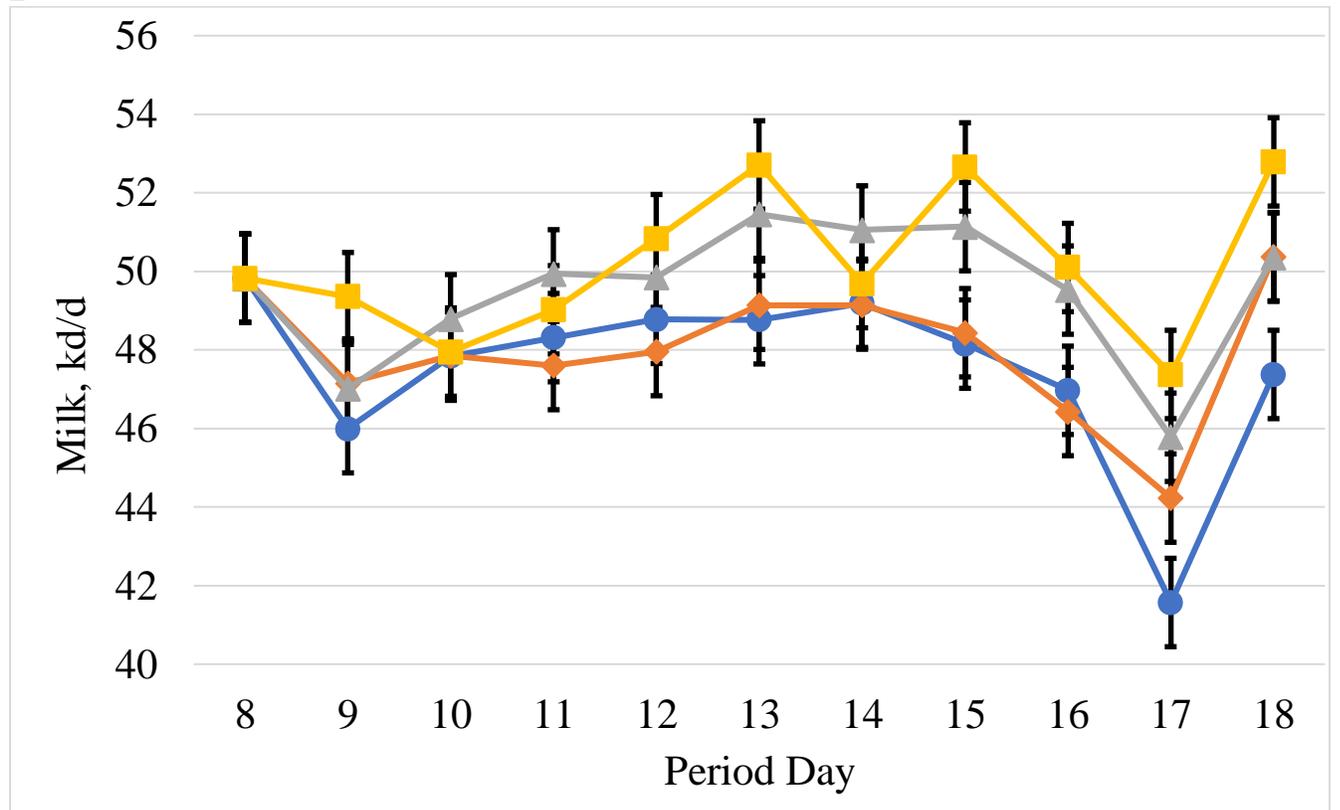
C



**Figure 6. 1.** Arterial Met (A), Lys (B), His (C) concentrations. Jugular infusion started on d 8 at approximately 1600 h. Day 7, 9, and 12 represent an average of two blood samples per cow whereas Day 16 represents average of 6 to 11 samples per cow. The CON treatment (blue line, circle) consisted of infused saline, the MKH treatment was infused Met, Lys, and His (orange line, diamond), the IL treatment was infused Ile and Leu (grey line, triangle) and the MKH + IL treatment was infused Met, Lys, His, Ile, and Leu (yellow line, square).



**Figure 6. 2.** Arterial Ile (A) and Leu (B) concentrations. Jugular infusion of treatments started on d 8 at approximately 1600 h. Day 7, 9, and 12 represent average of two blood samples per cow whereas Day 16 represents average of 6 to 11 samples per cow. The CON treatment (blue line, circle) consisted of infused saline, the MKH treatment was infused Met, Lys, and His (orange line, diamond), the IL treatment was infused Ile and Leu (grey line, triangle) and the MKH + IL treatment was infused Met, Lys, His, Ile, and Leu (yellow line, square).

**A****B**

**Figure 6. 3.** Milk protein concentration (A) and milk yield (B) by period day. Jugular infusion of treatments started on d 8 at approximately 1600. The CON treatment (blue line, circle) consisted of infused saline, the MKH treatment was infused Met, Lys, and His (orange line, diamond), the IL treatment was infused Ile and Leu (grey line, triangle) and the MKH + IL treatment was infused Met, Lys, His, Ile, and Leu (yellow line, square).

## 6.9. Supplemental Materials

**Table 6.S 1.** Composition of major dietary ingredients (DM basis)

Item	Corn Silage		Corn Grain		Alfalfa Hay		Wet Brewers		Grain Mix	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dry Matter	39.4	0.87	87.3	0.53	88.1	0.39	31.0	0.37	90.4	0.15
CP	7.83	0.47	8.05	0.25	19.7	1.25	35.6	2.57	19.9	1.96
ADF	21.7	0.53	4.45	0.13	30.8	2.09	22.4	1.06	18.8	1.11
NDFom	36.1	0.65	11.3	0.29	36.4	2.39	44.8	1.97	23.2	1.77
Lignin	2.78	0.17	2.50	0.09	7.76	0.50	7.19	0.78	1.63	0.59
NDFd30	57.7	1.39			34.6	1.75				
NDFd120	67.6	2.12			37.8	3.20				
NDFd240	69.8	2.11			40.7	3.75				
uNDF240	10.9	0.94			21.7	2.61				
ADICP	0.46	0.04	0.39	0.00	1.34	0.06			0.84	0.09
NDICP	0.59	0.03	0.94	0.03	2.09	0.19			1.72	0.07
Soluble CP	4.53	0.29	1.30	0.00	8.23	0.47			3.87	0.47
Ammonia	0.98	0.07	0.10	0.01	1.02	0.21				
Starch	40.4	0.60	71.8	0.37	2.53	0.34	4.68	1.27	9.38	0.83
7-hr Starch	77.9	0.69	55.1	1.16						
Fat	3.20	0.14	4.01	0.21	2.39	0.14	11.0	0.37	2.48	0.27
Fatty Acids	2.83	0.09	3.89	0.21	1.29	0.11				
Ash	2.85	0.36	1.16	0.08	9.17	0.62	4.33	0.20	12.9	1.46
Ca	0.15	0.01	0.03	0.01	1.42	0.10	0.31	0.02	2.05	0.08
P	0.22	0.01	0.29	0.01	0.28	0.01	0.55	0.04	0.58	0.09
Mg	0.18	0.01	0.11	0.01	0.25	0.02	0.17	0.02	0.51	0.08
K	0.87	0.06	0.40	0.02	3.05	0.32	0.10	0.04	2.39	0.20
S	0.13	0.01	0.11	0.01	0.30	0.03				
Na	0.01	-	0.02	0.01	0.07	0.03	0.02	0.01	1.28	0.17
sugar	1.08	0.15	2.75	0.06	10.6	0.97				
Silage acids	5.38	0.44								
Lactic acid	2.64	0.57								
Acetic acid	2.72	0.17								
Mn	18.3	2.06	8.00	1.41	42.0	6.38	55.5	7.00	85	11.3
Zn	28.3	2.63	25.3	2.75	26.3	5.68	110	11.1	109	13.2
Cu	6.00	0.82	3.25	0.50	9.25	0.96	14.8	2.87	32.5	3.32
Fe	44.0	11.3	30.0	4.08	199	44.5	155	13.5	715	131.2
pH	4.06	0.05	5.76	0.04						

<sup>1</sup>Ash corrected neutral detergent fiber

- <sup>2</sup>In-vitro digestibility percent of NDF at 30 h
- <sup>3</sup>In-vitro digestibility percent of NDF at 120 h
- <sup>4</sup>In-vitro digestibility percent of NDF at 240 h
- <sup>5</sup>In-vitro undigestible percent of NDF at 240 h
- <sup>6</sup>In-vitro digestibility percent of starch at 7 h

**Table 6.S 2.** Metabolizable amino acid supply as predicted by CNCPS v6.55 given observed DMI, infused AA, milk and component yields, and BW expressed as percentage of metabolizable protein, grams per mcal of metabolizable energy, and supply relative to AA requirement

Item	Treatment <sup>1</sup>			
	CON	MKH	IL	MKH+IL
MP allowable milk, kg/d	36.0	36.9	39.0	39.0
MP g/d, balance	-523	-520	-483	-541
Met, % MP	2.2	3.0	2.2	2.9
Lys, % MP	6.6	7.9	6.4	7.6
His, % MP	2.6	3.3	2.5	3.2
Ile, % MP	5.1	4.9	5.8	5.6
Leu, % MP	7.9	7.7	9.6	9.3
Met, g/Mcal ME	0.9	1.2	0.8	1.2
Lys, g/Mcal ME	2.5	3.1	2.5	3.0
His, g/Mcal ME	1.0	1.3	1.0	1.3
Leu, g/Mcal ME	3.0	3.0	3.7	3.7
Ile, g/Mcal ME	1.9	1.9	2.2	2.2
EAA, g/Mcal ME	18.0	19.1	19.0	20.1
Met g/d, balance	-19.3	-0.4	-20.3	-3.2
Lys g/d, balance	-46.8	-14.6	-49.7	-22.2
His g/d, balance	-6.2	12.0	-6.9	9.9
Ile g/d, balance	-23.9	-28.4	-3.9	-11.9
Leu g/d, balance	-87.0	-94.9	-40.8	-54.9
Lys/Met ratio	2.96:1	2.65:1	2.96:1	2.65:1

<sup>1</sup>Treatments: CON = infused 0.9% saline, MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys with 0.9% saline, IL = infused 22 g/d of Ile and 50 g/d of Leu with 0.9% saline, MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu with 0.9% saline.

**Table 6.S 3.** Effect of AA infusions on intake, milk production parameters, nutrient efficiency, and body weight (Main-effect treatment means)

Item	Treatment <sup>1</sup>				SEM	Main Effect ( <i>P</i> -value)		
	MKH		IL			MKH	IL	MKH*IL
	-	+	-	+				
<i>Intake</i>								
DMI, kg/d	25.5	25.7	25.4	25.7	0.28	0.45	0.31	0.90
Dietary CP intake, kg/d	3.87	3.90	3.86	3.90	0.04	0.45	0.31	0.90
Infused AA, g/d	36.0	114.9	39.5	111.4	0.03	<0.001	<0.001	0.53
Total CP intake, kg/d	3.90	4.01	3.90	4.01	0.04	0.01	0.01	0.99
<i>Milk production</i>								
Milk, kg/d	48.4	49.1	47.6	49.9	0.65	0.31	0.001	0.86
ECM, kg/d <sup>2</sup>	48.2	49.2	47.7	49.6	0.69	0.14	<0.01	0.93
Milk protein %	3.07	3.20	3.14	3.12	0.02	<0.001	0.45	0.09
Milk protein, g/d	1478	1560	1487	1551	22	<0.001	<0.01	0.28
Milk fat %	3.56	3.49	3.56	3.49	0.08	0.29	0.28	0.48
Milk fat, g/d	1709	1729	1696	1743	38	0.60	0.22	0.56
Lactose, %	5.05	4.98	5.02	5.01	0.02	<0.001	0.67	0.48
Lactose, g/d	2447	2448	2389	2507	36	0.98	0.002	0.97
MUN (mg/dL)	8.33	8.54	8.42	8.5	0.36	0.55	0.94	0.39
<i>Efficiency</i>								
Protein efficiency <sup>3</sup>	38.1	38.9	38.2	38.9	0.01	0.09	0.19	0.18
Gross feed efficiency <sup>4</sup>	1.89	1.89	1.86	1.92	0.03	0.99	0.07	0.68
BW, kg/d	671	675	670	676	4.84	0.52	0.24	0.97
BW change, kg/d	-13.6	-11.0	-13.3	-11.3	6.60	0.69	0.76	0.66

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>ECM=energy-corrected milk calculated as (12.82 x kg of fat) + (7.13 x kg of protein) + (0.323 x kg of milk).

<sup>3</sup>Protein efficiency calculated as milk protein yield (kg/d) / total CP intake (kg/d).

<sup>4</sup>Gross feed efficiency calculated as ECM yield (kg/d) / DMI (kg/d).

**Table 6.S 4.** Arterial amino acid plasma concentrations (main effects)<sup>1</sup>

Item	Treatments <sup>2</sup>				SEM	Effect (P-value)		
	MKH		IL			MKH	IL	MKH*IL
	-	+	-	+				
Arg	81.3	85.6	84.2	82.8	3.8	0.20	0.66	0.45
His	64.1	84.4	74.8	73.7	2.4	<0.001	0.65	0.93
Ile	142.8	136.7	132.2	147.3	8.4	0.32	0.02	0.02
Leu	234.5	214.2	195.2	253.6	15.5	0.03	<0.001	0.33
Lys	86.4	115.5	103.3	98.6	5.2	<0.001	0.20	0.05
Met	23.6	44.6	34.7	33.5	1.5	<0.001	0.52	0.52
Phe	47.2	43.5	45.4	45.4	1.8	<0.01	0.98	0.11
Thr	97.2	86.1	94.0	89.3	4.4	<0.001	0.10	0.69
Val	274.7	250.6	277.5	247.8	19.9	0.06	0.03	0.51
Ala	268.3	271.1	268.4	271.1	19.1	0.76	0.78	0.82
Asn	23.1	22.2	22.9	22.3	1.9	0.53	0.68	0.48
Asp	13.4	13.9	14.0	13.3	1.4	0.59	0.43	0.98
Gln	149.0	144.7	144.7	149.1	10.7	0.23	0.23	0.65
Glu	108.1	106.6	107.4	107.3	9.1	0.68	0.99	0.31
Gly	286.1	254.5	271.9	268.7	30.2	0.02	0.79	0.49
Pro	108.8	109.8	108.9	109.6	5.0	0.77	0.83	0.92
Ser	91.0	84.6	87.6	88.0	5.7	0.12	0.93	0.797
Tyr	35.3	29.9	33.6	31.6	2.4	<0.01	0.22	0.24
TAA <sup>3</sup>	2135	2112	2106	2141	44.6	0.61	0.45	0.53
EAA <sup>4</sup>	1055	1069	1051	1073	49.1	0.67	0.50	0.21
BCAA <sup>5</sup>	656	602	610	648	43.5	0.06	0.18	0.34
-MKH <sup>6</sup>	881	817	833	864	44.6	0.05	0.32	0.3
-IL <sup>7</sup>	675	715	717	673	25.6	0.03	0.02	0.21
NEAA <sup>8</sup>	1084	1044	1065	1063	48.9	0.19	0.95	0.81

<sup>1</sup>Data represent average AA concentrations for day 9, 12, and 16 of the experimental period.

<sup>2</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>3</sup>TAA = total amino acids (EAA + NEAA).

<sup>4</sup>EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val.

<sup>5</sup>BCAA = Ile, Leu, and Val.

<sup>6</sup>-MKH = Arg, Ile, Leu, Phe, Thr, and Val.

<sup>7</sup>-IL = Arg, His, Lys, Met, Phe, Thr, and Val.

<sup>8</sup>NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

**Table 6.S 5.** Venous amino acid plasma concentrations (main effects)<sup>1</sup>

Item	Treatments <sup>2</sup>				SEM	Effect ( <i>P</i> -value)		
	MKH		IL			MKH	IL	MKH*IL
	-	+	-	+				
Arg	40.2	43.8	44.0	39.9	3.1	0.27	0.21	0.88
His	50.4	67.1	61.6	55.9	3.5	<0.001	0.12	0.83
Ile	96.7	80.5	77.2	100.0	9.4	0.05	0.01	0.20
Leu	164.9	128.1	124.9	168.1	17.4	0.05	0.02	0.42
Lys	34.7	45.8	40.4	40.2	3.0	0.002	0.95	0.32
Met	8.2	23.9	15.3	16.8	1.7	<0.001	0.42	0.89
Phe	24.3	16.7	19.8	21.2	1.5	<0.001	0.36	0.26
Thr	60.1	48.6	56.4	52.2	5.1	0.04	0.42	0.8
Val	210.6	198.6	219.4	189.8	25.9	0.52	0.12	0.07
Ala	208.9	212.8	212.7	209.1	20.7	0.77	0.79	0.38
Asn	11.5	9.1	10.1	10.6	1.8	0.14	0.76	0.81
Asp	6.4	6.4	6.3	6.5	1.2	0.93	0.84	0.74
Gln	111.0	101.7	102.2	110.5	12.6	0.11	0.16	0.85
Glu	55.8	53.9	54.9	54.7	5.8	0.61	0.96	0.34
Gly	248.6	216.2	237.7	227.1	33.2	0.03	0.45	0.92
Pro	80.8	80.1	81.7	79.1	6.3	0.91	0.67	0.64
Ser	58.7	45.4	51.3	52.9	8.3	<0.01	0.69	0.54
Tyr	11.2	2.1	6.9	6.3	3.0	<0.001	0.77	0.77

<sup>1</sup>Data represent average AA venous concentrations for day 16 of the experimental period.

<sup>2</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

**Table 6.S 6.** Mammary plasma flow and net uptake of amino acids (main effects)<sup>1</sup>

Item	Treatment <sup>2</sup>				SEM	Effect ( <i>P</i> -value)		
	MKH		IL			MKH	IL	MKH*IL
	-	+	-	+				
Mammary plasma flow (MPF), L/h								
MPF, L/h	868	785	736	917	68.0	0.22	0.01	0.37
Net mammary uptake, mmol/h								
Arg	31.7	31.5	29.3	33.9	3.1	0.93	0.02	0.71
His	10.9	10.5	9.4	12.0	0.9	0.74	0.04	0.90
Ile	40.8	42.2	37.9	45.1	2.9	0.65	0.04	0.25
Leu	67.3	66.2	58.9	74.6	4.3	0.75	<0.001	0.85
Lys	38.7	51.0	44.6	45.8	3.1	<0.001	0.52	0.48
Met	11.8	13.3	12.9	12.2	1.3	0.22	0.51	0.78
Phe	19.3	20.3	19.2	20.5	0.9	0.02	<0.01	0.25
Thr	24.3	23.6	23.7	24.2	1.6	0.69	0.74	0.52
Val	52.5	48.5	49.3	51.7	4.7	0.31	0.54	0.50
Ala	32.4	34.8	31.9	35.3	8.9	0.72	0.62	0.62
Asn	7.7	9.0	7.9	8.9	0.7	0.12	0.23	0.59
Asp	3.9	3.9	4.4	3.4	0.8	0.95	0.38	0.91
Gln	28.4	32.2	28.8	31.8	2.8	0.12	0.22	0.8
Glu	38.9	33.7	33.0	39.6	3.9	0.21	0.12	0.43
Gly	15.7	19.7	12.6	22.7	4.0	0.43	0.06	0.98
Pro	17.1	17.5	16.3	18.2	2.2	0.85	0.44	0.37
Ser	18.5	23.5	20.2	21.8	2.7	<0.01	0.30	0.53
Tyr	18.5	19.9	19.0	19.4	0.6	<0.01	0.28	0.91
TAA <sup>3</sup>	571	673	649	595	52.3	0.12	0.40	0.5
EAA <sup>4</sup>	353	411	400	364	23.7	0.08	0.28	0.25
BCAA <sup>5</sup>	190	206	205	191	14.2	0.39	0.47	0.31
-MKH <sup>6</sup>	280	309	307	283	19.6	0.28	0.37	0.29
-IL <sup>7</sup>	226	270	264	232	16.8	0.05	0.15	0.31
NEAA <sup>8</sup>	218	262	249	230	31.2	0.22	0.59	0.85

<sup>1</sup>Data represent average AA concentrations for day 9, 12, and 16 of the experimental period.

<sup>2</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>3</sup>TAA = total amino acids (EAA + NEAA).

<sup>4</sup>EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val.

<sup>5</sup>BCAA = Ile, Leu, and Val.

<sup>6</sup>-MKH = Arg, Ile, Leu, Phe, Thr, and Val.

<sup>7</sup>-IL = Arg, His, Lys, Met, Phe, Thr, and Val.

<sup>8</sup>NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

**Table 6.S 7.** Mammary clearances (L/h) of amino acids (main effects)<sup>1</sup>

Item	Treatments <sup>2</sup>				SEM	Effect ( <i>P</i> -value)		
	MKH		IL			MKH	IL	MKH*IL
	-	+	-	+				
Arg	836.0	792.6	735.0	893.6	108.7	0.65	0.11	0.82
His	232.8	161.6	165.6	228.9	18.6	0.01	0.02	0.61
Ile	440.3	510.6	498.2	452.7	69.8	0.33	0.53	0.74
Leu	481.4	593.5	601.6	473.3	84.5	0.20	0.15	0.99
Lys	1206.3	1198.3	1151.7	1253.0	129.2	0.95	0.42	0.31
Met	1730.0	629.8	1108.3	1251.5	295.1	<0.001	0.43	0.46
Phe	849.0	1261.1	1017.4	1092.7	105.3	<0.001	0.30	0.06
Thr	409.4	511.7	444.3	476.9	47.9	0.11	0.60	0.99
Val	228.3	288.1	236.3	280.1	29.3	0.02	0.09	<0.01
Ala	174.3	185.6	165.3	194.5	54.1	0.73	0.40	0.23
Asn	722.5	929.1	814.5	837.1	141.1	0.16	0.88	0.84
Asp	672.3	742.4	773.1	641.5	176.5	0.76	0.56	0.99
Gln	276.5	336.4	308.6	304.3	48.8	0.12	0.91	0.75
Glu	672.2	596.4	629.8	638.8	97.3	0.48	0.93	0.48
Gly	43.9	93.5	58.6	78.9	24.0	0.01	0.26	0.1
Pro	228.3	241.1	207.2	262.2	39.7	0.79	0.26	0.44
Ser	425.0	606.7	426.6	605.2	123.9	0.07	0.07	0.81
Tyr	1680.4	-74.4	-661.1	2267.1	1299.7	0.31	0.10	0.17
TAA <sup>3</sup>	297	369	321	345	29.6	0.02	0.37	0.11
EAA <sup>4</sup>	437	491	442	486	36.0	0.22	0.31	0.48
BCAA <sup>5</sup>	325	423	381	367	46.0	<0.01	0.64	0.06
-MKH <sup>6</sup>	407	477	424	461	39.3	0.14	0.42	0.57
-IL <sup>7</sup>	438	478	414	503	30.5	0.31	0.03	0.37
NEAA <sup>8</sup>	235	274	234	275	34.6	0.26	0.24	0.47

<sup>1</sup>Data represent average AA concentrations for day 9, 12, and 16 of the experimental period.

<sup>2</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>3</sup>TAA = total amino acids (EAA + NEAA).

<sup>4</sup>EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val.

<sup>5</sup>BCAA = Ile, Leu, and Val.

<sup>6</sup>-MKH = Arg, Ile, Leu, Phe, Thr, and Val.

<sup>7</sup>-IL = Arg, His, Lys, Met, Phe, Thr, and Val.

<sup>8</sup>NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

**Table 6.S 8.** Effect of AA infusions on mammary AA uptake: milk protein output ratio (main effects)<sup>1</sup>

Item	Treatments <sup>2</sup>				SEM	Effect ( <i>P</i> -value)		
	MKH		IL			MKH	IL	MKH*IL
	-	+	-	+				
Arg	2.35	2.28	2.25	2.38	0.24	0.48	0.15	0.91
His	0.93	0.87	0.81	0.99	0.08	0.54	0.10	0.97
Ile	1.40	1.36	1.30	1.46	0.09	0.76	0.14	0.23
Leu	1.38	1.29	1.21	1.46	0.07	0.14	<0.01	0.98
Lys	1.05	1.32	1.20	1.17	0.07	<0.001	0.60	0.72
Met	0.95	1.02	1.02	0.94	0.09	0.49	0.39	0.71
Phe	1.01	0.99	0.99	1.01	0.01	0.30	0.07	0.57
Thr	1.01	0.94	0.99	0.96	0.05	0.26	0.66	0.46
Val	1.43	1.25	1.35	1.34	0.11	0.10	0.94	0.57
Ala	1.34	1.39	1.33	1.40	0.35	0.82	0.80	0.68
Asn	0.38	0.43	0.39	0.42	0.03	0.27	0.42	0.52
Asp	0.23	0.22	0.25	0.19	0.04	0.84	0.24	0.84
Gln	0.69	0.73	0.69	0.73	0.06	0.39	0.50	0.92
Glu	0.67	0.55	0.64	0.59	0.03	<0.01	0.13	0.83
Gly	0.94	1.17	0.79	1.33	0.23	0.44	0.08	0.94
Pro	0.30	0.29	0.29	0.30	0.03	0.90	0.74	0.39
Ser	0.47	0.57	0.51	0.53	0.07	0.02	0.62	0.75
Tyr	0.93	0.94	0.95	0.91	0.01	0.30	0.07	0.57
TAA <sup>3</sup>	0.90	0.90	0.88	0.92	0.03	0.89	0.36	0.96
EAA <sup>4</sup>	1.28	1.24	1.22	1.30	0.05	0.42	0.14	0.48
BCAA <sup>5</sup>	1.40	1.27	1.28	1.40	0.07	0.07	0.11	0.58
-MKH <sup>6</sup>	1.38	1.26	1.28	1.37	0.06	0.06	0.15	0.48
-IL <sup>7</sup>	1.22	1.22	1.21	1.23	0.06	0.98	0.62	0.86
NEAA <sup>8</sup>	0.60	0.62	0.60	0.62	0.05	0.73	0.73	0.59

<sup>1</sup>Data represents arterial and venous measurements (n=6 to 11 samples per cow) taken on d 16 and average milk AA yield from d 15 to d 17 between 1300 to 0100 h.

<sup>2</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>3</sup>TAA = total amino acids (EAA + NEAA).

<sup>4</sup>EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val.

<sup>5</sup>BCAA = Ile, Leu, and Val.

<sup>6</sup>-MKH = Arg, Ile, Leu, Phe, Thr, and Val.

<sup>7</sup>-IL = Arg, His, Lys, Met, Phe, Thr, and Val.

<sup>8</sup>NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, and Tyr.

## **CHAPTER 7: Effects of jugular infused methionine, lysine, and histidine or leucine and isoleucine on gene and protein expression and post-translational modification**

### **7.1. Abstract**

The objective of this study was to evaluate gene and protein expression and post-translation modifications in mammary and muscle tissue of dairy cows supplemented with two groups of AA in a 2 x 2 factorial. Four lactating cows were assigned to four jugular infusion treatments consisting of saline (CON), methionine plus lysine plus histidine (MKH), isoleucine plus leucine (IL), or MKH plus IL in 4 x 4 Latin square design. Periods were 18 d in length and comprised of 8 d of rest followed by 10 d of jugular infusion and tissue biopsies on d 18. Milk protein yield tended to increase with MKH and IL infusions independently and additively, with the MKH+IL treatment yielding a 9.6% increase versus the CON. Mammary and muscle proteomes were assessed for total and phosphorylated abundance. Total and site-specific phosphorylated abundances of mammalian target of rapamycin complex 1 (mTORC1), ribosomal protein S6 kinase (S6K1), ribosomal protein S6 (rpS6), and eukaryotic initiation factor 2a (eIF2a) were assessed by western immunoblotting. The treatments both independently affected or tended to affect the phosphorylation state of mTOR pathway related proteins in the udder. For the overall udder proteome, there was a tendency for a treatment interaction effect on total and phosphorylated abundance as well as the phosphorylation state. The MKH treatment also tended to affect the udder total abundance of proteins whereas IL treatment tended to affect the phosphorylation state of proteins. Univariate proteomic analysis revealed an enhanced phosphorylation state of mitogen-activated protein kinase 1 (MAP2K1) by MKH and IL treatments and insulin receptor substrate 1 (IRS1) by MKH treatment only which indicates negative mTOR feedback. Western immunoblotting indicated decreased total and phosphorylated abundance of S6K1 as well as phosphorylation state from MKH infusion. There

was a positive treatment interaction for total and phosphorylated abundance of rpS6, S6K1, and eIF2 $\alpha$  as well as the phosphorylation state of rpS6. No significant changes were observed in muscle for western immunoblotting and mRNA expression except for a small increase in phosphorylation state of eIF2 $\alpha$ . Overall, results indicate that supplementing MKH or IL affects protein expression and phosphorylation status of many proteins in the udder that correlated to milk protein yield increases, however, the changes in mTOR signaling proteins were generally opposite to the effects that are expected. The latter appears to be the result of negative feedback arising from infusion of MKH and IL alone and when infused in combination, less negative feedback, positive treatment interaction on mTORC1 targets, and greater milk protein yield.

## **7.2. Introduction**

Optimizing milk protein production in dairy cows is necessary for achieving maximal income over feed costs which directly relates to farm profitability. Common nutrition models (i.e., NRC, 2001, CNCPS v6.55) utilize fixed efficiencies for conversion of supplied AA into milk protein, growth, or maintenance use. This representation by nutrition models fails to consider the effects of regulation including the impact of AA as signaling molecules (Bradford et al., 2016). Nutrient responses are typically exhibited when supply is increased from low levels eventually reaching a plateau as nutrient supply saturates the response elements in the animal. Thus, over-supply provides no benefit whereas under-supply reduces production. Varying prices of milk protein (e.g., \$1.30 to \$4.50 from 2014-2018) and ingredient prices (i.e., soybean meal) necessitate varying dietary nutrient supplies to achieve optimal income-over feed costs. However, nutrition models lack the capability to predict nutrient supply driven changes in milk protein yield (Yoder et al., 2016). Incorporation into nutrition models of a representation of the effects of protein synthesis regulation on marginal responses will result in nutrient response

driven predictions, thereby providing farmers with the means for strategically varying dietary nutrients depending on milk component prices, feed prices, or environmental constraints.

Milk protein yield is regulated by transcription of milk protein genes, efficiency or rate of protein translation, abundance of translational machinery, and the number of epithelial cells (Cant et al., 2018). Of these factors, phosphorylation of enzymes regulating translation initiation and elongation rates via the mammalian target of rapamycin (mTOR) pathway is considered the most potent regulator of milk protein synthesis (Rius et al., 2010a, Toerien et al., 2010, Appuhamy et al., 2012, Appuhamy et al., 2014, Arriola Apelo et al., 2014c, Liu et al., 2017). Activation of mTOR is driven by changes in EAA supply, particularly Leu (Wolfson et al., 2016), energy (starch, acetate) (Rius et al., 2010a, Appuhamy et al., 2014), insulin, and other factors. mTOR serves to integrate signals arising from nutrient supply, endocrines, and oxidative state and regulate anabolic metabolism (Sabatini, 2017). These findings suggest that certain AA like Leu (most potent for regulating mTOR) can strategically be supplemented to increase milk protein yield independent of supply by enhancing protein translational machinery.

Demonstration of mTOR regulation of protein synthesis in long term studies in cows has been fleeting though. Studies of more than 5 d duration that altered EAA supplies elicited small to no changes in phosphorylation state of proteins related to mTOR pathway (Arriola Apelo et al., 2014a, Doelman et al., 2015a, Doelman et al., 2015b, Nichols et al., 2017). Additionally, while Leu and Ile are considered powerful activators of mTOR pathway, supplementing these EAA to dairy cows has not increased milk protein yield in many studies (Huhtanen et al., 2002, Korhonen et al., 2002, Appuhamy et al., 2011b, Curtis et al., 2018) and these EAA are not considered in ration formulation by common nutrition models (NRC, 2001; CNCPS v6.55). A 29% increase in milk protein yield from a complete mix of EAA infusion did

not affect the phosphorylation state for two substrates of mTOR; ribosomal protein S6 kinase 1 (S6K1), and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) which are directly involved in translation initiation (Nichols et al., 2017). This trial found that mRNA expression of certain genes (i.e., increased splicing of XBP1 and decreased DDIT3) indicated increased endoplasmic biogenesis and differentiation as the mechanism facilitating increased milk protein yield. However, supraphysiologic plasma concentrations of EAA were achieved with Met, Ile, and Leu elevated to concentrations of 117, 348, and 499  $\mu\text{M}$  plasma concentrations which are 388, 222, and 202% of typical plasma concentrations in dairy cows (Swanepoel et al., 2016). The cows also exhibited abnormally low milk fat concentrations (2.91%) (Nichols et al., 2017) which likely occurred because of high rumen outflow of trans-10, cis-12 CLA. This CLA causes endoplasmic reticulum stress and inhibition of SREBP1 in mouse adipocytes, which has been shown to be related to phosphorylation of eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) and activating transcription factor 4 (ATF4) expression (LaRosa et al., 2007). If the integrated stress response pathway (ISR) was activated, then mTOR pathway activation by EAA could have been blocked with increased Sestrin2 completely blunting Leu or Ile activation of mTOR (Wolfson and Sabatini, 2017).

In another trial of similar length, subtraction of Lys, His, Met, Phe, and Trp all numerically or statistically increased phosphorylation of S6K1 compared to infusing a complete mix of EAA (Doelman et al., 2015a, Doelman et al., 2015b). Supplementation of EAA also did not decrease phosphorylation of eIF2 $\alpha$  and in some cases increased it, which seems contrary given its role in the ISR pathway for sensing AA deficiencies (Doelman et al., 2015a, Doelman et al., 2015b, Nichols et al., 2017). In summary, short term studies in cells, cows, and goats definitely demonstrate that EAA activate the mTOR pathway and milk protein synthesis

(Appuhamy et al., 2012, Arriola Apelo et al., 2014d, Guo et al., 2017), however, results in longer term trials indicate that mTOR regulation may not be the primary regulator of milk protein yield.

Regulation of anabolic metabolism is complex, and the multitude of activators and inhibitors of the mTOR pathway (Sabatini, 2017) create multivariate complexity. In vivo trials encompass many more nodes of regulation than observed in cell culture, hence, a more holistic approach in a well-defined cell culture system evaluating proteomic responses to EAA might shed light on the regulation of milk protein synthesis in vivo. Advances in proteomics allow characterization of proteins and quantification of abundance and post-translation modifications which enable robust examination of underlying biological processes affecting metabolism (Distler et al., 2016, Li et al., 2017b). Western immunoblotting is at best considered semi-quantitative as a number of technical issues with the assay introduce variation (Gorr and Vogel, 2015). Such high analytical error may mask more subtle changes in the pathways regulating protein synthesis. The recent quantitative advances in proteomics have provided quantification inference that is considered vastly superior to western immunoblotting (Aebersold et al., 2013). Proteomic analysis of the udder under conditions of changing EAA supplementation may help elucidate the role the mTOR pathway plays in the regulation of long-term milk protein synthesis. If the latter is identified, then strategies for manipulation may be developed to improve N efficiency in dairy cows.

Our hypotheses were that proteomic evaluation of mammary and muscle tissues with and without EAA supplementation would identify regulatory proteins controlling milk protein yield. We expected enhanced phosphorylation of proteins associated with the mTOR pathway and possibly other previously unknown changes in the proteome. The objectives were to observe proteomic changes in relation to changes in EAA supply and milk protein yield in high

producing dairy cows. Additionally, transcriptomic and protein evaluation via PCR and western immunoblotting would be employed for comparison to proteomics.

### **7.3. Materials and Methods**

#### **7.3.1. Experimental Design and Treatments**

All animal procedures were approved by the Virginia Tech Animal Care and Use Committee. Complete details of the experimental design, diets, and treatments are reported in Chapter 6. Briefly, eight Holstein cows in second lactation were blocked by DIM (2 blocks) and randomly assigned to 1 of 4 treatment sequences within a 2 x 2 factorial arrangement in 2 orthogonally replicated 4 x 4 Latin squares. Period length was 18 d with an initial adaptation period of 28 d. Cows were fed a common diet for the entire study, including the adaptation period that was formulated to meet requirements for energy, rumen degradable protein, minerals, and vitamins but be deficient in metabolizable protein for a 680 kg Holstein cow producing 47 kg/d milk containing 3.6% fat and 3.1% protein. Cows in 1 block were biopsied on d 18 (n= 4 cows) to obtain samples of muscle and mammary tissue and are the subjects for the results reported herein. Treatments were jugular infusions of saline (**CON**), methionine, lysine, and histidine (**MKH**), isoleucine and leucine (**IL**), and the combination of MKH and IL (**MKH+IL**). The amount of jugular infused amino acids was 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys for MKH treatment and 22 g/d of Ile and 50 g/d of Leu for IL treatment. The rationale for selection of the AA infusion amounts was discussed in Chapter 6.

#### **7.3.2. Tissue Sample Collection**

On the last day of each period (d 18), tissue samples from the longissimus dorsi and the rear quarter of the udder were obtained by biopsy (average = 542 mg, SD = 139) (Toerien et al., 2010) immediately following the 12:30 pm milking. The biopsy site was alternated between sides of the rear udder and longissimus dorsi muscle for each period. Cows were restrained with

a halter and administered Banamine (1-2 mL/100 lbs), Xylazine (0.01-0.05 mg/kg), and 2% Lidocaine (3-8 mL) for general and local anesthesia. Muscle tissue was obtained using an 8 mm biopsy punch (Integra Miltex, #17A29, York, PA), and mammary tissue using a device described by Toerien et al. (2010). Immediately following collection (within 2 minutes), samples were sequentially rinsed 1x with room-temperature PBS, 2x with ice cold PBS, and 1x with ice cold PBS containing 1 mM NaF and 10  $\mu$ M Na<sub>3</sub>VO<sub>4</sub> to minimize kinase and phosphatase activity. Rinsed samples were placed in liquid N, transported to the laboratory, and stored at -80°C for further analysis.

Tissue samples were removed from -80°C storage to liquid N and weighed. Samples were cut into two pieces for processing, one for PCR analyses and the other for western immunoblotting and proteomic analysis. The PCR designated sample was placed in a buffer solution containing 3.54 M ammonium sulfate, 13.3 mM EDTA, and 16.7 mM sodium citrate to prevent RNA degradation. The proteomic sample was placed in 1 mL of ice-cold lysis buffer (20 nM HEPES, 2 mM EGTA, 50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM  $\beta$ -glycerophosphate, and Halt Protease and Phosphatase Single-Use Cocktail, Thermo Scientific, #78442) and the combination returned to liquid N. The frozen samples plus inhibitor solutions were ground into powder using a #6850 Freezer/Mill (SPEX Sample Prep, Metuchen, NJ) and a 2-min processing time at a power setting of 10 while immersed in liquid N. Upon removal from the mill, the additional lysis buffer was added to the sample to achieve a ratio of 1:7 (mg tissue / mL lysis buffer). The sample was thawed in warm water, mixed vigorously, and homogenized for 30 s using a TissueLyser. The lysed sample was placed on a rocker to maintain constant agitation for 1 h at 4° C, and centrifuged for 20 min at 12,000 rcf for 15 min at 4° C. The supernatant was collected and analyzed for total protein concentration in duplicate using

bicinchoninic acid (BCA) assay. The average concentration for the udder and muscle tissue was 3.3 mg/mL (SD = 0.97) and 5.2 mg/mL (SD = 1.07) respectively. Homogenates were stored at -80° C.

### 7.3.3. Western Immunoblotting Analysis

Aliquots of 20 ug of udder protein and 30 ug of muscle protein were combined with 2x Laemmli sample buffer (Bio-Rad, #161-0747) containing 10% 2-mercaptoethanol, incubated at 95°C for 5 min, placed on ice for 2 min, centrifuged at 16,000 rcf for 1 min at 20°C, and separated on 7.5 to 12% SDS-PAGE gels (Bio Rad Mini-PROTEAN TGX Gels) at 100 V for 90 min. Proteins were electrotransferred onto Immobilon-FL polyvinylidene fluoride (PVDF) membranes (Sigma-Aldrich, #IPFL00010), stained for total protein using REVERT Total Protein Stain (Licor, #926-11010), and scanned using an Odyssey Infrared Imaging System (LICOR Biosciences) for quantification of total protein. The stain was subsequently removed using REVERT Reversal Solution (Licor, #926-11013), washed vigorously with water, and blocked for 1 h at room temperature using Odyssey blocking buffer (TBS) (Licor, #927-50000). The blocked blots were incubated overnight at 4°C with primary rabbit and mouse antibodies in Odyssey blocking buffer containing 0.2% Tween-20 solution (Bio-Rad, #161-0781). Primary antibodies consisted of total and site-specific phosphorylated mTOR (Ser2448; mouse #4517 and rabbit #5536, respectively), p70 S6 kinase (Thr389; mouse #9206, rabbit #2708), ribosomal protein S6 (Ser235/236; mouse #2317 and rabbit #4858), eIF2 $\alpha$  (Ser51; mouse #2103 and rabbit #3597), 4E-BP1 (Thr37/46; mouse #sc-81149 and rabbit #2855), and the total form only of  $\alpha$ -tubulin (#2144). All primary antibodies were monoclonal except  $\alpha$ -tubulin which was polyclonal based. All primary antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA) except for the total form of 4E-BP1 which was acquired from Santa Cruz

Biotechnology Inc. (Dallas, TX). All antibodies were diluted 1:1,000 (vol/vol) except for the phosphorylated form of ribosomal protein S6, 1:2,000 (vol/vol). Following incubation, membranes were washed 4 times for 5 min each with Tris-buffered saline containing 0.1% Tween-20 solution and then incubated for 1 h at room temperature with goat anti-mouse (IRDye 680RD, LI-COR, #925-68070) and anti-rabbit (IRDye 800CW, LI-COR, #926-32211) secondary antibodies (diluted to 1:10,000 (vol/vol) in Odyssey blocking buffer (TBS), 0.2% Tween-20 solution, and 0.01% sodium dodecyl sulfate). Membranes were washed 4 times as described previously and scanned using an Odyssey Infrared Imaging System (LI-COR Biosciences). The signal intensity of phosphorylated and total forms of the proteins was quantified using Image Studio software from LI-COR (version 5.2.5). Samples were analyzed in triplicate, with block grouping by period and tissue type on each gel.

#### **7.3.4. Proteomic Analysis**

Protein samples were thawed and a volume containing 3 mg of protein was removed and subsequently brought to a final volume of 1.25 mL. Dithiothreitol (DTT) was added to a final concentration of 4.5 mM. Three  $\mu$ l ( $\geq 750$  units) of benzonase (Sigma E1014-25KU) was added and samples incubated with shaking at 37°C for 1 h. Iodoacetamide (IAA) was added to the cooled samples to achieve a final concentration of 10 mM, and the samples were incubated in the dark at room temperature for 30 min. DTT was added to quench unreacted IAA at a final concentration of 10 mM. The volume was brought to 10 mL using ice-cold acetone, samples were inverted 3X, and stored at -20°C overnight. Protein was recovered by centrifugation at 3696 x g in a chilled (8°C) swinging bucket rotor centrifuge for 15 min. Protein pellets were resuspended at a concentration of 5 mg/mL in freshly prepared 4 M urea containing 100 mM AmBic. Next, LysC (Wako) was added at a ratio of 1:130, and the digestion was carried out overnight at 37°C. The following day, the urea concentration was brought to 1.6 M by the

addition of 100 mM AmBic, trypsin (Pierce) was added at a ratio of 1:100, and the digestion was carried out for 4 h at 37°C. Digested samples were desalted using Oasis HLB 1 cc 10 mg extraction cartridges (Waters). A small aliquot was removed from each sample for analysis, samples were divided in half, and each half dried separately using a high vacuum line.

Phosphopeptides were enriched from one half of the samples using a High-Select Fe-NTA phosphopeptide enrichment kit (Thermo Scientific) and from the other half using a High-Select TiO<sub>2</sub> phosphopeptide enrichment kit (Thermo Scientific). Eluates were acidified and those originating from the same protein sample were combined. Acetonitrile was removed using a vacuum concentrator.

Unenriched and phosphopeptide-enriched samples were analyzed using an Orbitrap Fusion Lumos equipped with an Easy-nLC 1200 UPLC, and an Easy Spray nanospray source (Thermo Scientific). The column utilized for peptide separation was a PepMap RSLC C18 2 μm 100 A 75 μm x 25 cm (Thermo Scientific). Analysis of unenriched samples utilized a 180 min gradient from 2 to 45% solvent B and analysis of phosphopeptide-enriched samples utilized a 120 min gradient from 2 to 45% solvent B at a flow rate of 300 nL/min. Solvent A was 0.1% (v/v) formic acid in water and B was 20:80 water:acetonitrile containing 0.1% (v/v) formic acid. The column temperature was maintained at 55°C and the ion transfer tube at 275°C. Mass spectrometer conditions were as follows: 2200 V ion spray voltage, MS scans utilizing the orbitrap at 120000 resolution (60000 for phosphopeptides) for m/z 400-1500, an agc target of 400,000 with 50 ms max inject time, profile, positive, MSMS targets chosen using MIPS looking for peptide-like isotopic distribution with a z=2-5 and a minimum intensity of 20,000, dynamic exclusion for 15 sec, priority given to the most intense with a max cycle time of 4 sec (Top Speed mode), MSMS isolation window of 1.4 m/z (1.8 m/z for phosphopeptides), HCD at

30±3% (32±3% for phosphopeptides), detection using the orbitrap at 15000 resolution, normal m/z range with first mass at 150, agc target of 100,000 (200,000 for phosphopeptides) with a max inject time of 200 ms, centroid.

Initial data analysis utilized Thermo Proteome Discoverer 2.2 (Thermo Scientific) and Mascot server 2.6 (Matrix Science). Searches considered two possible missed cleavages with trypsin as the protease, carbamidomethylation of Cys residues as a fixed modification, and the following possible variable modifications: oxidation of Met, deamidation of Gln and Asn, pyroglutamate formation when Gln was at the N-terminus of a peptide, N-terminal acetylation of a protein, and phosphorylation of Ser, Thr and Tyr (for phosphopeptide-enriched samples). MS and MSMS mass tolerances were 10 ppm and 0.05 Da. False discovery rate was limited to less than 1% and phosphorylation site probability threshold was set to 75% confidence. Normalization and feature detection were performed separately for unenriched and phosphopeptide-enriched samples. Normalization was based on the assumption that total peptide amount was equal. Feature detection, mapping and retention time alignment used the Proteome Discoverer nodes Minora Feature Detector, Feature Mapper and Precursor Ions Quantifier to match peaks across runs. Matches were required to have a high PSM confidence in at least one run and be present in at least 20% of all runs.

### **7.3.5. Quantitative Real-Time PCR**

Total RNA was extracted using Qiagen RNeasy mini kits (Qiagen, Valencia, CA). For muscle samples, approximately 1 mL of TRIzol Reagent was utilized for initial digestion according to manufacturer's instructions (Invitrogen). Approximately 30-50 mg of tissue was incubated with 600 ul of lysis buffer plus 10 uL of B-mercaptoethanol per 1 mL of lysis buffer, and homogenized using a TissueLyser LT (Qiagen, Valencia, CA) for 2 minutes. The samples were centrifuged at 21,130 rcf for 3 min at 23° C and the supernatant collected and placed in a

genomic DNA eliminator column and subsequently centrifuged at 8,000 rcf for 30 s. Column effluent was collected and processed according to the RNeasy mini kit instructions. RNA purity and quantity were determined using a NanDrop ND-1000 spectrophotometer (NanoDrop Technologies; Rockland, DE). The mammary and muscle 260/280 ratios averaged 2.06 (SD=0.02) and 1.84 (SD=0.19), respectively.

Single-stranded cDNA was synthesized according to the method of Swank et al. (2013). Briefly, 2.0 ug of RNA was denatured in 11.3 uL of DNase/RNase free water for 5 min at 65° C, and placed on ice for 3 min. 4 uL of M-MLV, 2 uL of 0.1 M DTT, 1 uL of 10 mM dNTP, 1 uL of Oligo (dT) 20 primer, 0.5 m-MLV Reverse transcriptase, and 0.2 uL of RNAsin that was added to the sample, and reverse transcription was carried out on a heating block at 40° C for 1 h followed by 95° C for 10 min. The resulting cDNA was diluted 1:1 with DNase/RNase free water, aliquoted into tubes, and stored at -20° C.

Target genes and their respective primers are provided in supplemental material Table 1. Primer sequences were obtained from previous publications or designed using Primer3 (v. 0.4.0). Newly designed primer sequences were queried in the *Bos taurus* genome database maintained by the National Center for Biotechnology Information (NCBI) using the BLAST tool. Primer efficiency and melting curve analyses were conducted to detect primer dimer formation and genomic DNA contamination. Pooled samples of mammary and muscle were utilized to evaluate primers at dilutions of 1:1, 1:10, 1:100, and 1:1000. DNase/RNase free water was used as the diluent. Primer efficiency was determined as: efficiency percent =  $(10^{(-1/\text{slope})} - 1) * 100$ . Quantitative reverse transcription PCR (qPCR) was performed on all samples in triplicate using 1 uL of cDNA solution, 0.5 uL of a master mix for each forward and reverse primer, 5 uL of iQ SYBR Green Supermix (Bio-Rad; Hercules, CA), and 3 uL of RNAase/DNAase free water for a

total reaction volume of 20 uL. Assays were performed using an iQ5 Multicolor Real Time PCR Detection System with the following conditions; 1 cycle at 95°C for 5 min, 40 repeating cycles of 95°C for 10 s and either 55, 57.5, or 60°C for 45 s (annealing). In addition to the samples, null template (RNase/DNase free water) and null reverse transcriptase (no cDNA) blanks were included on each PCR plate. The average CV for CT values among duplicates was 3.1%. If the CV exceeded 10%, the sample was repeated or one of the triplicate samples was removed. Target genes of interest were normalized to the geometric mean of H3F3A and GAPDH (target gene CT – reference genes CT) =  $\Delta$  CT ((Vandesompele et al., 2002). Delta CT data were transformed to  $2^{(-\Delta CT)}$  to represent fold difference relative to the reference genes (Velayudhan et al., 2008) and standardized by dividing by the average CON delta CT value. The resulting data were not normally distributed (Shapiro-Wilk's Test), hence, they were natural log transformed prior to statistical analysis.

### **7.3.6. Statistical Analysis**

One cow had clinical mastitis during period 3, therefore, data for that period and period 4 were removed. Statistical analysis was conducted using R version 3.5.1 (R Core Team, 2018). For the western immunoblotting data, the phosphorylation intensity was divided by the total protein intensity within protein resulting in a phosphorylated to total expression ratio indicative of phosphorylation state. The total and phosphorylated protein intensity were also divided by the housekeeping protein intensity ( $\alpha$ -tubulin) to calculate total and phosphorylated expression. These expressions values were then standardized to the CON by dividing each sample intensity by the average intensity of the CON treatment. Western immunoblot and PCR data were fit to a model containing the fixed main effects of MKH and IL, the MKH by IL interaction, period and cow. Period and cow were considered fixed because the number of observations was too small

to estimate random effects and thus the results may be specific to these cows, and not the entire population (Yoder et al., 2013). Main effects and interactions were deemed significant at  $P < 0.05$  and a tendency at  $0.05 < P < 0.15$ . Post-hoc means-separation tests were conducted only when significant main or interaction effects were detected.

Proteomic analysis was done in duplicate and for some of the data analysis, the technical replicates were used for imputation and identification of outliers as previously described (Zhang et al., 2018). First, data were log transformed and missing data evaluated using the ProStaR *R* package (Wieczorek et al., 2017). If greater than 50% of expression values were missing for a single protein within a treatment, all observations for that protein were discarded. On average, 19.3 to 28.1% of the data within the four datasets (total protein udder, phosphorylated udder, total protein muscle, phosphorylated muscle) were missing.

Except for those proteins with all data removed, missing values were imputed (Wieczorek et al., 2017). A heatmap cluster was first used to evaluate if missing data were random or censored by treatment or sample (Zhang et al., 2018). In one mammary data sample from Period 2, the heatmap as well as later multivariate tests indicated the sample was likely an outlier with more missing data samples and multivariate relationships that were quite different the other samples., and thus the sample was removed from all further analyses. Missing values from the remaining samples appeared to be randomly distributed across treatments and samples. Having removed outliers, the *K* Nearest Neighbors clustering (ProStaR *R* package) algorithm was used to impute missing data points from the multivariate information within sample for the technical replicates (cluster size=10). Density maps were re-evaluated after imputation to ensure that distribution and variance of the dataset was not significantly changed. The imputed data set were then reverse log transformed, technical replicates were averaged, and the coefficient of

variation (CV) calculated. The average CV was 21.8 to 37.0% across 4 datasets. Protein samples exceeding 100% CV were removed from the dataset. 2.4 to 7.2% of the samples were removed. The total and phosphorylated enriched datasets were merged by protein within each tissue using the accession numbers, and the ratio of phosphorylated to total expression was calculated to infer the phosphorylation state. The resulting mammary dataset contained 3504 total and 1761 phosphorylated proteins, and 1144 proteins with calculated phosphorylation states. The muscle dataset contained 1047 total and 724 phosphorylated proteins, and 315 of these had calculated phosphorylation states.

Multivariate data analysis was conducted using Primer-e v7.0 software (<https://www.primer-e.com/>). Data were log transformed and the Euclidean distance was calculated for each protein between samples to derive a resemblance matrix that describes similarity and dissimilarity between samples (Legendre and Legendre, 2012). Calculation of distance for proteins with missing samples (samples previously removed) was corrected by multiplying the summation by a factor of  $(p/p')^{0.5}$  where  $p$  represents the total number of protein samples and  $p'$  is the actual number of protein samples (Clarke and Gorley, 2015). Non-metric multidimensional scaling (nMDS) using the resemblance matrix was conducted to observe the multidimensional similarity by cow and treatment (Figure 2 Supplemental Material). Insight from nMDS plots is straightforward, the closer the points, the more similar in multivariate dimensions (Clarke and Gorley, 2015). The stress index values were always below 0.10 for protein abundance values that were evaluated. This indicated the data could be well represented in reduced dimensions (Legendre and Legendre, 2012). The hierarchal structure of the data (cow and period effects), the number of proteins vastly exceeding the number of samples, and the assumption of multivariate normal distributions for each protein and homogeneity of variance

necessitated a nonparametric approach (Johnson and Field, 1993). Most non-parametric approaches (principle component analysis, cluster analysis, etc.) lack the ability to partition variability according to experimental designs (period and cow) while also assessing treatment or treatment interaction effects. Permutational multivariate analysis of variance (PERMANOVA) while not purely non-parametric, provides a solution for accounting for variance associated with treatment and experimental design factors (Anderson et al., 2008). The analysis and interpretation are analogous to ANOVA analysis. The derived Euclidean sum of squared distances variance represented in the resemblance matrix can be partitioned to treatment, period, and animal effects according to Huygens' theorem (Legendre and Legendre, 2012). This provides a means for calculating sums of squares, mean squared errors, and pseudo-F statistics for each effect (Anderson et al., 2008). In our case, the model contained the fixed effects of MKH ( $df = 1$ ), IL ( $df = 1$ ), their interaction ( $df = 1$ ), and the random effects of cow ( $df = 3$ ) and period ( $df = 3$ ). The distribution of the pseudo-F statistic is unknown given the presence of multivariate data, the possibility of non-normality in variables, and non-Euclidean dissimilarities (Anderson et al., 2008). To obtain a distribution for the F-statistic, the data were permuted by repeatedly varying the assignment of samples to groups.. In our case, 100,000 random permutations were conducted to derive F-statistic distributions which were adequate for achieving repeatable interpretation (Anderson et al., 2008). Homogeneity of variance for each effect was assessed by evaluating the dissimilarity of the Euclidean distances from the centroids as described by Anderson (2006). Random permutation of the residuals was conducted 100,000 times to obtain *P*-values. Significant treatment effects were evaluated visually in reduced dimensions by principal coordinates analysis using the derived resemblance matrix (Anderson et al., 2008).

Univariate proteomic analysis was conducted in R version 3.5.1 (R Core Team, 2018). The total, phosphorylated, and ratio of phosphorylated to total abundances were log transformed. The expression data were fit to a linear model containing the fixed effects of MKH (df = 1), IL (df=1), MKH x IL interaction (df=1), cow (df=3), and period (df=3). Period and cow were considered fixed effects given the small number of observations for estimating random effects and that inference was only for detecting differences in these cows, not the entire cow population. Proteins (phosphorylated abundance, total abundance, and ratio) significantly affected ( $P < 0.05$ ) by the main effects were identified. The raw sample data for these proteins were regressed on period and cow effects and the derived effects used to calculate period and cow adjusted sample data.

## **7.4. Results**

### **7.4.1. Production and Blood AA**

Intake and production results for the cows that were biopsied are listed in Table 1. The biopsy cows (n=4) were a subset of a larger animal trial that has been previously discussed (Chapter 6). For the biopsy cows only, milk protein concentration was increased for the MKH treatment ( $P < 0.01$ ) and protein yield tended to increase from MKH and IL ( $P = 0.14$  and  $P = 0.07$ ). The protein yield treatment differences however were smaller than observed differences in the larger trial, being 2.8, 4.4, and 9.8% higher versus the CON for MKH, IL, and MKH+IL treatments. Intake was unaffected by treatment.

Venous plasma AA concentrations for the biopsy cows are provided in Table 2 and should represent the concentrations that mammary epithelial cells were exposed to. Histidine, Lys, and Met all had increased venous concentrations on MKH treatment, being 38 % ( $P = 0.03$ ), 38 % ( $P = 0.02$ ), and 181 % ( $P < 0.01$ ) higher than the CON infusion (main effect comparison),

respectively. Isoleucine and Leu concentration increases were not significant, although there were numerical increases of 33% ( $P=0.38$ ) and 64% ( $P=0.18$ ), respectively.

#### **7.4.2. Protein Expression (Western Immunoblotting)**

Phosphorylated and total abundance, and the phosphorylated state of signaling proteins in udder tissue are presented in Figure 1. No treatment effects were observed for mTORC1 phosphorylated and total abundances or the phosphorylation state. Infusion of MKH decreased phosphorylated ( $P=0.01$ ) and total abundance of S6K1 ( $P=0.07$ ) as well as decreased the phosphorylation state ( $P<0.01$ ). Infusion of IL also tended to decrease the total abundance of S6K1 ( $P=0.14$ ). A treatment interaction tendency was also observed for phosphorylated ( $P=0.09$ ) and total abundances ( $P=0.09$ ) of S6K1 with the addition of MKH and IL negating the decreases observed with MKH and IL infusions alone. An interaction tendency between MKH and IL effects also was observed for the phosphorylated ( $P=0.14$ ) and total ( $P=0.07$ ) abundances, and phosphorylated state ( $P=0.06$ ) of rpS6. These were all numerically decreased by MKH and IL infusions alone (65% and 69%, 27% and 44%, and 70% and 57%, respectively). However, when combined, the infusions numerically increased these abundances by 13% and 7% and phosphorylation state by 2%. We observed a tendency for decreased expression of eIF2 $\alpha$  for both MKH ( $P=0.10$ ) and IL ( $P=0.12$ ). Additionally, there was an interaction between treatments for the phosphorylated ( $P=0.05$ ) and total abundance of eIF2 $\alpha$  ( $P=0.02$ ) as well as its phosphorylation state ( $P=0.10$ ). This interaction was similar to rpS6 in that the main effects individually decreased phosphorylated and total abundance whereas when combined, the abundances were increased. In general, for all of the proteins in the udder, it appeared that phosphorylated and total abundances decreased when a single group of AA was infused but increased when the AA infusions were combined. However, most of these trends were numerical.

Phosphorylated and total abundance, and phosphorylation state of signaling proteins in muscle tissue are presented in Figure 2. Infusion of Ile and Leu tended to decrease the total abundance of rpS6 by 9.5% ( $P=0.11$ ) but increased the phosphorylated state slightly ( $P=0.14$ ). The MKH treatment also tended to elicit an increase in the phosphorylated state of rpS6 ( $P=0.08$ ). The IL treatment also tended to increase the phosphorylated ( $P=0.14$ ) and total abundances ( $P=0.07$ ) of S6K1 by 9 and 12% respectively (inferred from main effect differences). Additionally, the IL treatment increased the phosphorylation state of eIF2a ( $P=0.03$ ). Overall, the tendency for more effects from the IL treatment were evident compared to MKH in skeletal muscle tissue signaling proteins.

#### **7.4.3. Udder Protein Expression (Proteomics)**

Results of the multivariate analysis of 3504 mammary protein expression changes are shown in Table 3. Methionine, Lys, and His infusion tended to affect the total abundance of proteins in the udder ( $P=0.09$ ) explaining 8% of the total variation. The interaction of the treatments though had a larger effect, explaining 14% of the variation in total protein abundance ( $P=0.01$ ) in the udder. Of the phosphorylated abundance ( $n=1761$ ), the interaction of the treatments also tended to have an effect ( $P=0.08$ ). For the phosphorylation state ( $n=1144$  proteins), Ile and Leu tended to have an effect ( $P=0.08$ ). Interestingly, the random effect of cow explained 43% ( $P<0.001$ ), 70% ( $P<0.001$ ), and 63% ( $P<0.001$ ) of the variation in total abundance, phosphorylated abundance, and the phosphorylation state of the mammary proteome.

Pathway analysis of mTOR related proteins (KEGG #bta04150 pathway) revealed that MKH tended ( $P=0.12$ ) to affect the abundance of the total form of mTOR pathway proteins while significantly affecting the phosphorylation state ( $P=0.04$ ) (Table 4). Infusion of Ile and Leu also tended to affect the phosphorylation state of the mTOR pathway ( $P=0.15$ ). There was also an observed tendency for an interaction between the treatments with the phosphorylation

state of the mTOR pathway in the udder ( $P=0.09$ ). However, as for the total data sets, cow explained much of the variance in all 3 measurements.

Univariate analysis of mTOR related proteins implicated 13 udder proteins as changing their total abundance with MKH infusion ( $P<0.15$ ) versus only 5 udder proteins being influenced by the IL infusion ( $P<0.15$ ) (Table 5). Notable changes from MKH included decreased abundance of 40S ribosomal protein S6 ( $P<0.001$ ), eukaryotic translation initiation factor 4E family member ( $P=0.15$ ), and ribosomal protein S6 kinase ( $P=0.03$ ). Infusion of Ile and Leu increased expression of regulator complex protein LAMTOR5 (related to mTOR complex sensing of AA) ( $P=0.03$ ). Phosphorylation abundance was affected for 2 proteins by both MKH and IL individually. One of those proteins was eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), with decreased expression observed with MKH and IL individually ( $P<0.01$  and  $P=0.05$ ). The phosphorylation state of mTOR related proteins was increased for 3 proteins by MKH and 2 proteins by IL. Again, one of those proteins was 4EBP1 with the phosphorylation state enhanced by IL infusion ( $P=0.03$ ).

Univariate analysis of the entire udder proteome revealed that total protein abundance was affected by MKH for 99 proteins ( $P<0.05$ ) whereas only 42 proteins were affected by IL ( $P<0.05$ ) (Supplemental Materials, Table 2). The most highly affected protein was similar for both MKH ( $P<0.001$ ) and IL ( $P<0.001$ ), being mannose-6-phosphate isomerase. For phosphorylated protein abundance, sixteen proteins were affected by MKH ( $P<0.05$ ) whereas 30 proteins were affected by IL ( $P<0.05$ ) (Supplemental Materials, Table 3). The phosphorylation state of 23 proteins was affected by MKH ( $P<0.05$ ) and 12 proteins by IL ( $P<0.05$ ) (Supplemental Materials, Table 4).

Milk proteins were also evaluated univariately (Table 6). Infusion of MKH decreased the phosphorylated form of alpha-lactalbumin ( $P=0.05$ ). Infusion of IL tended to decrease the total abundance of kappa-casein ( $P=0.07$ ) whereas a decrease was observed for beta-casein total abundance ( $P=0.04$ ). An interaction of treatments was observed for all five identified casein proteins total and phosphorylation abundances ( $P<0.12$ ). This interaction was similar in all five proteins with independent effects of MKH and IL decreasing abundances whereas the combination increased milk casein protein abundances. Alpha-lactalbumin also exhibited an interaction in both total and phosphorylated abundances ( $P<0.07$ ). No treatment differences were observed with phosphorylation state of the milk proteins listed in Table 6 ( $P>0.28$ ).

#### **7.4.4. Muscle Protein Expression (Proteomics)**

Multivariate analysis of 1047 muscle protein expression are shown in Table 3. Changes in the skeletal muscle proteome due to treatment were less evident compared to the udder. Only IL infusion tended to affect the total abundance of proteins in muscle ( $P=0.12$ ). The cow effect once again explained a significant proportion of the muscle proteome variance, accounting for 44% of total abundance variance ( $P<0.001$ ), 35% of phosphorylated abundance variance ( $P=0.08$ ) and 38% of the phosphorylation state variance ( $P=0.01$ ).

Univariate analysis of mTOR related proteins implicated two proteins as changing their total abundance with MKH infusion ( $P<0.10$ ) versus only one protein being influenced by IL infusion ( $P=0.10$ ) (Table 7). Eukaryotic translation initiation factor 4E was one of the proteins influenced by MKH infusion having decreased abundance ( $P=0.07$ ). No changes in the phosphorylated abundance of mTOR related proteins was observed in skeletal muscle. No significant changes were observed in the phosphorylation state of mTOR proteins, although a numerical increase in 4EBP1 was observed ( $P=0.16$ ).

Univariate analysis of the entire muscle proteome revealed 8 proteins with total abundance affected by MKH ( $P < 0.05$ ) whereas 15 proteins were affected by IL ( $P < 0.05$ ) (Supplemental Material, Table 5). The phosphorylated abundance of 3 proteins was affected by MKH ( $P < 0.05$ ) whereas 10 proteins were affected by IL ( $P < 0.05$ ) (Supplemental Material, Table 6). The phosphorylation state of 2 proteins were affected by MKH ( $P < 0.05$ ), and 4 proteins were affected by IL ( $P < 0.05$ ) (Supplemental Material, Table 7).

#### **7.4.5. mRNA Expression**

Two housekeeping gene candidates (GAPDH and H3F3A) were evaluated in mammary and muscle tissues and were not affected by treatment. Treatment had no effect on gene expression in the udder for 5 genes associated with mTOR pathway (EIF3K, MEN1, PRKAA1, RPS6 and YARS), 1 gene with ISR pathway (ATF4), 1 gene with AA transport (SLC7A5), 2 genes with cell turnover (BCL2 and CCND1), and 2 milk protein coding genes (CSN2 and LALBA) ( $P > 0.27$ ). In the muscle, gene expression was also not affected for 4 genes associated with the mTOR and ISR pathways (ATF4, EIF3K, RPS6, and YARS) ( $P > 0.28$ ).

#### **7.4.6. Method Comparison**

A comparison of the effects of treatment on expression of select gene products is presented in Table 10. The proteomic method resulted in more precise measurements of abundance for the five selected proteins than PCR and western immunoblotting. The decreases in CV were between 52.4 to 10.8 percentage units. Total abundance of rpS6, as an example, was not significantly affected by MKH infusion ( $P=0.75$  and  $P=0.54$ , respectively) when assessed by PCR and western immunoblotting. In contrast, a highly significant effect of MKH infusion ( $P < 0.001$ ) was detected using the proteomic results.

## **7.5. Discussion**

When the milk production data from all 8 cows used for this experiment were examined (Chapter 6), there were significant independent increases in milk protein yield with the MKH and IL treatments and these responses were additive. These findings are contradictory to the single limiting AA substrate theory (Mitchell and Block, 1946b). Our hypothesis was that these EAA could stimulate milk protein yield via regulation of protein translation initiation and that the effects would be independent, and additive as observed in rodents (Liu et al., 2017).

The most studied pathways regulating protein translation are mTOR and the integrated stress response networks (ISR). These regulatory pathways integrate information on AA supplies, hormonal factors (e.g. insulin), and energy status (e.g. ATP) to tightly regulate protein synthesis. We measured post-translation modification and abundance of proteins as well as mRNA abundance of genes associated with these pathways for inference.

### **7.5.1. Phosphorylation State of Mammary Proteins**

The lack of an increase in mTORC1 phosphorylation state and the two best characterized substrates of mTOR, S6K1, and rpS6 in udder tissue parallel the results of previous in-vivo cow trials (>5 d treatments) (Arriola Apelo et al., 2014a, Doelman et al., 2015b, Bajramaj et al., 2017, Nichols et al., 2017). The MKH and IL interaction effect on increasing rpS6 does directionally agree with the 9.6% increase in milk protein yield observed in this trial. According to previous and current results, correlation of enhanced S6K1 phosphorylation state to greater milk protein yield is weak if not negative in long term trials. Previously, a 29% increase in milk protein yield resulted in no change in phosphorylation state of S6K1 (Nichols et al., 2017). Supplementation of imbalanced mixes of EAA (devoid of Lys, His, Met, Phe, or Trp) elicited an increase in phosphorylation state of S6K1 compared to saline infusion and in some cases also compared to complete EAA infusion (Doelman et al., 2015a, Doelman et al., 2015b). Proteomic analysis of

4EBP1 in the udder, a substrate of mTOR for translation initiation revealed a decrease in phosphorylation state to IL infusion, again, directionally opposite of what would be expected.

The common markers of activated mTOR pathway in our study did not provide an obvious mechanism for explaining the increased milk protein yield. While the theory that anabolic signaling upregulation from specific EAA (i.e. Leu) predominates results from in vitro, rats, and short-term dairy research (Toerien et al., 2010, Arriola Apelo et al., 2014d, Liu et al., 2017), long term documentation in other species besides dairy also remains elusive. Feeding 4 d old piglets 70% of their protein requirements for 8 d with supplemental Leu increased phosphorylation state of S6K1 and 4EBP1 but failed to increase weight gain or muscle protein synthesis (Manjarin et al., 2016). Consumption of EAA enriched with Leu (40% Leu) in 66 y old women increased muscle protein synthesis similarly to whey protein but failed to elicit a difference in phosphorylation state of S6K1 and 4EBP1 (Bukhari et al., 2015). Bolus dosing whey protein to young men demonstrated a transient increase in muscle protein synthesis for 60-90 min coinciding with enhanced activity of S6K1 and 4EBP1 (Atherton et al., 2010). However, muscle protein synthesis decreased despite continued activation of S6K1 and 4EBP1 demonstrating that enhanced anabolic signaling was unable to long term upregulate protein synthesis. Some argue that given a review of the literature, supplementation of Leu or BCAA in humans does not cause long term upregulation of anabolic activity or protein synthesis as often speculated (Wolfe, 2017).

One potential reason for the lack of response is negative feedback loops on mTORC1 activation, which is necessary to prevent out-of-control anabolic activity. Besides the commonly known substrate limitation of protein mis-folding and uncharged tRNAs (IRS pathway, phosphorylation of eIF2 $\alpha$ ), phosphorylation of S6K1 can inhibit activation by Akt and growth

factors on mTOR. One specific example is that activated S6K1 increases phosphorylation of insulin receptor substrate 1 (IRS1) at specific sites that promote degradation of IRS1 (Haruta et al., 2000, Um et al., 2004). Additionally, activated S6K1 reduces mRNA expression of IRS1 (Harrington et al., 2004). Interestingly, we found increased phosphorylation state of IRS1 ( $P < 0.001$ ) from MKH infusion and a numerical increase from IL infusion. Activation of IRS1 if occurring in phosphorylation sites from S6K1 infers negative feedback is occurring. Decreased IRS1 intracellularly will depress sensitivity to insulin and activation of Akt and mTORC1 (Dibble et al., 2009). The increased activation of mitogenic-activated protein kinase isoform 2 (MAP2K1) by both MKH ( $P = 0.08$ ) and IL ( $P = 0.06$ ) treatments also support the notion of Akt downregulation and promotion of insulin resistance. Interestingly, phosphorylation state of MAP2K1 was affected by a negative treatment interaction ( $P = 0.03$ ) which coincides with the 9.6% increase in milk protein yield with MKH+IL infusion. In human isolated skeletal muscle cells in vitro, increased expression of MAP2K1, MAP2K4, and MAP4K4 resulted in insulin resistance (Bouzakri and Zierath, 2007). Our findings of decreased lactose concentration with cows that upregulated milk protein concentration the most (MKH treatment) correlate to the increased insulin resistance that might be occurring from upregulation of IRS1 and MAP2K1 that we observed. Hence, we may be observing long term increased negative feedback thereby explaining our lack of observed increased S6K1, mTORC1, and rpS6 activity. This negative feedback maybe indicative of the significant multivariate effects (PERMANOVA) we observed by MKH and IL individually and the treatment interaction on mTOR pathway phosphorylation state in the udder. Our multivariate findings do not indicate directionality in whether mTOR pathway proteins have enhanced or decreased phosphorylation state. However, as discussed, the univariate analysis reveals upregulation of IRS1 and MAP2K1 and downregulation of 4EBP1

phosphorylation status supporting our hypothesis of a negative feedback to our treatments that increased extracellular EAA supplies. When milk protein yield was increased by 9.6%, the excess of AA relative to demand was likely less, hence, the decreased MAP2KI phosphorylation state observed with MKH+IL infusion and lessened negative feedback on mTORC1. This finding also may explain the positive treatment interactions for S6K1, rpS6, and 4EBP1 given reduced negative feedback from MAP2K1.

### **7.5.2. Phosphorylation State of Muscle Proteins**

In the skeletal muscle, increased phosphorylation state of rpS6 was observed from MKH infusion. This protein is phosphorylated by S6K1 and activates ribosomal biogenesis as it is important for rRNA synthesis, post-transcriptional modifications, and assembly of ribosomal proteins (Chauvin et al., 2014). Previously, feeding rumen-protected Met for 10 weeks increased skeletal muscle phosphorylation of rpS6 but not S6K1 (Sadri et al., 2016a) which is similar to what we observed. Others measured only S6K1 in muscle and observed no effects from infusing a complete mix of EAA (Nichols et al., 2017). The 181% rise in mammary venous concentrations of Met obviously would increase Met extracellularly for skeletal muscle cells. This appeared to have activated increased ribosomal biogenesis via rpS6 which might result in greater pull of AA by extra-mammary tissues.

In contrast to minor anabolic activation by MKH, infusion of Ile and Leu tended to decrease rpS6 phosphorylation state while increasing phosphorylation state of eIF2 $\alpha$  in muscle tissue. Many have documented the sensitivity of muscle tissue to Leu and its upregulation of mTOR signaling pathway (Kimball et al., 2000). Perhaps, decreased intracellular availability of limiting AA with the IL treatment such as Met and Lys (Met was 9.1  $\mu$ M and Lys was 32.0  $\mu$ M in mammary venous plasma) led to increased uncharged tRNA abundance in muscle tissue. The latter would increase phosphorylation of eIF2 $\alpha$  as was observed and decrease rpS6 activity. An

activated eIF2 $\alpha$  is an inhibitor of eIF2B by blocking the release of GDP which prevents methionyl-tRNA recruitment and active ternary complex for protein synthesis (Holcik, 2015). Further support of downregulated mTOR exists with the numerical decrease in rpS6 mRNA abundance we observed in the muscle tissue. In summary, it appears that minor activation of muscle mTOR pathway occurred with MKH infusion whereas infusion of IL resulted in a minor decrease. However, multivariate analysis of the phosphorylation state of the mTOR pathway in muscle suggest no effects of treatment. Given that these were high producing cows, insulin sensitivity in the muscle was probably low to support homeorhesis state (Bauman and Currie, 1980). Hence these tissues likely were less sensitive to increased AA supplies for stimulation since the insulin arm of mTOR regulation likely had a low signal.

### **7.5.3. Abundance of Mammary Proteins**

The decreased mammary total abundance of S6K1 despite an increase in milk protein yield was also observed by a previous study (Doelman et al., 2015a). Decreased mammary S6K1 phosphorylation abundance has also been observed in complete EAA infusion mixtures compared to EAA infusion mixtures lacking 1 to 3 EAA (Doelman et al., 2015a, Doelman et al., 2015b). S6K1 in a number of longer-term trials (>5 d) appears sensitive to dietary nutrition manipulation (Doelman et al., 2015a, Doelman et al., 2015b, Nichols et al., 2017). However, the directional changes in abundance and phosphorylation state often do not match expected changes given observed milk protein yield changes. The proteomic analyses mimicked the western immunoblotting findings for S6K1. One reason for the mismatch may be negative feedback on anabolic activation by S6K1. Two serine kinases, JNK1 and IKK2, transmit signals from the TNF- $\alpha$  pathway and regulate stability of S6K1. Decreased IKK2 signal results in ubiquitinylation and degradation of S6K1 despite mTORC1 activation (Zhang et al., 2013). Regulation of S6K1 does not occur solely through the mTORC1 network as ubiquitinylation

and turnover of S6K1 provide another source of regulation. As discussed earlier, we also observed increased activation of IRS1 and MAP2K1 which would also negatively regulate mTORC1. Another possible reason is that decreased intracellular AA availability of other EAA depressed mTORC1 signaling via phosphorylation of eIF2 $\alpha$  from uncharged tRNAs. We did observe an arterial decrease in concentration of EAA not infused. However, we also observed a numerical decrease in phosphorylation state of eIF2 $\alpha$  which is contrary to expectations when AA become limiting.

The treatment positive interaction effects for S6K1 and rpS6 on total and phosphorylation abundances point towards enhanced translation initiation to support the 9.6% increase in milk protein yield. Phosphorylation abundance of 4EBP1 followed a similar pattern as S6K1 and rpS6 but was not significant. These western immunoblotting findings were confirmed by proteomic analyses with significant treatment interactions for both S6K1 and rpS6 total abundance. For proteomic analysis of 4EBP1 phosphorylation abundance, a similar trend was observed as western immunoblotting except that MKH and IL infusions had a significant negative effect versus only a numerical effect with western immunoblotting. A treatment interaction resulted in a positive effect on phosphorylated abundance of 4EBP1 as well. However, despite the positive interactions for the observed total and phosphorylation abundances of these proteins, the abundances were not different from the CON consisting of infused saline. Supplementation of the two groups alone did increase milk protein yield but apparent negative feedback reduced mTOR signaling. When supplemented together, the greatest effect on milk protein yield was observed (9.6%) and seemed to occur by positive interaction of many mTOR related proteins. This positive treatment interaction was likely spurred by less excess of intracellular AA relative to demand. Excess AA and subsequently phosphorylation of S6K1 will

down-regulate mTOR by negative feedback on IRS1 via phosphorylated MAP2K1 (Manning, 2004). We observed a decrease in S6K1 activity from the treatment interaction. The significant treatment interaction on the multivariate analysis of total and phosphorylated abundances for all proteins analyzed also suggests a relationship between the treatments. While directionality cannot be determined from the multivariate analysis, these infused EAA acting as signals to affect protein expression and phosphorylation is evident. That MKH affected mammary total protein abundances versus no effect of IL infusion coincides with more univariate changes observed with MKH infusion. Of the EAA infused, Met increased extracellularly 181%, far exceeding the rise of other infused EAA, which, if proportional to intracellular Met concentrations, may have resulted in more changes in the proteome than the other treatment, IL. Methionine can be transformed to be a methyl donor in the one-carbon pathway. Methionine adenosyltransferase enzyme catalyzes the reaction of Met and ATP to form the methyl donor S-adenosylmethionine (SAM) (Gu et al., 2017). We greatly increased Met supplies which likely translated into altered one-carbon metabolism.

Inhibition of protein translation was reduced given the observed decrease in total abundance of eIF2 $\alpha$  by both MKH and IL treatment infusions alone. This suggests less uncharged tRNAs intracellularly were present allowing inference that AA supply versus AA demand was more balanced. Previously, no changes in abundances of eIF2 $\alpha$  were observed from complete mix of EAA infusion (Doelman et al., 2015a, Nichols et al., 2017) but the milk protein production in our study was much higher despite a similar stage of lactation (49% and 29% higher respectively). There was a positive treatment interaction with total and phosphorylated abundances with eIF2 $\alpha$ . This might infer increased prevalence of uncharged tRNAs when milk protein was increased by 9.6%. We observed decreases in plasma EAA

concentrations that were not infused. That greater activation of ISR pathway coincided with greater mTOR pathway activation and milk protein yield. This might suggest greater anabolic state resulting in more demand for intracellularly AA that is also resulting in a more active ISR pathway to balance this demand. We did not observe a greater phosphorylation state of eIF2 $\alpha$  which suggests that despite greater abundance of the total and phosphorylated forms (positive interaction), a strong negative signal to downregulate protein synthesis was not evident.

#### **7.5.4. Abundance of Muscle Proteins**

In skeletal muscle, minor changes were observed with total and phosphorylation abundances of proteins as indicated by western immunoblotting and proteomics. Previously, supplementation of a complete mix of EAA or addition of Met and His did not change S6K1 total or phosphorylated abundances (Sadri et al., 2016b, Nichols et al., 2017). Changes in anabolic signaling in muscle to Leu have been well documented and in our case we observed slight S6K1 total abundance increase without a change in the phosphorylation state. The observed decrease of rpS6 total abundance which is a phosphorylated target of S6K1 does not match with expectations of an enhanced mTOR activation. Decreasing MP supplies to lactating cows over a 10 wk feeding trial actually increased total abundance of rpS6 while numerical decreasing S6K1 abundance (Sadri et al., 2016a). No treatment multivariate effects in the mTOR pathway were observed. However, a tendency for a change in muscle total abundance was observed from IL treatment. Our results tend to suggest that Ile and Leu had more impact on protein abundances in muscle. Some have suggested these EAA direct AA towards muscle by stimulating protein synthesis (Appuhamy et al., 2011b, Curtis et al., 2018). Infusion of 150 g/d of BCAA depressed milk protein yield and caused a decrease in non-BCAA essential AA plasma concentrations indicating possibly enhanced muscle draw for these AA. In our experiment, we observed an increase in milk protein yield from infusion of Ile and Leu which suggests that AA

were not being siphoned off to muscle to an extent that limited milk protein yield. However, we tended to see changes in abundance of total and phosphorylated more so than Met, Lys, and His highlighting apparent Ile and Leu affinity for muscle.

#### **7.5.5. Milk Proteins**

The decrease in three casein proteins from IL infusion versus no change in MKH corresponds with the observed milk protein concentrations changes. In the larger animal trial (Chapter 6), the milk protein concentrations were 3.10, 3.19, 3.04, and 3.21 for CON, MKH, IL, and MKH+IL treatments. Interestingly, it appears these concentrations differences correlate to observed intracellular concentrations of kappa-casein, beta-casein, and alpha-S1-casein. There was also positive interaction of MKH and IL for total and phosphorylated abundance of 5 milk casein and whey proteins which correlates with the 9.6% increase in milk protein yield. Export of milk proteins requires phosphorylation for packaging into vesicles (Akers, 2002). The phosphorylation state of casein and whey proteins were unchanged. This indicates that this step for cellular export packaging was unchanged or not inhibited despite up to a 9.6% increase in milk protein export by the cells. In general, proteomic analyses appeared to possess sensitivity to match observed changes in milk protein content with cellular milk protein content.

Previously, we observed a decreased lactose concentration in cows infused with MKH. As discussed in Chapter 6, the lactose concentrations were 5.04, 4.99, 5.05, and 4.97 in the CON, MKH, IL, and MKH+IL with the MKH effect being highly significant ( $P < 0.001$ ). Interestingly, this corresponded to decreased phosphorylation abundance of alpha-lactalbumin from MKH infusion. Phosphorylation of this protein triggers lactose disaccharide formation of galactose and glucose via allosteric activation of galactosyl transferase (Akers, 2002). This infers that the slight change in lactose concentration could mechanistically be traced to measured change in a key enzyme in lactose synthesis and demonstrates the precision inference of proteomic analysis.

In a rat study, a link between activated MAPK pathway (MAP2K2 protein) and phosphorylated IRS1 receptor and down regulation alpha-lactalbumin as part of insulin resistance has been demonstrated (Gao et al., 2018). The enhanced phosphorylation state of IRS1, MAP2K1, and also an associated protein mitogen-activated protein kinase from MKH infusion negatively affected phosphorylation of alpha-lactalbumin thereby decreasing lactose concentration slightly in the MKH infused cows. This suggests cows that are fed Met, Lys, and His supplies according to CNCPS v6.55 recommendations without adequate supply of other EAA may lead to negative feedback on mTOR pathway and decreased concentrations of lactose. Lactose is important for osmolarity of milk (Akers, 2002) hence fairly constant in milk concentration. Therefore, increasing milk yield requires greater lactose synthesis. In our case, we demonstrate that if MAPK pathway is activated, lactose synthesis may be hampered and this may limit any increase in milk production from what are considered limiting EAA. The lack of response in milk yield to Met and Lys is often observed (Appuhamy et al., 2011b, Sinclair et al., 2014, Zanton et al., 2014). This in part might be due to excess supplies of Met and Lys depressing lactose synthesis machinery, though this is highly speculative.

#### **7.5.6. mRNA Abundance**

The lack of change for casein encoding mRNAs abundances correspond to findings in a similar trial where milk protein yield was increased by 29% but no change in mRNA abundance of casein genes was observed (Nichols et al., 2017). Milk protein gene transcription is regulated by prolactin, insulin, and cortisol signals with strong activation at the onset of lactation (Rosen et al., 1999). Nutritional interventions do not appear to affect milk protein gene expression (Cant et al., 2018). We also did not observe changes in mRNA abundance of genes in the udder related to mTOR and ISR pathways (i.e. RPS6, ATF4, SLC7A5, YARS, PRKAA1, MEN1) or cell turnover (i.e. CCND1 and BCL2). Again, previous studies have also not shown changes in

mRNA abundance of these genes in mammary tissue. For example, infusion of a complex EAA mix or simply feeding a higher dietary CP diet increased milk protein yields by 29% and 34% respectively but no changes in mRNA abundance of genes related to mTOR and ISR pathways or cell turnover were observed (Nichols et al., 2017, Curtis, 2018). We did not observe a change in MEN1 which encodes for the protein Menin. Menin is thought to attenuate effects of insulin and prolactin effects (Agarwal et al., 2005). In Mac-T cells, overexpression of MEN1 downregulated mTOR, S6K1, and 4EBP1 (Li et al., 2017a).

Proteomic analysis demonstrated enhanced precision compared to western immunoblotting which agrees with previously commentary of benefits of recent strides in proteomics (Aebersold et al., 2013). Previous studies have indicated a minor role for mTOR pathway proteins (S6K1, 4EBP1, rpS6) in regulating long term milk protein yields and these findings arise from lack of significant treatment effects and in some cases, directionality expression changes that do not agree with increased milk protein yield (Arriola Apelo et al., 2014a, Doelman et al., 2015a, Doelman et al., 2015b, Nichols et al., 2017, Cant et al., 2018). A broader representation herein reveals treatment effects on mTOR pathway (multivariate) and that negative feedback might be occurring when milk protein yield is stimulated. Maintaining a new state of protein expression is not only a function of translation or transcription but factors such as degradation and temporal treatment differences on transcription, translation, folding, modification, and degradation (Vogel and Marcotte, 2012, Cheng et al., 2016). Measuring mRNA abundance is also weakly correlated to protein expression state (30-40% in mammalian cells) and time-course for treatment effects reveals more of pulse like response to achieving a new steady state expression of protein (Vogel and Marcotte, 2012). Proteomics in our study demonstrates a valuable tool for insight into how nutrients affect protein expression and post-

translation modifications that appears to be superior to western immunoblotting and real-time PCR.

The increase in milk protein yield independently by two groups of EAA appears to be related to changes in protein expression and phosphorylation state within the mTOR pathway. These increases appeared to result in negative feedback and in the case of MKH infusion, seemed to affect insulin sensitivity and lactose synthesis. We balanced the MKH treatment diet according to NRC (2001), CNCPS v6.55, and industry recommendations. Given the numerical decrease in mammary plasma flow (Chapter 6), decreased lactose concentration, decreased S6K1, rpS6, and 4EBP1 abundances as well as phosphorylation state of  $\alpha$ -lactalbumin, and increased negative feedback by MAP2K1 and IRS1 observed from the MKH treatment, perhaps the EAA were supplied in excess. While greater milk protein yield was obviously observed with MKH treatment, no changes in milk yield, negative feedback, and low marginal efficiency to milk protein (Met 9%, Lys 13%, His 9%) draws into question the recommendation merit. However, when combined with Ile and Leu supplementation, this negative feedback was reduced (MAP2K1 decreased), greater milk yield achieved (3 kg/d), increased marginal efficiency (Met 20%, Lys 31%, His 20%) and an additive effect on milk protein yield. Regulation of milk and milk protein yield is highly multivariate, and nutrients are more than substrates with ability to affect protein expression and activity (post-translation modification). Attention to more EAA than Met, Lys, and His supplies when formulating lactating cow diets, particularly low protein diets, is warranted. Sizeable milk and milk component responses to EAA supplementation in some studies (i.e. 5.4 kg/d over 10 wk trial (Noftsker and St-Pierre, 2003) versus minor to no responses (Sinclair et al., 2014, Zanton et al., 2014) support previous insight that attention to more EAA than Met and Lys is necessary (Hanigan et al., 2000, Chandler and Cawood, 2010).

Delineation of supplementation EAA recommendations also should not be solely based on substrate balance but should consider signaling effects of EAA.

## **7.6. Conclusions**

Supplementation of two groups of EAA independently affected udder mTOR pathway proteins in a multivariate manner and correlated to independently increased milk protein yield also being observed from these two groups of EAA. Western immunoblotting and proteomic analysis in concert revealed decreased expression and phosphorylation state of direct mTORC1 targets, i.e. S6K1, rpS6, and 4EBP1 from supplementation of these two groups of EAA. The one EAA group, Met, Lys, and His, when supplemented at industry recommendations, increased MAP2K1 and IRS1 phosphorylation states which leads to insulin resistance which in our study may have provided negative feedback on mTOR signaling. Decreased phosphorylation of  $\alpha$ -lactalbumin and lactose concentrations also occurred from infusion of Met, Lys, and His which might be linked to activated MAP2K1. Positive treatment interactions were observed for mTORC1 targets (i.e. S6K1, rpS6, and 4EBP1) indicating possibly less negative feedback in maintaining the new anabolic resulting state of 9.6% increased milk protein yield. Minor changes were observed in skeletal muscle from both groups of EAA and in a multivariate sense, only infusion of Ile and Leu demonstrated an effect on protein expression. The negative feedback and directionally reversed changes in mTORC1 downstream targets demonstrate the complexity of long-term regulation of milk protein yield and challenge of studying mTOR regulation on milk protein yield. This study implicates two groups of EAA as functioning beyond substrates to affect protein expression and post translation modifications of proteins in the udder of lactating dairy cows. Greater milk protein yield stimulation occurred with supplementation of both groups of EAA and this was facilitated by positive treatment interactions of mTORC1 signaling proteins

and decreased negative feedback. The latter finding highlights importance of other EAA beyond Met, Lys, and His in lactating dairy cow nutrition.

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**Table 7. 1.** Effect of AA infusions on intake and production of lactating cows. Data are presented as least-square treatment means (n=4 cows).

Item	Treatments <sup>1</sup>				SEM	Effect (P-value)		
	CON	MKH	IL	MKH + IL		MKH	IL	MKH*IL
DMI, kg/d	24.4	24.7	24.6	25.0	0.92	0.64	0.60	0.91
Milk, kg/d	44.4	44.8	46.8	47.2	1.70	0.69	0.07	0.99
ECM, kg/d <sup>2</sup>	44.2	44.8	46.7	47.1	1.85	0.68	0.09	0.97
Milk protein %	3.18	3.25	3.13	3.32	0.05	<0.01	0.75	0.14
Milk protein, g/d	1410	1449	1472	1548	55	0.14	0.07	0.61
Milk fat %	3.53	3.46	3.58	3.41	0.17	0.29	0.99	0.66
Milk fat, g/d	1548	1557	1648	1626	95	0.92	0.23	0.8
Lactose, %	5.03	5.00	5.06	4.98	0.05	0.04	0.79	0.38
Lactose, g/d	2236	2244	2377	2364	96	0.97	0.09	0.87

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>ECM=energy-corrected milk calculated as (12.82 x kg of fat) + (7.13 x kg of protein) + (0.323 x kg of milk).

**Table 7. 2.** Venous plasma amino acid concentrations in lactating dairy cows subjected to AA infusion (least-square treatment means)<sup>1</sup>Venous plasma amino acid concentrations in lactating dairy cows subjected to AA infusions (least-square treatment means)<sup>1</sup>

uM	Treatments <sup>2</sup>				SEM	Effect ( <i>P</i> -value)		
	CON	MKH	IL	MKH + IL		MKH	IL	MKH*IL
Arg	44.8	47	36.4	48.6	7.7	0.26	0.58	0.42
His	57.1	68.7	42.7	68.9	8.0	0.03	0.34	0.30
Ile	70.4	87.8	123.7	86.7	29.1	0.71	0.38	0.34
Leu	103.1	106.5	200.0	143.0	47.6	0.52	0.18	0.49
Lys	30.7	43.2	33.3	45.5	5.6	0.02	0.55	0.98
Met	8.0	25.5	10.3	25.7	4.7	<0.01	0.68	0.72
Phe	22.5	13.6	24.2	15.8	3.6	0.03	0.52	0.92
Thr	60.8	48.6	45.0	48.5	11.6	0.63	0.42	0.39
Val	193.8	206.8	194.7	161.9	52.6	0.45	0.32	0.91
Ala	217.4	221.6	212.5	214.2	35.2	0.91	0.82	0.96
Asn	12.8	9.7	8.8	11.6	3.4	0.95	0.69	0.23
Asp	5.6	4.8	5.5	6.5	2.3	0.96	0.71	0.67
Gln	97.7	85.5	96.0	102.7	13.2	0.71	0.35	0.23
Glu	51.4	53.6	60.1	51.5	9.8	0.70	0.70	0.53
Gly	290.5	250.7	262.4	255.5	49.3	0.47	0.73	0.60
Pro	85.0	80.3	69.4	86.1	11.2	0.57	0.65	0.33
Ser	58.1	44.6	52.6	46.7	14.7	0.33	0.87	0.68
Tyr	11.1	-1.4	5.8	0.3	4.2	0.05	0.63	0.36

<sup>1</sup>Data represent average venous AA concentrations sampled from the subcutaneous mammary abdominal vein on d 16 of the experimental period.

<sup>2</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

**Table 7. 3.** Effect of amino acid infusions on expression and post translational modification of proteins in mammary and muscle tissue assessed by permutational multivariate ANOVA.

Effect <sup>1</sup>	Total <sup>2</sup>		Phosphorylated <sup>2</sup>		Ratio <sup>2</sup>	
	R <sup>2</sup>	P-value	R <sup>2</sup>	P-value	R <sup>2</sup>	P-value
<i>Mammary</i> <sup>3</sup>						
MKH	0.08	0.09	0.03	0.41	0.04	0.31
IL	0.03	0.52	0.05	0.20	0.08	0.08
MKH*IL	0.14	0.01	0.07	0.08	0.07	0.13
Cow	0.43	<0.001	0.70	<0.001	0.63	<0.001
Period	0.29	0.01	0.10	0.34	0.13	0.32
<i>Muscle</i> <sup>4</sup>						
MKH	0.05	0.45	0.04	0.96	0.04	0.92
IL	0.08	0.12	0.09	0.33	0.07	0.47
MKH*IL	0.05	0.35	0.04	0.95	0.05	0.69
Cow	0.44	<0.001	0.35	0.08	0.38	0.01
Period	0.21	0.12	0.19	0.77	0.16	0.85

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>Total represents the protein abundance in total form, phosphorylated represents abundance of total form that is phosphorylated, and the ratio is phosphorylated abundance divided by total.

<sup>3</sup>Mammary dataset contained 3504 proteins in total form, 1761 proteins in phosphorylated form, and the ratio of 1144 proteins.

<sup>4</sup>Muscle dataset contained 1047 proteins in total form, 724 proteins in phosphorylated form, and the ratio of 315 proteins.

**Table 7. 4.** Effect of amino acid infusions on expression and post translational modification of mTOR pathway proteins (KEGG #bta04150) in mammary and muscle tissue assessed by permutational multivariate ANOVA

Effect <sup>1</sup>	Total <sup>2</sup>		Phosphorylated <sup>2</sup>		Ratio <sup>2</sup>	
	R <sup>2</sup>	P-value	R <sup>2</sup>	P-value	R <sup>2</sup>	P-value
<i>Mammary</i> <sup>3</sup>						
MKH	0.08	0.12	0.02	0.87	0.07	0.04
IL	0.04	0.35	0.00	1.00	0.04	0.15
MKH*IL	0.06	0.20	0.08	0.37	0.05	0.09
Cow	0.48	<0.001	0.61	0.05	0.74	<0.001
Period	0.22	0.13	0.07	0.95	0.13	0.07
<i>Muscle</i> <sup>4</sup>						
MKH	0.06	0.40	0.03	0.88	0.12	0.20
IL	0.07	0.32	0.15	0.17	0.11	0.23
MKH*IL	0.03	0.85	0.04	0.80	0.01	0.85
Cow	0.48	0.02	0.30	0.36	0.35	0.19
Period	0.11	0.78	0.14	0.88	0.14	0.56

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>Total represents the protein abundance in total form, phosphorylated represents abundance of total form that is phosphorylated, and the ratio is phosphorylated abundance divided by total.

<sup>3</sup>Mammary dataset contained 34 proteins in total form, 13 proteins in phosphorylated form, and the ratio of 10 proteins.

<sup>4</sup>Muscle dataset contained 9 proteins in total form, 8 proteins in phosphorylated form, and the ratio of 3 proteins.

**Table 7. 5.** Effects of AA infusions on total and phosphorylated protein abundance and post translational phosphorylation state of mTOR pathway proteins (KEGG #bta04150) in mammary tissue (n=4)<sup>1</sup>

Item <sup>2</sup>	Accession <sup>3</sup>	Least Square Means				SEM	Effect (P-value)		
		CON	MKH	IL	MKH+IL		MKH	IL	MKH*IL
<i>Total<sup>4</sup></i>									
40S ribosomal protein S6	Q5E995	30.7	29.0	29.6	29.8	0.08	0.00	0.41	0.00
Calcium-binding protein 39	Q29RI6	27.2	27.3	27.1	26.9	0.19	0.46	0.54	0.54
CAP-Gly domain containing linker protein 1	E3W9A2	25.0	24.7	24.8	24.4	0.15	0.04	0.61	0.70
Eukaryotic translation initiation factor 4E family member 2	Q0II31	24.5	23.5	24.1	24.2	0.19	0.15	0.62	0.17
Eukaryotic translation initiation factor 4E	Q9N0T5	28.3	27.5	27.7	27.9	0.15	0.13	0.96	0.10
Eukaryotic translation initiation factor 4E-binding protein 1	Q0P5A7	25.3	24.4	24.8	25.3	0.16	0.20	0.43	0.04
Growth factor receptor-bound protein 2	Q3T0F9	27.4	27.2	27.3	27.0	0.03	0.00	0.87	0.25
Inhibitor of nuclear factor kappa-B kinase subunit beta	Q95KV0	22.0	22.4	23.3	22.7	0.28	0.28	0.08	0.33
Insulin receptor substrate 1	F1MYE0	24.4	22.9	23.4	23.1	0.15	0.01	0.78	0.05
KRAS proto-oncogene, GTPase	E1BMX0	25.9	25.8	26.0	25.4	0.17	0.34	0.80	0.28
MAP2K1 protein	Q0VD16	28.8	28.2	28.4	28.5	0.07	0.02	0.91	0.03
MAP2K2 protein	Q17QH2	26.1	26.1	26.0	26.0	0.29	0.66	0.89	0.90
Mitogen-activated protein kinase 1	P46196	29.4	29.4	29.4	29.1	0.05	0.02	0.12	0.05
Mitogen-activated protein kinase	E1B8P9	27.6	28.1	28.0	27.8	0.12	0.74	0.79	0.10
Non-specific serine/threonine protein kinase	E1B9D1	26.0	26.1	26.3	25.9	0.10	0.16	0.48	0.20
Nucleoporin SEH1	A7YY75	23.0	23.9	23.7	22.8	0.11	0.31	0.81	0.03
Phosphatidylinositol 3-kinase regulatory subunit alpha	P23727	25.4	26.4	24.8	24.7	0.50	0.84	0.12	0.40
Protein kinase C alpha type	P04409	27.0	28.6	28.2	28.8	0.53	0.15	0.61	0.52
Protein SEC13 homolog	Q3ZCC9	27.9	26.8	27.3	27.4	0.14	0.03	0.44	0.03
Ragulator complex protein LAMTOR1	Q3T0D8	25.0	23.5	24.6	24.6	0.43	0.77	0.62	0.33
Ragulator complex protein LAMTOR2	Q3T132	23.8	23.3	23.3	23.8	0.36	0.65	0.77	0.30
Ragulator complex protein LAMTOR3	Q17QQ1	26.3	25.3	25.6	25.4	0.35	0.51	0.98	0.47
Ragulator complex protein LAMTOR5	Q3SZ68	24.7	24.4	24.9	24.7	0.10	0.26	0.03	0.63

Ras-related GTP binding C	Q0VD29	24.9	25.2	24.6	24.3	0.20	0.43	0.08	0.44	
Ribosomal protein S6 kinase	A4IFF4	25.8	24.5	25.1	25.3	0.14	0.03	0.95	0.02	
SLC3A2 protein	Q08DL0	24.5	22.5	23.5	22.3	0.94	0.23	0.96	0.76	
Transforming protein RhoA	P61585	30.2	29.8	29.9	29.8	0.08	0.06	0.69	0.14	
V-type proton ATPase catalytic subunit A	P31404	28.9	28.9	28.8	28.8	0.07	0.98	0.76	0.91	
V-type proton ATPase subunit B, brain isoform	P31408	28.3	27.9	27.7	28.0	0.13	0.53	0.24	0.12	
V-type proton ATPase subunit C 1	P21282	21.6	22.8	22.5	22.5	0.42	0.43	0.91	0.26	
V-type proton ATPase subunit D	F1N270	25.6	25.1	25.1	24.9	0.13	0.08	0.45	0.28	
V-type proton ATPase subunit E 1	P11019	27.1	26.5	26.7	26.5	0.14	0.08	0.65	0.27	
V-type proton ATPase subunit F	Q28029	25.9	24.9	25.7	25.4	0.28	0.12	0.46	0.34	
V-type proton ATPase subunit G 1	P79251	26.2	26.1	25.8	26.0	0.16	0.82	0.18	0.52	
<b><i>Phosphorylated<sup>4</sup></i></b>										
CAP-Gly domain containing linker protein 1	E3W9A2	23.1	24.7	25.0	24.0	0.21	0.44	0.40	0.01	
Eukaryotic translation initiation factor 4E-binding protein 1	Q0P5A7	29.5	28.4	28.6	29.1	0.05	0.00	0.05	0.00	
Insulin receptor substrate 1	F1MYE0	25.4	24.5	24.7	25.5	0.06	0.08	0.08	0.00	
MAP2K1 protein	Q0VD16	24.1	25.1	24.8	24.5	0.26	0.46	0.42	0.15	
Mitogen-activated protein kinase 1	P46196	22.5	24.3	24.2	23.2	0.30	0.53	0.22	0.03	
Mitogen-activated protein kinase	E1B8P9	21.2	22.1	22.2	21.6	0.28	0.97	0.58	0.17	
Non-specific serine/threonine protein kinase	E1B9D1	22.1	22.2	22.2	22.4	0.45	0.84	0.86	0.97	
Protein kinase C alpha type	P04409	26.1	26.5	26.8	26.6	0.21	0.83	0.25	0.32	
RPTOR independent companion of MTOR complex 2	F1MHN4	20.6	18.6	19.2	18.5	2.26	0.66	0.86	0.85	
Transforming protein RhoA 1	P61585	21.8	22.9	22.5	23.7	0.75	0.83	0.69	0.98	
<b><i>Ratio<sup>4</sup></i></b>										
CAP-Gly domain containing linker protein 1	E3W9A2	2.7	4.6	4.8	4.2	0.25	0.28	0.34	0.03	
Eukaryotic translation initiation factor 4E-binding protein 1	Q0P5A7	8.7	8.8	8.5	8.5	0.15	0.66	0.03	0.90	
Insulin receptor substrate 1	F1MYE0	5.6	6.2	5.9	7.1	0.09	0.00	0.22	0.06	
MAP2K1 protein	Q0VD16	0.0	1.3	0.8	0.5	0.23	0.08	0.06	0.03	
Mitogen-activated protein kinase 1	P46196	-2.3	0.1	-0.4	-0.9	0.44	0.10	0.74	0.04	
Mitogen-activated protein kinase	E1B8P9	-1.8	0.1	-0.8	-1.0	0.57	0.36	0.52	0.19	

Non-specific serine/threonine protein kinase	E1B9D1	0.7	1.7	1.1	1.5	0.69	0.31	0.55	0.73
Protein kinase C alpha type	P04409	3.7	3.6	3.7	2.9	0.68	0.53	0.45	0.64
Transforming protein RhoA	P61585	-3.8	-2.8	-2.8	-1.8	0.66	0.51	0.72	1.00

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>Data are expressed as log transformed abundance of proteins.

<sup>3</sup>UniProt accession number.

<sup>4</sup>Total represents the protein abundance in total form, phosphorylated represents abundance of total form that is phosphorylated, and the ratio is phosphorylated abundance divided by total.

**Table 7. 6.** Effects of AA infusions on expression and post translational modification of casein related proteins in mammary tissue of lactating dairy cows<sup>1</sup>

Item <sup>2</sup>	Accession <sup>3</sup>	Least Square Means				SEM	Effect ( <i>P</i> -value)		
		CON	MKH	IL	MKH+IL		MKH	IL	MKH*IL
<b><i>Total</i><sup>4</sup></b>									
Alpha-S2-casein	P02663	34.3	33.2	32.7	34.0	0.26	0.60	0.48	0.03
Kappa-casein	A0A140T8A9	33.6	33.0	32.1	33.4	0.18	0.45	0.07	0.02
Beta-casein	P02666	34.6	33.7	32.7	34.2	0.17	0.53	0.04	0.01
Alpha-S1-casein	P02662	36.1	35.7	35.1	36.3	0.14	0.12	0.14	0.02
Alpha-lactalbumin	P00711	33.3	32.8	32.0	33.3	0.24	0.48	0.32	0.06
<b><i>Phosphorylated</i><sup>4</sup></b>									
Kappa-casein	A0A140T8A9	26.6	24.6	25.0	25.4	0.37	0.42	0.62	0.07
Alpha-S1-casein	P02662	37.0	36.2	35.8	36.6	0.17	0.81	0.16	0.04
Alpha-S2-casein	P02663	34.2	33.2	33.3	34.2	0.17	0.42	0.29	0.02
Beta-casein	P02666	35.4	34.2	33.9	35.1	0.33	0.82	0.74	0.11
Alpha-lactalbumin	P00711	28.4	26.8	27.5	27.5	0.17	0.05	0.59	0.03
<b><i>Ratio</i><sup>4</sup></b>									
Kappa-casein	A0A140T8A9	-2.3	-1.7	-1.4	-2.4	1.09	0.93	0.83	0.54
Alpha-S1-casein	P02662	5.4	6.0	5.8	5.3	0.51	0.89	0.48	0.39
Alpha-S2-casein	P02663	4.5	5.5	5.6	5.3	0.45	0.39	0.90	0.29
Beta-casein	P02666	5.5	6.3	6.4	6.1	0.75	0.65	0.93	0.62
Alpha-lactalbumin	P00711	-0.26	0.09	0.83	-0.42	0.76	0.80	0.87	0.40

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>Data represents log transformed abundance of proteins.

<sup>3</sup>UniProt accession number.

<sup>4</sup>Total represents the protein abundance in total form, phosphorylated represents abundance of total form that is phosphorylated, and the ratio is phosphorylated abundance divided by total.

**Table 7. 7.** Effects of AA infusions on expression and post translational modification of mTOR pathway proteins (KEGG #bta04150) in muscle tissue<sup>1</sup>

Item <sup>2</sup>	Accession <sup>3</sup>	Least Square Means				SEM	Effect (P-value)		
		CON	MKH	IL	MKH+IL		MKH	IL	MKH*IL
<b>Total<sup>4</sup></b>									
Calcium-binding protein 39	Q29RI6	24.2	24.6	23.9	23.8	0.25	0.63	0.10	0.41
Eukaryotic translation initiation factor 4E	Q9N0T5	24.4	23.9	23.9	23.5	0.20	0.07	0.49	0.71
Eukaryotic translation initiation factor 4E-binding protein 1	Q0P5A7	23.4	22.8	22.8	23.0	0.20	0.24	0.25	0.19
Growth factor receptor-bound protein 2	Q3T0F9	25.4	25.3	25.5	25.5	0.11	0.85	0.60	0.83
MAP2K1 protein	Q0VD16	25.7	25.3	25.4	25.6	0.21	0.54	0.85	0.28
MAP2K2 protein	Q17QH2	22.9	22.5	22.5	22.0	0.39	0.37	0.63	0.94
Mitogen-activated protein kinase 1	P46196	22.8	22.7	23.0	22.4	0.13	0.09	0.72	0.15
RAF proto-oncogene serine/threonine-protein kinase	A7E3S4	26.5	27.1	26.3	26.5	0.32	0.29	0.38	0.54
Transforming protein RhoA	P61585	23.6	24.3	24.2	24.3	0.34	0.49	0.67	0.63
<b>Phosphorylated<sup>4</sup></b>									
CAP-Gly domain containing linker protein 1	E3W9A2	25.0	25.1	25.3	25.3	0.14	0.44	0.93	0.65
Eukaryotic translation initiation factor 4E-binding protein 1	Q0P5A7	27.7	27.8	27.1	27.6	0.32	0.30	0.55	0.62
Insulin receptor substrate 1	F1MYE0	21.9	22.3	22.1	22.0	0.20	0.80	0.73	0.28
Mitogen-activated protein kinase 1	P46196	21.7	23.0	23.7	23.6	0.67	0.38	0.29	0.44
Mitogen-activated protein kinase	E1B8P9	21.1	21.3	21.7	22.9	0.82	0.65	0.41	0.67
Non-specific serine/threonine protein kinase	E1B9D1	20.8	20.8	21.4	22.1	0.55	0.63	0.23	0.64
Protein kinase C alpha type	P04409	23.0	22.0	23.1	23.3	0.81	0.62	0.42	0.57
<b>Ratio<sup>4</sup></b>									
Mitogen-activated protein kinase 1	P46196	3.6	4.9	5.3	5.9	0.65	0.22	0.26	0.63
Eukaryotic translation initiation factor 4E-binding protein 1	Q0P5A7	8.9	9.6	8.9	9.3	0.37	0.16	0.90	0.74

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>Data represents log transformed abundance of proteins.

<sup>3</sup>UniProt accession number.

<sup>4</sup>Total represents the protein abundance in total form, phosphorylated represents abundance of total form that is phosphorylated, and the ratio is phosphorylated abundance divided by total.

**Table 7. 8.** Effect of amino acid infusions on relative mRNA abundance [expressed as  $\log 2^{(-\Delta Ct)}$ ] of milk protein genes and genes coding for proteins regulating protein synthesis in mammary tissue (least-square treatment means)

Item <sup>2,3</sup>	Treatment <sup>1</sup>				SEM	Effect ( <i>P</i> -value)		
	CON	MKH	IL	MKH+IL		MKH	IL	MKH*IL
ATF4	4.79	3.16	2.61	1.87	2.53	0.70	0.78	0.87
BCL2	4.01	5.00	5.46	4.40	0.83	0.79	0.68	0.31
CCND1	4.47	3.64	3.42	3.36	0.81	0.58	0.65	0.67
CSN2	4.72	2.85	3.26	2.69	1.01	0.44	0.42	0.57
EIF3K	4.20	4.44	4.09	4.22	0.50	0.91	0.50	0.91
LALBA	4.77	3.23	3.76	3.82	0.99	0.76	0.94	0.48
MEN1	4.44	4.45	4.41	3.95	0.74	0.65	0.97	0.77
PRKAA1	3.48	1.57	4.33	0.01	1.74	0.29	0.95	0.54
RPS6	4.11	4.34	3.78	4.35	0.38	0.81	0.47	0.69
SLC7A5	4.78	6.74	6.86	6.87	1.71	0.62	0.58	0.61
YARS	4.52	3.73	4.60	3.55	0.68	0.28	0.99	0.86

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>Gene expression data was standardized to the average control  $2^{(-\Delta Ct)}$ , multiplied by 100, and then log transformed.

<sup>3</sup>ATF4 = activating transcription factor 4, BCL2 = apoptosis regulator, CCND1 = cyclin D1, CSN2 = beta-casein, EIF3K = eukaryotic translation initiation factor 3 subunit K, LALBA = alpha lactalbumin, MEN1 = menin, PRKAA1 = AMPK alpha 1, RPS6 = ribosomal protein S6, SLC7A5 = solute carrier family 7 member 5, YARS = tyrosyl-tRNA synthetase.

**Table 7. 9.** Effect of amino acid infusion treatment on relative mRNA abundance [expressed as  $\log 2^{(-\Delta Ct)}$ ] of protein synthesis and regulation related genes in muscle tissue (least-square treatment means)

Item	Treatment				SEM	Effect ( <i>P</i> -value)		
	CON	MKH	IL	MKH+IL		MKH	IL	MKH*IL
ATF4	3.37	0.34	2.40	1.59	2.98	0.44	0.59	0.68
EIF3K	4.19	4.60	4.44	4.27	0.69	0.95	0.46	0.65
RPS6	3.11	2.46	4.19	3.36	0.88	0.51	0.28	0.90
YARS	-0.22	-0.90	0.91	0.50	1.50	0.90	0.46	0.92

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>Gene expression data was standardized to the average control  $2^{(-\Delta Ct)}$ , multiplied by 100, and then log transformed.

<sup>3</sup>ATF4 = activating transcription factor 4, EIF3K = eukaryotic translation initiation factor 3 subunit K, RPS6 = ribosomal protein S6, YARS = tyrosyl-tRNA synthetase.

**Table 7. 10.** Comparison of measured mRNA abundance by PCR, total protein abundance by western immunoblotting, and total protein abundance by proteomics

Item <sup>2,3</sup>	Treatments <sup>1</sup>				SEM	CV % <sup>4</sup>	Effect ( <i>P</i> -value)		
	CON	MKH	IL	MKH + IL			MKH	IL	MKH*IL
CSN2									
PCR	4.72	2.85	3.26	2.69	1.01	29.9	0.44	0.42	0.57
Proteomic	34.6	33.7	32.7	34.2	0.17	0.52	0.53	0.04	0.01
LALBA									
PCR	4.77	3.23	3.76	3.82	0.99	25.4	0.76	0.94	0.48
Proteomic	33.3	32.8	32.0	33.3	0.24	0.73	0.48	0.32	0.06
S6K1									
Western	0.84	0.53	0.57	0.61	0.07	11.3	0.07	0.14	0.09
Proteomic	25.8	24.5	25.1	25.3	0.14	0.56	0.03	0.95	0.02
rpS6									
PCR	4.11	4.34	3.78	4.35	0.38	9.2	0.81	0.47	0.69
Western	1.00	0.73	0.56	1.07	0.19	24.1	0.54	0.24	0.07
Proteomic	30.7	29.0	29.6	29.8	0.08	0.28	<0.001	0.41	<0.001
4EBP1									
Western	0.93	0.45	0.32	0.92	0.28	52.6	0.67	0.68	0.16
Proteomic	29.5	28.4	28.6	29.1	0.05	0.18	<0.001	0.05	<0.001

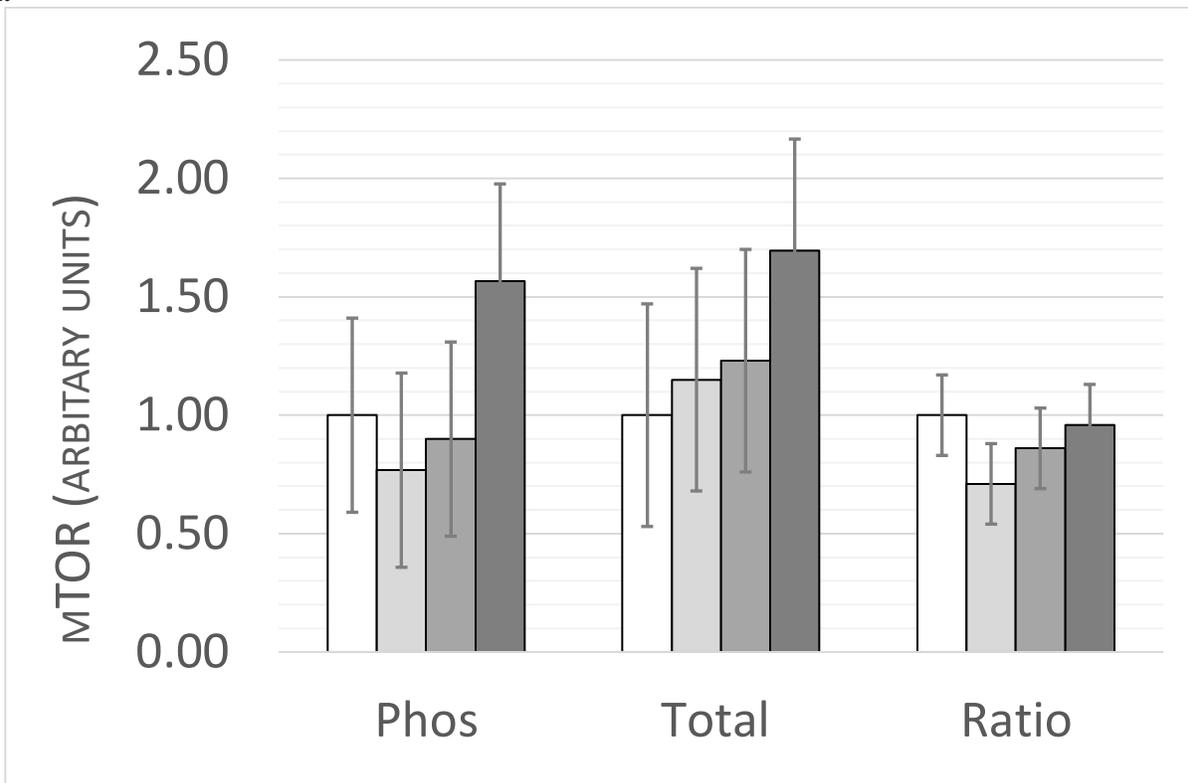
<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>Gene/Proteins: CSN2= B-casein, LALBA=α-Lactalbumin, S6K1= ribosomal protein S6 kinase, rpS6= ribosomal protein S6, 4EBP1 = Eukaryotic initiation factor 4E-binding protein 1.

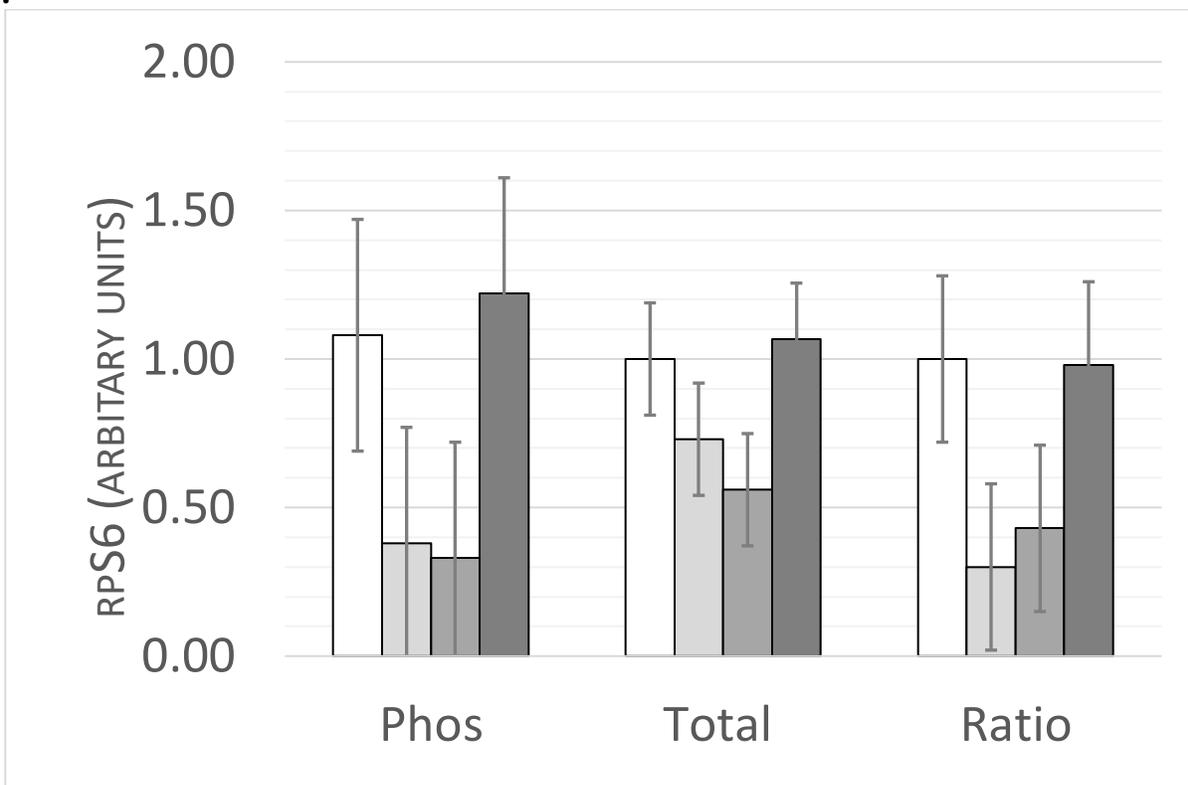
<sup>3</sup>Method of analysis is PCR, Western, or Proteomic which represent mRNA abundance in arbitrary units (log form), total protein abundance standardized to α-tubulin in arbitrary units, and total protein arbitrary units (log form).

<sup>4</sup>Coefficient of variation percent calculated by dividing SEM by the average least square mean.

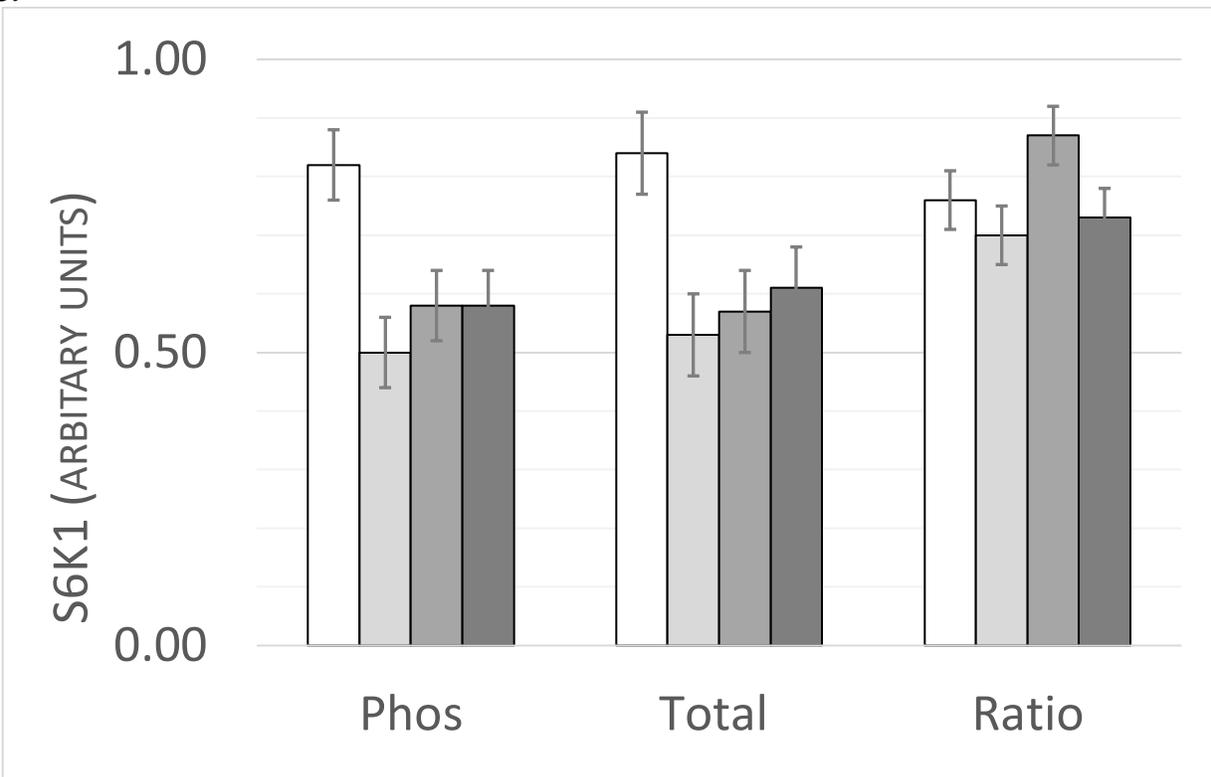
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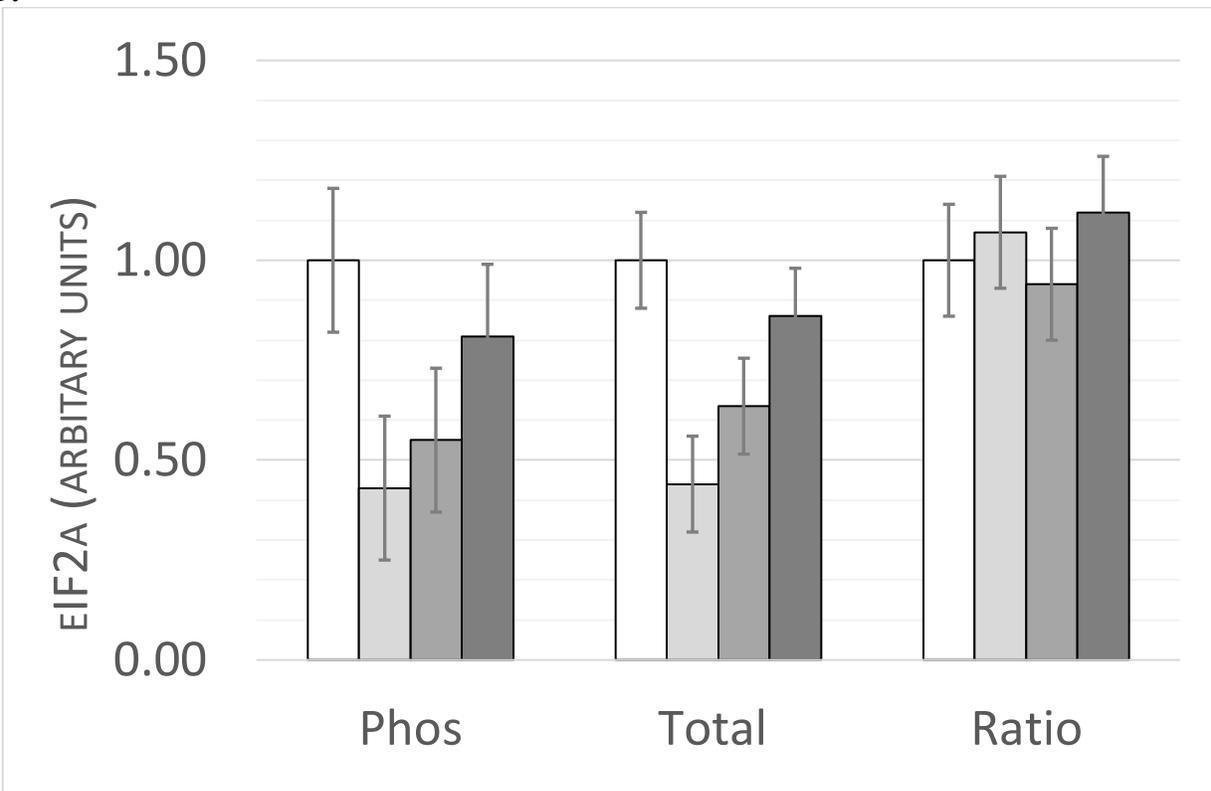
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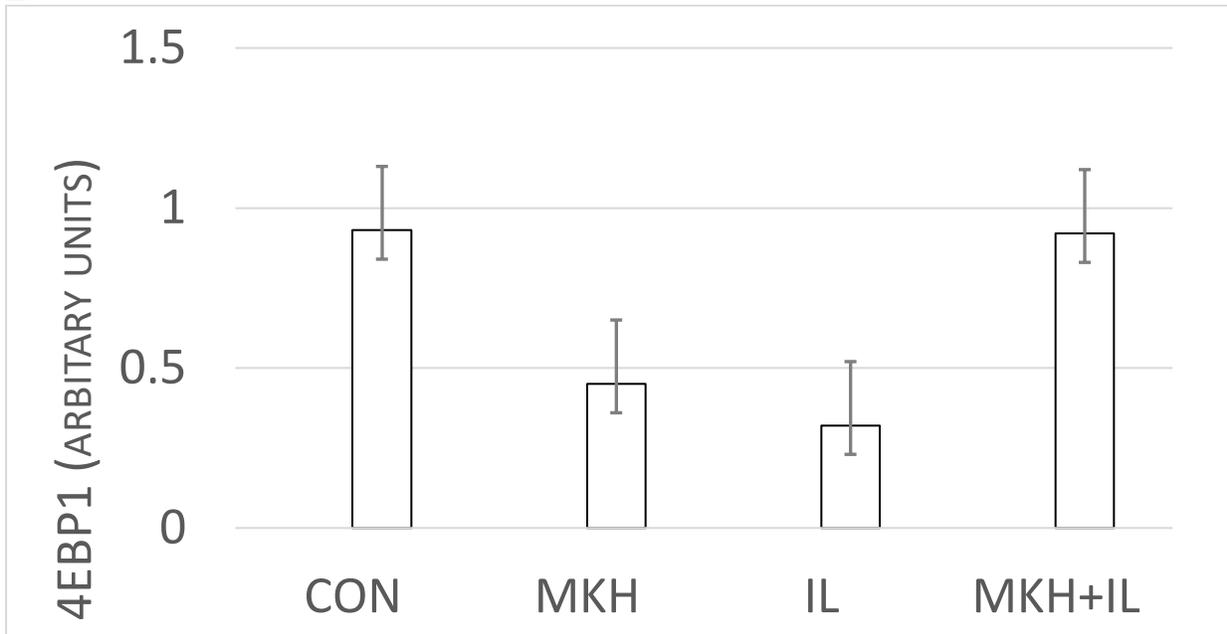
C.



D.

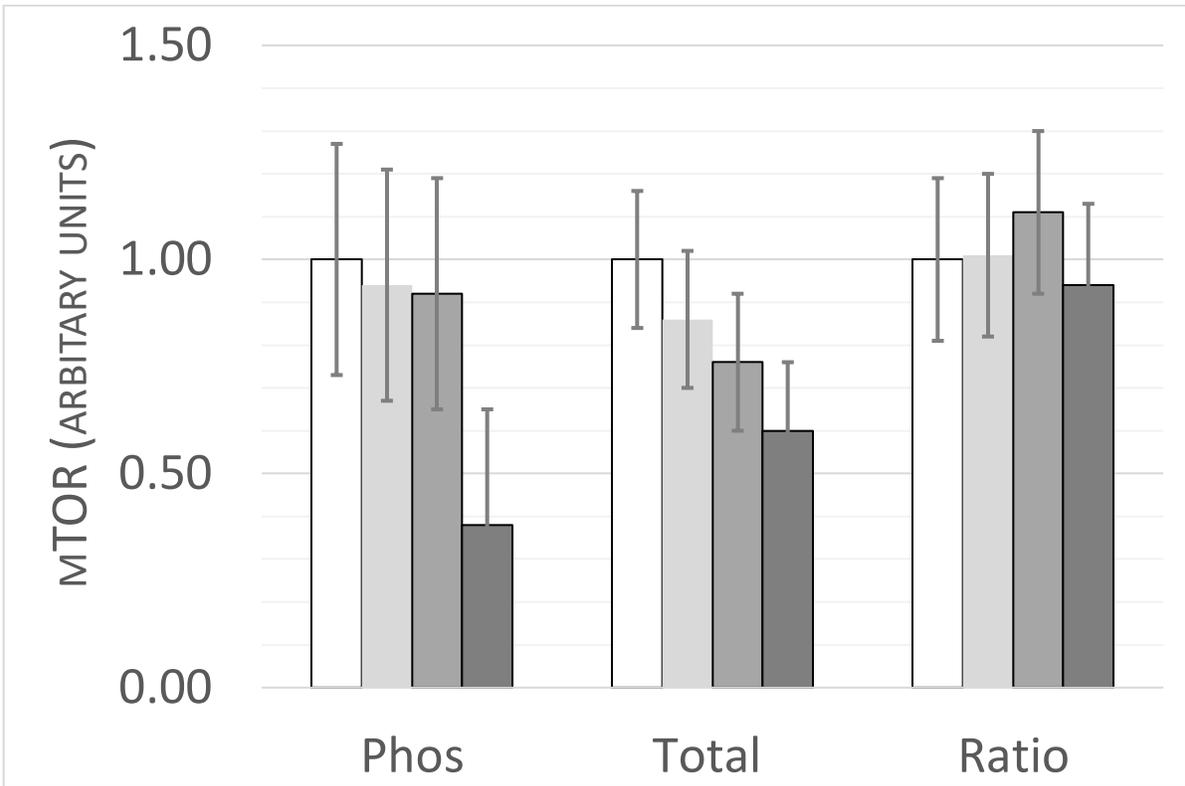


**E.**

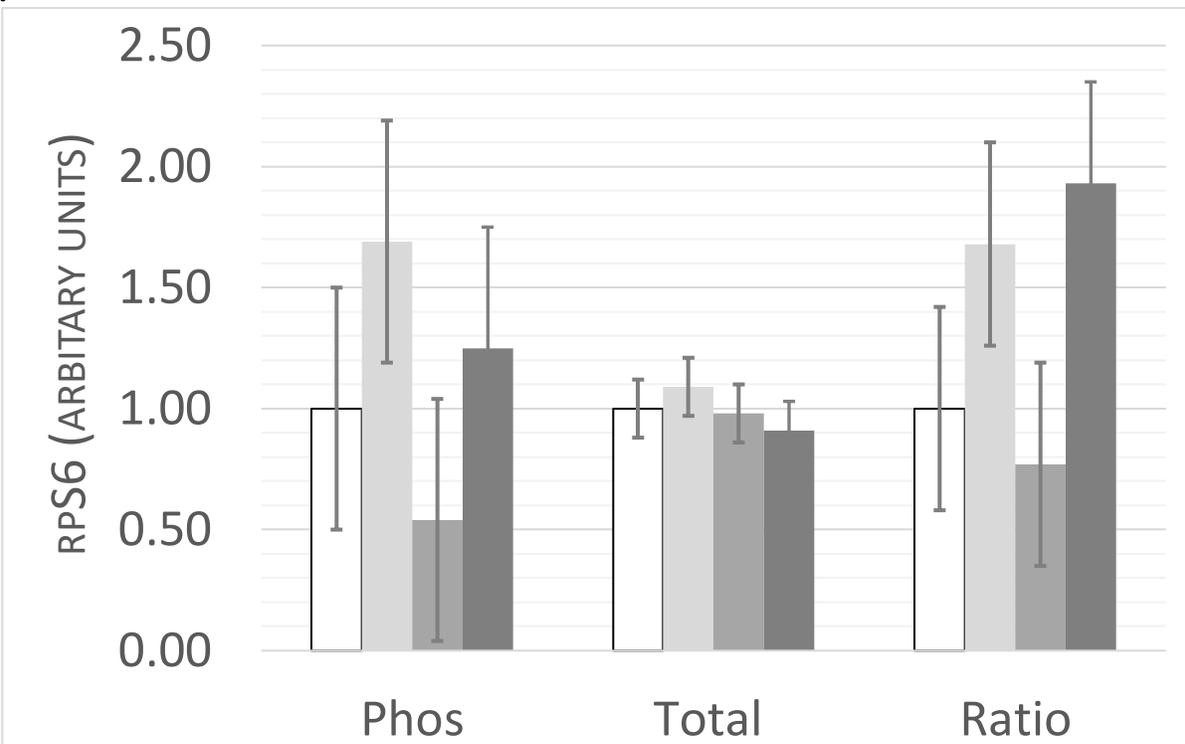


**Figure 7. 1.** Phosphorylation abundance, total abundance, and the ratio of these abundances determined by western immunoblotting for the following proteins in mammary tissue from dairy cows supplemented with AA; (A) mTORC1, (B) rpS6 = ribosomal protein S6, (C) S6K1 = p70 S6 kinase, (D) eIF2 $\alpha$ =eukaryotic initiation factor 2 a subunit, (E) 4EBP1 = eukaryotic initiation factor 4E binding protein 1. Treatments were the following; CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

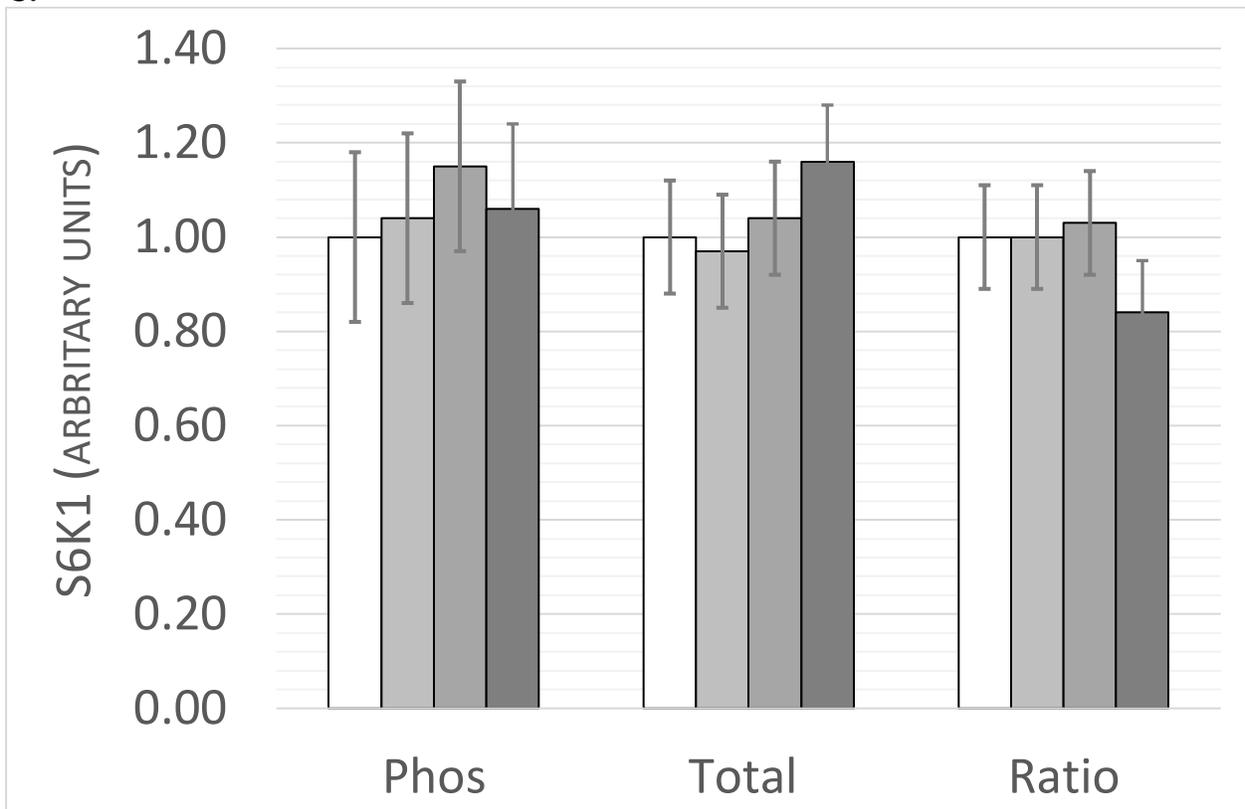
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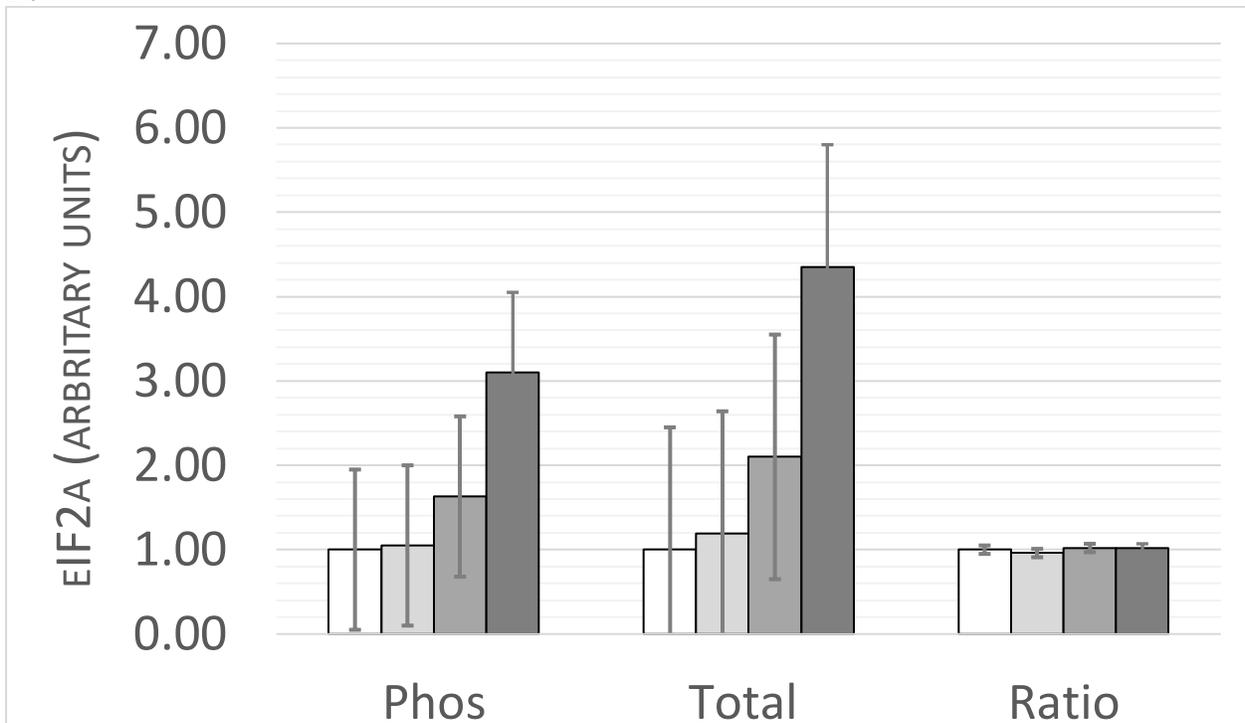
B.



C.



D.



**Figure 7. 2.** Phosphorylation abundance, total abundance, and the ratio of these abundances determined by western immunoblotting for the following proteins in muscle tissue from dairy cows supplemented with AA; (A) mTOR, (B) rpS6 = ribosomal protein S6, (C) S6K1 = p70 S6

kinase, and (D) eIF2 $\alpha$ =eukaryotic initiation factor 2  $\alpha$  subunit. Treatments were the following; 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

## 7.9. Supplemental Material

**Table 7.S 1.** Genes and respective primer sequences analyzed using quantitative real-time PCR in mammary and muscle tissue

Gene	Protein	Forward Primer	Reverse Primer	Accession #
LALBA	Alpha Lactalbumin	AGGTGTTCCGGGAGCTGAAA	GTGGTACAGACCCATTCAGGC	FJ232912.1
CSN2	Beta-Casein	CAGGCCTTTCTGCTGTACCA	CAAAAGTGAGGAGGGGGCAT	XM_015471671.2
RPS6	Ribosomal Protein S6	CGGGAACGATAAGCAGGGTT	CCCTTACTCAGTAGCAGGCG	NM_001015548.2
PRKAA1	AMPK Alpha 1	AGCCCTTCCTTCTCTTGCTC	AGGATGCCTGAAAAGCTTGA	NM_001109802.2
ATF4	Activating Transcription Factor 4	CCGAGATGAGCTTTCTGAGC	AGCATCCTCCTTGCTGTTGT	NM_001034342.2
BCL2	BCL2	ACAGCATCGCCCTGTGGATG	GTGCCTTCAGAGACAGCCAG	NM_001166486.1
H3F3A	H3 Histone Class 3	CCTCTACTGGAGGGGTGAAGA	CAGACGCTGGAAGGGAAGTT	NM_001014389.2
MEN1	Menin	GGTGCCTTGTGTGGGATGTAAG	GCGGGAATAGCGTTTTCTGG	NM_001076161.3
SLC7A5	Solute carrier family 7 member 5	GATCGGGAAGGGTGACGTAG	ACATGACGCCCAAGTGGTAG	BC126651.1
YARS	Tyrosyl-tRNA Synthetase	AAGTTGCTGGATCCCATCCG	TTGAGGGATCTGGGTAGGCA	NM_174220.2
CCND1	Cyclin D1	ATCAGATGTGACCCGGACTG	TCAGATGTTACGTCACGCA	NM_001046273.2

**Table 7.S 2.** Effects of AA infusions on select protein abundance in mammary tissue of lactating dairy cows<sup>1</sup>

Item <sup>2</sup>	Least Square Means					Effect ( <i>P</i> -value)		
	CON	MKH	IL	MKH+IL	SEM	MKH	IL	MKH*IL
40S ribosomal protein S6	30.7	29.0	29.6	29.8	0.12	0.00	0.41	0.00
40S ribosomal protein S9	31.5	30.2	30.8	31.0	0.11	0.01	0.08	0.00
60S ribosomal protein L28	29.2	27.8	28.3	28.4	0.15	0.01	0.88	0.01
AARS protein	29.1	28.8	29.0	28.9	0.05	0.01	0.56	0.13
Acyl-CoA binding domain containing 3	27.3	26.5	26.9	26.7	0.09	0.00	0.32	0.04
Acylamino-acid-releasing enzyme	25.9	25.5	26.2	25.7	0.06	0.00	0.02	0.15
Adenosylhomocysteinase	32.9	32.8	33.0	32.4	0.09	0.01	0.39	0.03
ADP-ribosylation factor GTPase-activating protein 2	27.4	26.5	26.8	27.0	0.06	0.00	0.67	0.00
ADP-ribosylation factor GTPase-activating protein 3	28.9	26.9	27.4	28.0	0.13	0.00	0.52	0.00
Aminoacyl tRNA synthase complex-interacting multifunctional protein 2	27.8	27.6	27.3	27.7	0.03	0.65	0.00	0.00
Aminoacyl tRNA synthetase complex interacting multifunctional protein 1	28.9	27.6	28.1	28.3	0.10	0.00	0.46	0.00
Annexin OS=Bos taurus	29.3	29.6	29.9	29.0	0.06	0.01	0.15	0.00
AP-3 complex subunit delta-1	29.0	28.3	28.6	28.6	0.07	0.00	0.71	0.01
Arachidonate 15-lipoxygenase	29.4	26.7	27.7	28.5	0.10	0.00	0.00	0.00
Asparagine synthetase [glutamine-hydrolyzing]	30.3	27.9	28.8	29.3	0.14	0.00	0.31	0.00
ATP-dependent 6-phosphofructokinase, liver type	31.1	30.3	30.7	30.6	0.07	0.00	0.99	0.01
ATP-dependent RNA helicase DDX19B	27.3	26.7	27.0	26.7	0.07	0.00	0.51	0.08
Bis(5'-nucleosyl)-tetrphosphatase [asymmetrical]	25.4	25.5	25.1	25.9	0.10	0.01	0.37	0.03
C7orf55-LUC7L2 readthrough	27.2	27.1	27.2	26.7	0.03	0.00	0.00	0.00
Catenin beta-1	27.8	27.6	28.1	27.2	0.10	0.01	0.48	0.03
Cation-transporting ATPase	23.0	22.5	23.2	21.0	0.27	0.01	0.31	0.04
Chloride intracellular channel protein 4	31.5	31.1	31.2	31.1	0.07	0.01	0.05	0.19
Clusterin OS=Bos taurus	27.0	30.1	29.1	27.4	0.12	0.00	0.03	0.00
Coatomer subunit beta	31.5	30.6	30.8	31.1	0.08	0.01	0.61	0.00
Coatomer subunit beta'	30.9	29.9	30.1	30.5	0.08	0.01	0.40	0.00

Coatomer subunit delta	31.0	30.1	30.2	30.6	0.03	0.00	0.04	0.00
Coatomer subunit gamma	31.5	30.7	30.8	31.3	0.03	0.00	0.39	0.00
Colony stimulating factor 2 receptor beta common subunit	23.1	22.8	23.1	22.3	0.06	0.00	0.16	0.02
COMM domain-containing protein 3	24.1	24.8	23.8	24.5	0.07	0.00	0.01	0.93
CTP synthase 2 OS=Bos taurus	27.5	26.4	26.6	26.9	0.09	0.01	0.37	0.00
Cyclin-dependent kinase 2-associated protein	20.2	21.2	22.1	21.0	0.17	0.32	0.01	0.01
Cytoplasmic aconitate hydratase	32.1	31.7	31.4	32.1	0.02	0.06	0.00	0.00
Cytosolic arginine sensor for mTORC1 subunit 1	22.4	22.2	23.1	21.9	0.04	0.00	0.28	0.00
D-3-phosphoglycerate dehydrogenase	29.6	27.5	28.4	28.7	0.15	0.01	0.32	0.00
D-aminoacyl-tRNA deacylase 1	22.4	22.7	22.1	20.9	0.14	0.01	0.00	0.01
DEAD-box helicase 46 OS=Bos taurus	24.7	24.5	24.7	24.0	0.09	0.00	0.04	0.06
Desmin OS=Bos taurus	27.9	28.2	27.8	28.1	0.04	0.00	0.13	0.53
EH domain containing 4	28.1	27.3	27.7	27.8	0.03	0.00	0.01	0.00
Elongation factor 1-delta	32.2	31.4	31.9	31.8	0.07	0.01	0.03	0.01
Elongator complex protein 3	24.0	23.5	23.4	23.5	0.07	0.01	0.01	0.01
Eukaryotic initiation factor 4A-II	30.0	29.4	29.7	29.7	0.08	0.01	0.78	0.02
Eukaryotic translation initiation factor 2 subunit 2	28.9	27.9	28.2	28.5	0.09	0.01	0.44	0.00
Eukaryotic translation initiation factor 4H	29.2	29.1	29.5	29.3	0.06	0.19	0.01	0.65
Farnesyl pyrophosphate synthase OS=Bos taurus	29.8	28.3	28.6	29.3	0.09	0.01	0.67	0.00
FAT atypical cadherin 3	22.7	24.6	22.7	22.8	0.19	0.03	0.00	0.01
Fatty acid-binding protein 5 OS=Bos taurus	30.8	29.5	29.8	30.5	0.09	0.01	0.40	0.00
GDP-mannose pyrophosphorylase A	28.6	27.6	27.7	28.2	0.06	0.01	0.19	0.00
Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	32.5	30.3	30.8	31.9	0.06	0.00	0.06	0.00
Growth factor receptor-bound protein 2	27.4	27.2	27.3	27.0	0.05	0.00	0.87	0.25
Heat shock 70kD protein binding protein	30.3	30.1	30.3	30.0	0.07	0.01	0.61	0.28
Hydroxysteroid dehydrogenase-like protein 2	27.0	26.7	25.9	27.5	0.08	0.01	0.03	0.00
Hypoxanthine phosphoribosyltransferase	25.4	24.5	24.9	25.1	0.06	0.00	0.06	0.00
Intercellular adhesion molecule 1 OS=Bos taurus	21.5	23.5	22.5	22.1	0.14	0.01	0.22	0.00
Interferon induced protein 44 OS=Bos taurus	24.2	25.0	25.6	23.3	0.03	0.00	0.16	0.00

Iron-sulfur cluster assembly 2 homolog, mitochondrial	24.6	23.1	23.5	24.0	0.10	0.01	0.03	0.01
La ribonucleoprotein domain family member 7	24.0	23.6	24.0	23.1	0.17	0.01	0.20	0.16
LKAAEAR motif containing 1 OS=Bos taurus	23.3	21.9	23.3	22.2	0.27	0.01	0.28	0.48
Malignant T-cell-amplified sequence OS=Bos taurus	27.7	27.5	27.6	27.3	0.06	0.01	0.09	0.30
Mannose-6-phosphate isomerase	25.6	25.3	25.7	24.6	0.00	0.00	0.00	0.00
Mapk-regulated corepressor-interacting protein 1	23.2	22.8	23.0	22.2	0.08	0.00	0.09	0.08
Mitochondrial import inner membrane translocase subunit Tim10	26.8	26.7	25.9	26.1	0.10	0.14	0.01	0.16
MTHFD1 protein OS=Bos taurus	29.5	29.1	29.3	29.1	0.07	0.01	0.81	0.16
N-terminal kinase-like protein	27.8	25.7	26.4	27.1	0.17	0.01	0.69	0.00
NIF3-like protein 1	27.7	27.4	27.4	27.5	0.04	0.01	0.02	0.01
NLR family pyrin domain containing 1	24.7	24.1	23.3	24.4	0.07	0.21	0.00	0.00
Nuclear factor 1 B-type	27.2	27.2	27.0	26.6	0.07	0.01	0.03	0.03
Nucleoside diphosphate kinase B	32.4	31.5	31.8	32.2	0.06	0.01	0.24	0.00
Nucleosome assembly protein 1-like 4	27.5	27.6	27.5	27.3	0.03	0.01	0.01	0.03
Peptidylprolyl isomerase	30.5	30.0	30.1	30.0	0.07	0.00	0.10	0.03
Perilipin	29.5	28.1	28.6	29.0	0.09	0.00	0.55	0.00
Phosphatidylethanolamine-binding protein 1	32.4	32.2	32.4	32.1	0.02	0.00	0.63	0.40
Potassium channel tetramerization domain containing 12	28.6	28.7	29.0	28.2	0.06	0.00	0.70	0.00
Prostaglandin-H2 D-isomerase	23.0	25.4	24.3	23.0	0.15	0.01	0.08	0.00
Protein phosphatase 1B	25.5	24.4	25.3	24.8	0.10	0.00	0.10	0.04
Protein transport protein sec16	27.5	26.3	26.7	27.1	0.04	0.00	0.03	0.00
Protein VAC14 homolog	25.0	26.1	25.7	25.7	0.11	0.01	0.53	0.01
Pyridoxal-dependent decarboxylase domain-containing protein 1	30.1	28.7	28.8	29.6	0.03	0.00	0.02	0.00
RB binding protein 9, serine hydrolase	24.4	23.6	24.4	23.7	0.09	0.00	0.43	0.59
Reticulon	28.5	27.8	28.3	27.9	0.06	0.01	0.05	0.08
Rho guanine nucleotide exchange factor 16	27.0	25.1	25.7	26.5	0.15	0.01	0.18	0.00
Ribosomal protein L7 like 1	19.9	21.0	20.4	20.9	0.05	0.00	0.01	0.02
SEC23 interacting protein	28.6	27.3	27.6	27.9	0.06	0.00	0.10	0.00
SEC24 homolog D, COPII coat complex component	28.8	28.0	28.1	28.2	0.07	0.01	0.06	0.00

Sequestosome 1	22.7	23.1	23.0	22.3	0.04	0.00	0.01	0.00
Signal recognition particle subunit SRP68	29.1	28.0	28.3	28.7	0.09	0.01	0.29	0.00
Signal transducing adaptor family member 2	24.9	24.3	24.4	24.2	0.03	0.00	0.02	0.00
Sorbitol dehydrogenase	26.2	24.1	24.3	25.4	0.04	0.00	0.00	0.00
Spectrin beta chain	29.3	27.9	28.3	29.0	0.06	0.00	0.02	0.00
Synapse associated protein 1, SAP47 homolog (Drosophila)	27.0	25.4	25.9	26.4	0.10	0.00	0.92	0.00
Synaptobrevin homolog YKT6	28.4	26.8	27.3	27.8	0.08	0.00	0.49	0.00
T-complex protein 1 subunit delta	29.9	29.6	29.7	29.6	0.05	0.01	0.76	0.08
Target of myb1 like 2 membrane trafficking protein	25.4	25.5	24.8	25.6	0.06	0.16	0.00	0.00
Tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1	20.9	21.4	20.6	20.9	0.06	0.00	0.01	0.15
Tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1	20.9	21.4	20.6	20.9	0.06	0.00	0.01	0.15
Threonine--tRNA ligase, cytoplasmic	31.4	29.8	30.5	30.5	0.17	0.01	0.64	0.01
Thrombospondin 3	22.6	24.1	24.9	23.8	0.18	0.09	0.01	0.00
TPSB1 protein	23.0	23.6	24.6	25.0	0.07	0.59	0.00	0.17
Trafficking protein particle complex subunit	24.6	24.0	24.4	24.6	0.03	0.00	0.01	0.00
Transmembrane 9 superfamily member	27.4	24.5	25.5	26.1	0.18	0.00	0.11	0.00
Transmembrane protein 263	27.8	26.1	26.8	26.9	0.03	0.00	0.01	0.00
Transthyretin	27.5	28.5	29.4	27.9	0.12	0.10	0.00	0.00
Trinucleotide repeat containing 18	26.4	26.3	26.5	25.6	0.14	0.01	0.10	0.04
Tryptophan--tRNA ligase, cytoplasmic	32.9	31.9	32.2	32.2	0.09	0.01	0.13	0.01
Tumor protein D52-like 1	28.3	27.2	27.4	28.0	0.05	0.00	0.28	0.00
Ubiquitin-conjugating enzyme E2 H	24.2	23.3	24.0	23.0	0.22	0.01	0.99	0.84
Ubiquitin-like modifier-activating enzyme 3	24.8	24.5	25.2	24.5	0.08	0.00	0.07	0.06
Uncharacterized protein	24.1	22.3	23.7	23.0	0.17	0.00	0.04	0.03
Uroporphyrinogen decarboxylase	27.0	27.3	26.6	27.3	0.07	0.00	0.02	0.03
Valyl-tRNA synthetase	29.9	29.1	29.3	29.4	0.06	0.00	0.54	0.00
Zeta-crystallin	28.9	28.9	28.5	28.4	0.09	0.07	0.01	0.54

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>Table contains only proteins significantly affected by one of the treatment main effects ( $P < 0.01$ ).

<sup>3</sup>Least square means represent log transformed abundance.

**Table 7.S 3.** Effects of AA infusions on select phosphorylated protein abundance in mammary tissue of lactating dairy cows<sup>1</sup>

Item <sup>2</sup>	Least Square Means				SEM	Effect (P-value)		
	CON	MKH	IL	MKH+IL		MKH	IL	MKH*IL
40S ribosomal protein S20	23.6	23.3	23.4	21.7	0.01	0.01	0.04	0.01
Actin-related protein 2/3 complex subunit 4	24.4	24.4	21.5	23.4	0.12	0.80	0.00	0.01
Alpha-actinin-1	23.0	23.1	25.5	24.6	0.13	0.03	0.01	0.09
EML3 protein	24.1	23.7	24.5	24.9	0.13	0.09	0.01	0.07
Eukaryotic translation initiation factor 4E-binding protein 1	29.5	28.4	28.6	29.1	0.05	0.00	0.05	0.00
Formin binding protein 1 like	26.2	24.5	25.4	26.1	0.06	0.00	0.01	0.00
G3BP stress granule assembly factor 2	25.6	25.4	26.2	26.1	0.05	0.01	0.00	0.31
Heat shock cognate 71 kDa protein	27.4	27.1	27.4	27.0	0.05	0.01	0.12	0.80
Heat shock protein beta-1	28.8	29.5	29.8	29.4	0.05	0.03	0.01	0.00
IKAROS family zinc finger 2	21.4	22.3	23.4	23.0	0.17	0.07	0.01	0.10
KN motif and ankyrin repeat domains 3	24.3	25.2	25.7	25.2	0.05	0.78	0.00	0.00
Lysine methyltransferase 2B	19.8	21.1	21.8	21.6	0.12	0.24	0.01	0.01
Mediator of RNA polymerase II transcription subunit 1	22.4	24.3	24.8	24.7	0.15	0.04	0.01	0.01
Membrane-associated progesterone receptor component 1	27.2	26.9	28.2	27.9	0.10	0.07	0.00	0.86
Methylosome protein 50	22.2	24.0	25.7	24.4	0.06	0.51	0.00	0.00
MICAL-like protein 1	24.0	23.6	24.3	24.5	0.03	0.09	0.00	0.01
NIMA related kinase 1	22.8	22.1	23.6	22.8	0.10	0.01	0.01	0.96
Nuclear mitotic apparatus protein 1	26.0	26.5	27.7	28.2	0.15	0.13	0.00	0.90
Phosphoinositide kinase, FYVE-type zinc finger containing	22.4	22.7	23.1	23.5	0.05	0.19	0.01	0.82
Proline, glutamate and leucine rich protein 1	23.4	24.2	24.7	24.1	0.15	0.34	0.01	0.04
Protein NDRG2	27.6	27.3	27.9	28.4	0.07	0.33	0.01	0.02
Protein phosphatase 1 regulatory inhibitor subunit 14A	26.4	26.7	27.4	27.6	0.04	0.24	0.00	0.62
RAB3A interacting protein (Rabin3)	26.0	25.8	25.9	25.7	0.06	0.01	0.60	0.71
Rap guanine nucleotide exchange factor 1	21.7	22.7	23.5	22.8	0.10	0.20	0.01	0.01
RNA-binding protein 14	25.5	26.4	26.6	26.6	0.07	0.22	0.01	0.01

Scribbled planar cell polarity protein	26.3	26.2	27.0	26.7	0.10	0.17	0.01	0.65
SEL1L ERAD E3 ligase adaptor subunit	24.7	20.2	21.7	24.8	0.22	0.01	0.01	0.01
Septin-11	22.6	23.5	23.6	23.3	0.08	0.67	0.01	0.03
Serine/threonine-protein kinase RIO3	24.1	24.7	22.5	22.8	0.00	0.00	0.00	0.00
Synaptopodin	23.6	25.4	25.8	24.7	0.14	0.68	0.01	0.00
Thymosin beta-4	25.0	27.1	26.7	26.7	0.07	0.01	0.07	0.01
Transcription elongation factor SPT5	21.9	23.9	24.1	23.1	0.03	0.00	0.08	0.00
TRIM3 protein	23.4	24.1	24.7	24.5	0.07	0.78	0.01	0.04
UTP--glucose-1-phosphate uridylyltransferase	24.8	22.8	23.6	24.2	0.01	0.02	0.01	0.01
Xanthine dehydrogenase/oxidase	19.5	23.0	20.5	21.0	0.00	0.00	0.01	0.00

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>Table contains only proteins significantly affected by one of the treatment main effects ( $P < 0.01$ ).

<sup>3</sup>Least square means represent log transformed abundance.

**Table 7.S 4.** Effects of AA infusions on select proteins phosphorylation state in mammary tissue of lactating dairy cows<sup>1</sup>

Item	Least Square Means				SEM	Effect (P-value)		
	CON	MKH	IL	MKH+IL		MKH	IL	MKH*IL
Adenine phosphoribosyltransferase	-1.5	-1.8	-2.7	-2.5	0.24	0.67	0.01	0.42
ADP ribosylation factor guanine nucleotide exchange factor 2	2.9	4.0	3.8	3.5	0.02	0.00	0.01	0.00
Alpha-actinin-1	-3.9	-4.1	-1.5	-2.1	0.18	0.09	0.01	0.43
Ataxin 2	5.0	6.5	6.1	7.3	0.12	0.00	0.94	0.49
Caveolae-associated protein 3	4.6	5.8	5.6	6.2	0.17	0.01	0.18	0.19
Cofilin-1	-3.7	-2.8	-3.3	-3.1	0.11	0.01	0.02	0.09
Cortactin	3.4	4.6	4.3	4.3	0.13	0.01	0.30	0.01
CRK like proto-oncogene, adaptor protein	2.7	4.6	4.5	4.2	0.14	0.00	0.48	0.00
Dynactin subunit 2	-1.6	-1.1	-1.4	-1.4	0.00	0.01	0.00	0.00
Gephyrin	2.1	3.5	3.8	3.2	0.04	0.01	0.00	0.00
GIT ArfGAP 2	4.7	5.3	6.4	6.5	0.17	0.12	0.01	0.25
Golgi brefeldin A resistant guanine nucleotide exchange factor 1	3.2	4.3	4.7	4.2	0.11	0.01	0.60	0.00
GTP-binding protein	4.0	5.0	4.7	4.7	0.06	0.00	0.76	0.00
HECT domain E3 ubiquitin protein ligase 1	6.0	5.0	7.3	6.4	0.15	0.01	0.01	0.88
Histone deacetylase 1	4.7	5.7	5.7	5.7	0.08	0.00	0.12	0.01
Insulin receptor substrate 1	5.6	6.2	5.9	7.1	0.09	0.00	0.22	0.06
Kinesin family member 21A	5.9	5.9	6.7	7.3	0.00	0.00	0.02	0.01
Microtubule associated protein 1B	4.3	5.2	5.1	5.7	0.14	0.01	0.59	0.45
N-acetyl-D-glucosamine kinase	0.8	1.3	0.7	0.0	0.11	0.05	0.00	0.01
Potassium channel tetramerization domain containing 12	0.5	2.1	1.9	1.8	0.09	0.00	0.11	0.00
PPFIA binding protein 1	3.6	3.7	3.0	4.6	0.00	0.01	0.00	0.00
Protein LSM14 homolog A	6.2	7.3	7.5	7.8	0.15	0.01	0.19	0.09
Protein phosphatase 6 regulatory subunit 3	3.7	5.1	5.4	4.7	0.12	0.01	0.28	0.00
Reticulon	2.2	3.7	3.4	3.7	0.16	0.01	0.46	0.04
SH3 domain containing 19	5.9	7.2	7.1	8.4	0.22	0.01	0.82	0.91

Structural maintenance of chromosomes protein	-1.2	2.8	2.6	1.0	0.01	0.00	0.01	0.00
Uncharacterized protein	3.5	4.1	3.8	4.7	0.12	0.01	0.38	0.32

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>Table contains only proteins significantly affected by one of the treatment main effects ( $P < 0.01$ ).

<sup>3</sup>Least square means represent the log of ((phosphorylated abundance / total abundance)\*100).

**Table 7.S 5.** Effects of AA infusions on select proteins total abundance in muscle tissue of lactating dairy cows<sup>1</sup>

Item <sup>2</sup>	Least Square Means					Effect (P-value)		
	CON	MKH	IL	MKH+IL	SEM	MKH	IL	MKH*IL
60 kDa heat shock protein, mitochondrial	27.0	26.7	27.4	27.7	0.08	0.79	0.00	0.03
60S ribosomal protein L26	24.2	23.9	23.6	23.8	0.05	0.11	0.00	0.01
Aminoacyl tRNA synthase complex-interacting multifunctional protein 2	21.0	19.6	20.2	22.4	0.21	0.10	0.01	0.00
Basic leucine zipper and W2 domains 1	22.8	21.8	21.5	21.4	0.12	0.00	0.00	0.02
Calpain-2 catalytic subunit	24.8	24.6	25.2	25.0	0.09	0.11	0.01	0.81
Carboxymethylenebutenolidase homolog	29.7	29.7	29.5	29.5	0.05	0.45	0.01	0.46
Elongation factor 1-beta	25.8	26.2	26.5	26.4	0.04	0.01	0.00	0.04
Galectin-1	28.7	28.4	28.7	28.4	0.06	0.01	0.24	0.68
Methanethiol oxidase	24.0	23.6	24.7	25.5	0.04	0.01	0.01	0.01
Myosin-7	33.2	33.2	32.6	32.1	0.10	0.08	0.00	0.14
Peptidylprolyl isomerase	23.4	23.3	23.7	24.3	0.13	0.14	0.01	0.09
Peroxiredoxin-2	30.1	30.1	30.3	30.4	0.01	0.25	0.00	0.01
Protein phosphatase 1 regulatory subunit 3A	25.7	25.5	25.8	25.9	0.08	0.87	0.01	0.12
Protein phosphatase 1 regulatory subunit 7	22.6	23.4	22.9	23.3	0.05	0.00	0.04	0.02
Ras GTPase-activating protein-binding protein 1	22.3	22.3	22.0	21.1	0.14	0.08	0.01	0.05
Ribosomal protein L34	23.6	23.7	23.6	24.2	0.07	0.01	0.07	0.03
Tripartite motif containing 66	25.7	25.3	25.9	25.1	0.11	0.01	0.06	0.15

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>Table contains only proteins significantly affected by one of the treatment main effects ( $P < 0.01$ ).

<sup>3</sup>Least square means represent log transformed abundance.

**Table 7.S 6.** Effects of AA infusions on select proteins phosphorylation abundance in muscle tissue of lactating dairy cows<sup>1</sup>

Item <sup>2</sup>	Least Square Means <sup>3</sup>				SEM	Effect (P-value)		
	CON	MKH	IL	MKH+IL		MKH	IL	MKH*IL
Alpha-2-macroglobulin	23.8	23.4	25.3	25.3	0.29	0.37	0.01	0.66
Amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	28.7	28.5	29.1	29.0	0.10	0.10	0.01	0.74
Catalase	21.3	20.7	22.9	24.3	0.08	0.89	0.00	0.02
Cyclin dependent kinase 16	22.5	22.3	22.2	22.1	0.04	0.08	0.00	0.40
L-lactate dehydrogenase A chain	30.0	30.2	32.6	32.1	0.07	0.22	0.00	0.04
Malic enzyme	23.4	23.4	23.2	23.2	0.05	0.16	0.01	0.73
Mitogen-activated protein kinase kinase kinase MLT	22.0	21.8	22.5	22.3	0.11	0.13	0.01	0.80
Myocyte enhancer factor 2D	23.4	23.6	23.0	23.4	0.02	0.01	0.00	0.02
Myozenin-1	19.2	20.6	24.2	23.4	0.13	0.00	0.00	0.01
PDZ and LIM domain 1	22.2	22.6	21.1	21.9	0.15	0.05	0.01	0.27

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>Table contains only proteins significantly affected by one of the treatment main effects ( $P < 0.01$ ).

<sup>3</sup>Least square means represent log transformed abundance.

**Table 7.S 7.** Effects of AA infusions on select proteins phosphorylation to total ratio in muscle tissue of lactating dairy cows<sup>1</sup>

Item <sup>2</sup>	Least Square Means <sup>3</sup>				SEM	Effect (P-value)		
	CON	MKH	IL	MKH+IL		MKH	IL	MKH*IL
40S ribosomal protein S3	2.55	0.93	1.16	0.61	0.22	0.06	0.01	0.07
Amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	0.32	0.16	0.65	0.72	0.05	0.07	0.00	0.11
Fructose-bisphosphate aldolase	3.47	3.66	2.84	3.04	0.11	0.07	0.01	0.96
L-lactate dehydrogenase A chain	-1.30	-1.00	1.18	0.78	0.13	0.30	0.01	0.18
Peptidyl-prolyl cis-trans isomerase FKBP1A	-2.1	-2.2	-2.2	-1.4	0.16	0.01	0.37	0.03
Phosphoprotein enriched in astrocytes 15	8.8	9.2	8.4	9.6	0.09	0.01	0.66	0.03

<sup>1</sup>Treatments: 1) CON = infused 3 liters of 0.9% saline; 2) MKH = infused 21 g/d of Met, 20 g/d of His, and 38 g/d of Lys; 3) IL = infused 22 g/d of Ile, and 50 g/d of Leu; 4) MKH+IL = infused 21 g/d of Met, 20 g/d of His, 38 g/d of Lys, 22 g/d of Ile, and 50 g/d of Leu.

<sup>2</sup>Table contains only proteins significantly affected by one of the treatment main effects ( $P < 0.01$ ).

<sup>3</sup>Least square means represent the log of ((phosphorylated abundance / total abundance)\*100).

## CHAPTER 8: Conclusion

Post absorptive metabolism of dietary protein into milk protein is not well described in dairy nutrition models resulting in poor predictions of milk protein production to changing protein supply. This partially constrains diet formulation with respect to successfully balancing diets for protein and amino acids, maximizing income over feed costs, and improving N efficiency.

The gross N efficiency of milk production is 25% which if increased would reduce dietary feed costs, improve environmental sustainability, and place protein efficiency closer to other species such as lactating sows (e.g., 43%) (Millet et al., 2018). The US dairy trend of increased herd size and location concentration into smaller geographic areas to maximize profits (i.e., 25% of US dairy production located in only 13 counties or 0.41% of US counties) (USDA, 2015) may put N emissions by dairy under increased scrutiny. Dietary protein costs comprise 45% of total feed costs or \$2.99 per cow/day typically. This is based on a cow producing 31.8 kg/d of 3.6% fat, 3.0% protein, and 5.7% other solids milk in the Northeast region of the US using feed prices over the last five years (2013-2017) (St-Pierre, 2017). If N efficiency was to be improved to 35% and production maintained, feed costs associated with protein would be reduced by \$0.30 per cow per day and cost of production by \$0.43 per cwt of milk.

The overarching objective of this dissertation was to increase our understanding of how the udder takes up AA and milk protein production regulation when AA supplies are varied or specific EAA are supplemented. Our findings may contribute to future modeling efforts for improving nutrition models, which will enable more robust dietary formulation for maximizing income over feed costs and reducing N emissions.

The NRC 2001 and CNCPS models suggest milk protein production is regulated by the single limiting AA theory and that a unique optimal AA profile exists. Our in vitro results indicate that multiple distinct AA profiles can equally upregulate signaling proteins related to translation initiation (i.e., mTORC1, rpS6, and 4EBP1). Previous research has demonstrated that enhanced phosphorylation of these proteins correlates to increased milk protein synthesis (Toerien et al., 2010, Appuhamy et al., 2012, Arriola Apelo et al., 2014d, Castro et al., 2016). Multiple linear regression of intracellular AA concentrations and phosphorylation state of the signaling proteins suggest the importance of Met, Ile, Leu, and Thr. This means as long as the AA profile supplied to mammary epithelial cells contains sufficient amounts of one or more of these EAA, activation of translational machinery can occur. These findings confirm earlier research and that identification of a single optimal EAA profile for dairy cows is somewhat a fleeting objective.

As demonstrated in our first experiment and previous research, intracellular AA concentrations influence activation of protein translation initiation. Intracellular AA concentrations are dictated by net AA uptake. Therefore, how changing extracellular AA supplies affect net uptake is important for predicting milk protein production. We developed a method for assessment of the bi-directional transport for 14 AA. This method was then used to evaluate AA concentrations representing 16, 100, 186, and 271% of cow plasma AA concentrations. Efflux of EAA averaged 69% of the unidirectional uptake for extracellular AA concentrations that were similar to in-vivo. This demonstrates substantial bi-directional transport and a mechanism to manage intracellular AA shortages by simply reducing the mass action efflux. Most AA exhibited non-saturable uptake kinetics at extracellular AA concentrations similar to in vivo. Arginine, Pro, and Val uptake was saturable at in vivo AA concentrations

with the calculated Michaelis-Menten  $k_m$  being below typical *in vivo* concentrations. Our results indicate that for most AA, transport capacity or competitive inhibition does not occur at *in vivo* AA concentrations and can be represented by mass action kinetics. Further research into Arg, Pro, and Val is warranted, particularly since empirical research has indicated that increasing Val dietary supplies decreases milk protein production (Hanigan et al., 2018). Net AA uptake should likely not be represented as linear function of AA supplies as we observed quadratic increases in most intracellular AA concentrations to increasing AA supplies. This means that when AA supplies are reduced (i.e., feeding a low protein diet), net AA uptake by the udder for most AA will not decrease proportionally to the supply reduction. Instead, efflux will decrease, thereby increasing the proportion of AA extracted on a net basis by the udder from arterial supplies. In summary, incorporation of varying absorbed EAA to milk protein efficiency seems warranted and AA transport might be one mechanism that contributes to this varying efficiency.

Conflicting reports exist in the literature whether EAA exert control on protein translation via the mTOR pathway. Leucine and Ile have been demonstrated to have a potent effect on upregulating protein translation in numerous *in vitro* experiments (Appuhamy et al., 2012, Arriola Apelo et al., 2014c). In contrast, *in vivo* supplementation of Leu or BCAA have not increased milk protein production (Korhonen et al., 2002, Appuhamy et al., 2011b, Kassube et al., 2017, Curtis et al., 2018). We sought to test Leu and Ile supplementation in high producing dairy cattle (>45 kg/d) for effects on milk protein production and mTOR pathway regulation. Additionally, we tested well documented limiting EAA, Met, Lys, and His, and how these EAA increase milk protein production. Supplementing the two groups of EAA: 1) Met, Lys, and His and 2) Ile and Leu, independently and additively increased milk protein yield. These outcomes were associated with changes in blood flow in the udder and AA net uptake of various AA

demonstrating that once demand is increased, mechanisms exist to increase AA delivery. If the single-limiting AA theory were regulating milk protein production, increases in milk protein yield from two groups of EAA as observed is contradictory to this theory. This suggests milk protein production is not regulated solely by substrate supplies. Nutrient signaling in the udder proteome partially regulated the changes in milk protein yield. Negative feedback within the mTOR pathway when milk protein yield was independently stimulated by the two EAA groups suggest complexity of inference on whether mTOR pathway is upregulated or not when evaluating long term effects on milk protein production. Positive treatment interactions from supplementation of the two EAA groups suggest less negative mTOR pathway feedback when both groups are supplemented in conjunction and when milk protein yield was maximal. We conclude that EAA do exert effects on mTOR pathway associated proteins to regulate milk protein yield, however, interpretation is complex as directional changes in protein expression is contrary to what would be expected from positive activation of the mTOR pathway to support greater milk protein yield.

Nutritionists should consider the following findings from this research. First, the single limiting AA theory likely does not regulate milk protein production. Secondly, other EAA beyond Met, Lys, and His should be considered during formulation for optimizing dairy cattle performance, particular when low CP diets are administered. Thirdly, attempting to identify a unique optimal EAA profile for feeding dairy cows is somewhat a moot objective. Lastly, some EAA exert control over protein expression and activation which can change the metabolism efficiency of converting absorbed protein into milk protein.

Strategies that nutritionists may want to contemplate are as follows. First, place less emphasis on model protein or AA requirements as the efficiency of use will vary and this is not

considered. The udder has much more plasticity for meeting the AA demands for milk protein production than inferred by nutrition models. Secondly, consider that more EAA than Met, Lys, and His are important for optimizing milk protein production, hence crystalline AA should not be compared to feed protein on a single AA basis. Supplementation of high quality RUP feed protein has more value than simply Met, Lys, and His when balancing for AA. Lastly, place less emphasis on common nutrition model predictions when formulating diets regarding protein supplementation. Instead, consider biological findings such as energy stimulating milk protein production or the inherent flexibility that will result in efficiency of use changes. Pondering the latter results in more flexibility in protein supplementation during formulation and may suggest supplementing protein supplies below model requirements. The latter may be necessary when milk protein prices are low to achieve maximum income over feed costs. Along with this recommendation, observe cow responses to provide continuous feedback and learning on how best to supplement protein to dairy cows.

### **8.1. References**

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