Relationships between circulating plasma concentrations and duodenal flows of essential amino acids in lactating dairy cows

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ABSTRACT

The objective of this study was to better define essential AA (EAA) requirements in lactating dairy cows through examination of the relationship between plasma essential AA concentration (p[EAA]) and predicted duodenal flow of essential AA (EAA_{duo}). Our hypothesis was that at a given level of milk protein output, p[EAA] would remain steady in response to increasing EAA_{duo} until the EAA requirement was met, at which point p[EAA] would increase rapidly in response to greater duodenal flow of EAA until p[EAA] reached a plateau as other body processes degraded excess EAA to avoid toxicity. Thus, the requirement of each EAA would be fulfilled when p[EAA] increased rapidly. To investigate this hypothesis, we compiled a literature database that included 102 studies with 420 treatment means that reported p[EAA], dietary nutrient content, body weight, and milk production. A second database was produced to validate relationships developed in the first database and included 32 studies with 98 treatment means. All relationships were evaluated as regression equations with study as a random factor. Breed, days in milk, body weight, and milk protein production had no effect on the plasma concentration of any EAA. Other than metabolizable protein supply, nutritional content of the rations did not affect p[EAA]. Only p[Arg] was affected by parity, with primiparous cows having higher concentrations of Arg than older cows. No break points in the relationship between p[EAA] versus EAA_{duo} were detected as either steep increases or plateaus. Plasma Arg, Ile, Lys, Thr, and Val concentrations were best associated with their respective EAA_{duo} as quadratic equations, whereas His, Leu, Met, and Phe were associated only linearly. Adding a quadratic term improved the adjusted R² or decreased the root mean square error marginally (<2.0%). Thus, we conclude that the main effect of EAA_{duo} on p[EAA] is linear. Abomasal

or duodenal infusions of Met, Lys, His, Lys+Met, and casein revealed that Met or Lys infused alone increased the plasma concentration of the infused EAA and lowered the concentration of other EAA, particularly His. Infusion of Lys+Met or His alone was associated with increases in concentrations of these EAA without affecting others. We conclude that over a wide range of protein intakes in lactating cows, plasma levels of EAA increase linearly with duodenal flow. No evidence was found that EAA requirements are reflected in blood plasma concentrations.

Key words: plasma amino acid, dietary amino acid

INTRODUCTION

A better understanding of the metabolism of EAA by the lactating dairy cow is necessary to improve efficiency of their transfer to milk protein, thereby increasing N efficiency and reducing the amount of N excreted into the environment. Milk protein secretion is the major net user of absorbed AA (Lapierre et al., 2012b). The direct precursors of mammary milk protein synthesis are preformed free AA extracted from blood arterial supply by lactating mammary cells (Linzell, 1974). Broderick et al. (1974) have proposed that variations of plasma essential AA concentration (p[EAA]) could be used as a method of identifying limiting EAA, similar to the methodology used in humans and monogastric animals (Garlick, 1980). This method assumes that an EAA concentration builds up in blood plasma only after the requirement for all uses has been fulfilled. Indeed, although it has been reported in both monogastrics (e.g., humans; Moore et al., 2009) and in dairy cows (e.g., Raggio et al., 2006) that increased supply of EAA increased oxidation of Leu as well as increased p[Leu]. This suggests that the point where a specific EAA concentration begins to increase could indicate the supply at which this EAA requirement is fulfilled, but this was not detected by Broderick et al. (1974) due either to the range of EAA supply relative to the requirements or to the limited number of animals.

Received October 21, 2014.

Accepted March 2, 2015.

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Although many studies with dairy cows reported changes in p[EAA] when evaluating the effects of dietary protein changes or additions of rumen protected AA, few studies simultaneously measured duodenal EAA flow (**EAAduo**) and p[EAA] (e.g., only 2 out of the 40 studies in the database used by Pacheco et al., 2012). This severely limits the number of studies available to examine the relationship between EAAduo and p[EAA] in lactating dairy cows. Also, in addition to the fact that collecting duodenal flow information requires invasive and costly surgery, the latter may also in itself produce biases in the measurements. Recently the models used in several ration balancing programs to predict the duodenal flow of protein and EAA have been shown to yield predictions with acceptable accuracy and precision (Pacheco et al., 2012). Therefore, using predictions of duodenal flow rather than direct measurement would make it possible to use the data from a large number of existing studies reporting p[EAA]. In turn, this could allow for more clearly defined relationships between EAAduo and p[EAA] and may ultimately help to better define EAA requirements. Furthermore, few studies have specifically examined potential factors (e.g., cow breed, BW, stage of lactation, parity, and nutrient density of the diet) that may potentially alter the relationships between AA supply and p[EAA]. For example, it has been suggested that conversion of EAA to milk protein by the mammary gland is negatively affected by increased DIM (Yeo et al., 2003), although this observation has not been confirmed by others (Dalbach et al., 2011). Likewise, it has been postulated that greater dietary energy density increases the transfer of Met from duodenal flow (**Metduo**) into milk protein (Rulquin and Delaby, 1997), but higher energy did not increase milk protein to a greater extent in response to elevated Met supply (Patton, 2010). A better understanding of the relationship between EAAduo and p[EAA] might help to explain these apparent contradictions. To date, a comprehensive review of the relationship between AA supply and p[EAA] and factors that may affect this relationship has not been performed in lactating dairy cows.

Our hypothesis was that over a wide supply of EAA and a large number of animals, p[EAA] in blood plasma would increase slightly or not at all until requirements were met, at which point the concentration would begin to increase significantly. This point of rapid change was postulated as indicating the AA requirement for lactating cows. Therefore, the objectives of the present study were to investigate the relationship between predicted EAAduo and p[EAA] using the meta-analysis approach to detect any nonlinear change in the relationship between p[EAA] and EAAduo (break points) as well as to examine the potential effect of cow characteristics

(breed, DIM, BW, and milk protein yield) and dietary factors as modifiers of the relationship between p[EAA] and EAAduo.

MATERIALS AND METHODS

The developmental database was collected from a search of lactating cow studies in the *Journal of Dairy Science* between 1982 and 2012 that reported p[EAA], BW, DMI, and enough ration details so that the nutritional content could be sufficiently reconstructed. Studies from the *Journal of Dairy Science* were selected for the developmental database because it appeared as though most studies that reported p[EAA] were published here. Studies outside these years, in other refereed journals, or that were initially missed were reserved for the validation database. Studies selected for both databases included feeding trials (including those using rumen-protected AA: **RPAA**) and infusion experiments wherein a test protein was infused into the abomasum or into the duodenum. When EAA or proteins were infused, the whole quantity of infused EAA was added to the total EAAduo. No distinction was made between abomasal and duodenal infusions; however, studies that infused directly into the bloodstream were excluded. The developmental database contained studies that reported EAA concentration only in plasma because we did not want potential biases in EAA measurement between serum and plasma to affect development of associative equations. Studies that reported AA concentration in whole blood were excluded, whereas studies that reported AA concentration in serum were used solely in the validation database. The final database used for the developmental analysis contained 104 studies that represented 420 treatment means (Appendix A), whereas the validation database contained 32 studies and 106 treatment means (Appendix B).

Diets were entered into the AminoCow Dairy Ration Evaluator, version 3.5.2 (Evonik Industries AG, Hanau, Germany) for estimating daily EAAduo and MP using the approach previously reported (Patton, 2010; Pacheco et al., 2012). AminoCow was chosen over NRC (2001) because of a more comprehensive feeds library, a slightly more accurate prediction of EAAduo, and author familiarity. Briefly, when ingredient analysis was provided, the corresponding ingredient from the feed database was adjusted to this analysis. For studies that reported the nutrient content of some feeds and not others, the nutrient content for those analyzed were entered and those unanalyzed ingredients were adjusted to agree with the total reported ration analyses. For those studies where only the total ration analysis was reported, the ingredients were adjusted by first adjusting the forages to a maximum of $\pm 20\%$ of the program

default for a nutrient. If this level of adjustment could not match the reported analyses, the concentrate portion of the ration was then adjusted until nutritional agreement with the reported total nutrient content in the study was obtained. For the developmental database, 40 of 106 studies (37.7% of total studies) and 156 of 420 rations (37.1% of total) reported only total nutrient content of the diets, whereas 66 (62.2%) studies representing 264 treatment means (62.8%) reported nutritional content of ingredients as well as nutritional content of total diets. The validation database percentages were similar with 11 of 32 studies (34.4%) representing 42 of 106 (37.1%) dietary means reporting dietary nutrient composition, and 66 (62.2%) studies and 264 diets reporting both nutrient composition of diets as well as individual ingredients. The program option to adjust the microbial protein production for a deficiency of MP was always employed, with the exception that when predicted MP deficiency was greater than 600 g, the decrease was maintained at that level as suggested by the studies of Pacheco et al. (2012). Recorded ration nutrients included DMI (kg/d), NE_l (MCal/kg), total NE_l intake (MCal/d), CP, NDF, ash, crude fat, and NFC (% of DM) as well as RUP and RDP (% CP). Net energy of lactation was calculated according to NRC (2001) by selecting this option in the AminoCow program. Also included in the database were the duodenal flows of Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val (g/d) as estimated by the AminoCow model. Thus, individual EAA are presented as duodenal flow with no digestibility or bio-availability calculated. For calculation of MP supply, digestibility of both essential and nonessential individual AA was assumed to be 80% for intact proteins, 90% for RPAA with level of protection as specified by the product manufacturer, and 100% for infused AA (Lapierre et al., 2012b). As defined for the AminoCow model, MP is comprised of RUP of individual feeds $\times$ digestibility (including RPAA) plus microbial true protein $\times$ 80% digestibility and an estimation of endogenous protein based on 1.9 g of N/kg of DMI (Swanson, 1977). The grams of endogenous protein flow are calculated using the calculation of NRC (2001) as $DMI \text{ (kg)} \times 1.9 \text{ (g of N)}$. The contribution to digestible protein was calculated as $DMI \text{ (kg)} \times 1.9 \text{ (g of N)} \times 6.25 \times 80\% \text{ digestibility}$ (AminoCow Help, Evonik AG). In addition to total MP flow, a calculated variable was constructed taking into account the amount of AA secreted as milk protein. This variable (**MTP_MPY**) is defined as grams of MP supply per day $\div$ true milk protein output per day. Amino acids as % of MP (**AA%MP**) were calculated as $\text{duodenal flow of AA} \times \text{digestion coefficients}$ referenced above $\div$ $MP \times 100$. To assess the effect of increasing duodenal microbial crude protein (**MCP**)

supply on p[EAA], microbial digestible protein as a % of total MP (**MDP_MP**) was calculated by the following formula: $MDP\%MP = (MCP \times 0.8) / MP \times 100$.

Cow data included from individual studies were breed, parity, DIM, BW, milk production, milk fat, and milk protein concentration (%). Unless explicitly stated as true protein (**TP**), it was assumed that milk protein was reported as milk CP, and milk CP was converted to TP as $\text{milk CP} \times 0.93$ (NRC, 2001). Total milk protein yield was calculated as $\text{milk yield} \times \text{true milk protein \%} \times 0.01$. Also recorded from individual studies was the average DIM, which was recorded as the DIM at study initiation plus half the study length unless explicitly stated that the period when blood samples were taken was not reported as the average of the study periods. The parity of the experimental cows was recorded as primiparous, multiparous ($\geq$ second lactation) or mixed parities (primiparous + multiparous). Finally, p[EAA], recorded as μM , included Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val. The database contained samples collected from jugular and tail vessels as well as some from the mammary artery. Finally, smaller databases were constructed combining studies from both the development and the validation databases, which included only studies with infusion of casein (21 comparisons) or increased supply of a single AA via infusion or feeding as RPAA. Data in these studies were for His (8 comparisons), Lys (11 comparisons), Met (34 comparisons), or both Lys and Met (41 comparisons). Insufficient numbers of other studies were available with a single addition or deletion of EAA to develop a rigorous database for other EAA. These databases were used to test the effect of a single AA addition to the duodenal flow on the plasma concentration of the added and other EAA.

Outlier values for p[EAA] were assessed by the method of Gill (1976) using $P < 0.01$ as the critical value before an observation was deleted from the database. We chose this method because we hoped to observe break points in plasma concentrations, even if they occurred at extreme ends of the database but still in physiological ranges. This method purposely results in exclusion of a limited number of studies. In our case, eliminated values were away from the mean by more than $3.6 \times$ the standard deviation.

Associations among variables in the database were assessed as mixed linear regressions using the JMP edition (version 10) of SAS, employing the Fit Model specification with the REML method and with study specified as a random effect. Other variables were specified as fixed effects (St-Pierre, 2001). Nonsignificant factors were removed from the regression equation in a stepwise manner until all remaining terms except the intercept were significant. For p[EAA], linear and

quadratic terms were evaluated, whereas for other associations only linear associations were tested. Because it has been shown that D-Met is more slowly metabolized than L-Met, and therefore D-Met accumulates more in plasma compared with L-Met (Lapierre et al., 2012a), separate regression equations were developed for rations where Met was sourced from protein or where Met was sourced as DL-Met either from RPMet or infusion of DL-Met. Besides regressions for EAAduo versus p[EAA], equations were developed for p[EAA], which included not only EAAduo, but also cow factors and ration factors as described earlier. Where mean differences are presented, probability of difference was assessed using the Tukey honestly significant difference test. Comparisons between equations resulting from associations in the development database with values observed in the validation database were compared using the techniques of Bibby and Toutenburg (1977). Probabilities ≤ 0.01 will be referred to as highly significant, $0.01 < P \leq 0.05$ will be referred to as significant, and probabilities of $0.05 < P \leq 0.10$ will be referred to as almost significant in the text.

RESULTS

Features of the Databases

The development database is described in terms of the stated purpose of each experiment, experimental design, cow breed, parity, and number of cows in Table 1. The validation database is similarly described in Table 2. Characteristics associated with cows and rations for both databases are detailed in Table 3. Overall, the data are representative of the range of milk production, BW, DIM, and dietary ingredients observed in intensive commercial dairy operations worldwide. Experiments were not weighted in this analysis for 2 reasons: (1) most the studies ($n = 73$) were of crossover designs, which gives an unfair advantage to these studies when weighted (Lean et al., 2009), and (2) when sufficient studies are included, weighting does not change conclusions (Pacheco et al., 2006). We attempted to determine whether sampling site (coded as mammary artery, jugular vein, or caudal vessels) had an effect on p[EAA]. No effect of site was observed for any EAA except for p[Arg], which displayed both an effect of sampling site (86.4 ± 3.1 , 81.8 ± 6.4 , and 70.0 ± 4.3 for coccygeal, arterial, and jugular samples, respectively; $P < 0.05$ for coccygeal vs. jugular samples) and a site $\times$ Argduo interaction. A total of 13 p[EAA] values were removed from the developmental database as identified as outliers (1 observation for Arg, 4 for His, 1 for Ile, 3 for Leu, 2 for Lys, and 2 for Met) as well as 1 Met value for the validation database. Other data from these experi-

ments remained in the database. All statistics for the development database were applied with these values being excluded. The development database contained 8 studies and 43 diets with Ayrshire cows, 5 studies and 15 diets with Jersey cows, and 2 studies with 6 diets for mixed breed studies; the remaining studies and diets were with Holstein cows. Non-Holsteins represented 14.4% of the studies and 15.2% of treatment means. A total of 1,576 individual cows were sampled. Only primiparous cows were used in 6 studies, whereas 39 studies used both first and older parities, and 57 studies used only mature cows. The validation database contained 2 studies and 9 diets with Ayrshire cows, 2 studies and 4 diets with Norwegian red cows, 1 study and 4 diets with Brown Swiss cows, and 1 study and 5 diets with mixed breeds. For this database, non-Holsteins represented 18.2% of studies and 20.1% of treatment means. A total of 450 individual cows are represented in the validation database. None of the studies used only primiparous cows; 17 studies used mixed parities and 15 used mature cows.

Because serum samples were included in the validation database to secure sufficient numbers for an adequate test, a determination for differences in both EAAduo and p[EAA] between plasma and serum samples across both developmental and validation databases and within the validation set alone was undertaken. Only p[Arg] was different between plasma and serum samples: $82.9 \pm 2.4 \mu M$ versus $132.0 \pm 6.0 \mu M$ ($P < 0.001$) for both databases combined, and $70.1 \pm 17.3 \mu M$ versus $123.3 \pm 9.1 \mu M$ ($P < 0.001$) for the validation database alone for plasma and serum samples, respectively. For this reason, only Arg data from plasma samples in the validation database were evaluated.

In general for the developmental database, EAAduo displayed a CV varying between 18 and 24%, and with the exception of p[Met] from both protein and DL-Met sources, the p[EAA] displayed a CV of 10 to 16% (Table 4). Plasma Phe and Thr appear to be held in particularly tight ranges, whereas His, Leu, and Val and especially Met show a wider variation in plasma concentration. Breed had no effect for any p[EAA], nor were p[EAA] different between treatments administered from changes in the diet or from infusions. The only effect of parity on p[EAA] was for p[Arg], where it was observed that primiparous cows had a greater concentration ($104.5 \mu M$, $P = 0.002$) compared with mixed parities ($81.2 \mu M$) and multiparous cows ($78.5 \mu M$). In general BW, parity and DIM had no effect on p[EAA]. Although the validation database displayed about the same mean predicted duodenal flow with about the same standard deviation, and the resultant plasma concentrations were also approximately equal, the CV for p[EAA] was larger than the development

database due to a wider observed range and a lower number of studies.

Duodenal AA Flows and Plasma AA Concentration: Developmental Database

Relationships between predicted individual duodenal AA flow and respective p[EAA] concentration were generally high (Table 5), although the root mean square error (**RMSE**) as a percent of the mean was always greater than 10%. Of the EAAduo studied, 5 were related to p[EAAduo] only in a linear manner (Arg, His, Leu, and Met from DL-Met supplementation, and Phe), and 5 were related in both a linear and quadratic manner (Ile, Lys, Met from protein sources, Thr, and Val). The slope of the linear equation for DL-Met sources was approximately twice that of L-Met sources. Over all EAA when both linear and quadratic equations were significant, only a slight statistical efficiency, measured by adjusted R^2 (**adjR²**), RMSE, or RMSE as % mean observed value, was gained by adding the squared term compared with the linear term (Table 5). For the linear regression equations, the intercept as a percent of mean represented over 50% of the mean for the association of duodenal flow with p[Arg], p[Thr], and p[Phe] and almost 50% for p[His], p[Ile], and p[Met] from protein sources. When considering only the linear equations, Val had the largest slope value (1.26), whereas Phe displayed the smallest (0.15). Relationships between p[EAA] and their respective duodenal flow also displayed rather low slopes for Arg, Lys, and Met from protein sources. However, besides Val, only Met from DL sources displayed a slope greater than 0.50.

To explore the possibility that duodenal EAA flow expressed as % of MP (EAA_MP) would show more significant relationships to p[EAA] than EAAduo, linear regressions with EAA_MP were developed and the results compared with EAAduo (Table 6). In general, associations between EAA_MP and p[EAA] were remarkably similar to those for EAAduo and p[EAA] with the exception of p[Ile] which revealed no association to Ile_MP. When expressed as EAA_MP, Arg, Lys, Met from DL, and Val displayed significant quadratic effects. Only Lys and Val displayed quadratic effects for both EAAduo and EAA_MP, whereas His, Leu, and Thr displayed linear associations only in both systems. As when expressed as EAAduo, for EAA_MP Phe displayed the lowest slope parameter, and Val displayed the largest. In general, however, the intercept term was much smaller for significant associations when duodenal EAA were expressed as EAA_MP than as total flow in grams per day. Likewise, adding a quadratic term made little difference in increasing **adjR²** or decreasing RMSE

except for Lys whose associated equation was increased more than 2% when the quadratic term was added.

Because MP was highly correlated with EAAduo for all EAA except Met from DL sources (Table 7), regression models for p[EAA] were constructed without terms for MP or other EAA included. Likewise we found that consumption variables, such as DMI, and NE_l consumption were highly correlated with EAAduo, and therefore these variables were also not evaluated in the equations developed. Milk production and milk true protein yield (**MPY**) were correlated with EAAduo to a moderate degree ($R^2 < 0.55$). These variables were left in subsequent associative equations in hope of finding more relevant association. Correlation between MP and the flow of each individual EAA as well as individual p[EAA] was always highly significant (Table 7). Terms for MTP_MP and MDP_MP were included in evaluations for subsequent factors in regression equations.

Effect of Increasing AA Demand for Milk Protein Production on Plasma Concentration

Increased production of milk true protein was not associated with increased plasma concentration of Arg, His and Met from DL-Met (Table 8). The p[Lys] was associated linearly whereas the other EAA were associated quadratically (Figure 1). For Ile, Leu, and Val (the branched-chain EAA), as MTP_MP increases, p[EAA] concentration declines, reaching a nadir of 76.6, 82.9, and 141.4 μM for Ile, Leu and Val, respectively, whereas p[Met] and p[Phe] reach their low points at 15.8 and 34.2 μM , respectively. Table 8 also suggests that p[Thr] is maintained between a minimum of 79.0 μM and a maximum of 97.6 μM . The p[Thr] remains constant until the MTP_MP reaches 40%, at which time it decreases at a gradually increasing rate. Plasma concentrations of Met from intact proteins and Phe decrease in parallel with Lys in spite of the quadratic nature of the best equations.

Effect of Increasing Percentage of Microbial Protein

Associated with each 1% increase in calculated MDP_MP was a 28-g decrease in MP flow to the duodenum ($P < 0.001$). In turn, this led to a highly significant ($P < 0.001$) decrease in Argduo (−1.19 g), Hisduo (−0.81 g), Ileduo (−1.28 g), Leuduo (2.78 g), Lysduo (−1.55 g), Metduo (−0.65 g), Pheduo (−1.55 g), Thrduo (−1.18 g), and Valduo (−1.77 g) for each percent increase in MDP_MP. Likewise, increasing MDP_MP was negatively associated with all p[EAA] except p[Arg] and p[Thr]. This association was linear for all EAA except for His, which was associated in a

Table 1. Characteristics of studies included in the developmental database

First author	Year	Breed	Parity	Infusion	Study design ¹	Cows sampled	Study objective was to determine: ²
Awawdeh	2007	Holstein	3		4 × 4 LS	4	Lys digestibility in soybean meals
Bach	2000	Holstein	3		4 × 4 LS	4	Effects of high and low quality of protein
Bertrand	1998	Jersey	2		RCB	24	RPMet and RPLys effects on production
Berthiaume	2001	Holstein	2		RCB	15	Degradability and digestibility of RPMet
Berthiaume	2006	Holstein	2		3 × 3 LS	6	Effect of RPMet on splanchnic metabolism
Blauwikel	1997	Holstein	3		4 × 4 LS	4	Effect of gossypol RPLys
Blouin	2002	Holstein	2		2 × 2 LS	6	MP effects on splanchnic flux
Blum	1999	Mixed	2		SB	6	Compare 2 RPMet supplements
Borucki Castro	2008	Holstein	3		4 × 4 LS	8	Effect of soybean protein on N metabolism
Boucher	2007	Holstein	2		4 × 4 LS	4	Effect of urea levels on microbial protein
Bremmer	1997	Jersey	2		4 × 4 LS	12	Effect of RPAA and somatotropin
Brito	2008	Holstein	2		SB	16	Nitrogen metabolism in cows fed alfalfa
Broderick	1986	Holstein	3		SB	6	Expeller vs. solvent soybean meal
					4 × 4 LS	20	Effect of soybean meal types
Broderick	1993	Holstein	3		4 × 4 LS	12	Urea vs. true protein
Cabrita	2011	Holstein	3		8 × 8 LS	18	Effect of MP balance on performance
Cant	1993	Holstein	1	Yes	4 × 4 LS	4	Abomasal vs. ruminal casein infusion
Carvalho	2006	Holstein	2		RCB	20	Effect of palm kernel meal vs. distillers dried grains on performance
					RCB	18	Effect of dried distillers grain on Lys metabolism
Chan	1997	Holstein	2		4 × 4 LS	4	Fat and protein flow to duodenum
Christen	2010	Holstein	2		4 × 4 LS	12	Distillers grain vs. other protein sources
Christensen	1994	Holstein	3		4 × 4 LS	4	Effect of RPMet and RPLys on nutrient flows
Christensen	1996	Holstein	2		4 × 4 LS	4	Fat and niacin on nutrient flows to duodenum
Colin-Schoellen	1994	Holstein	2		4 × 4 LS	16	RPMet and RPLys interaction with energy level
			2		4 × 4 LS	12	RPMet and RPLys interaction with energy level
Dalbach	2011	Holstein	2		RCB	16	HMBi on nitrogen metabolism at 4 DIM
Dalbach	2011				RCB	16	HMBi on nitrogen metabolism at 15 DIM
	2011				RCB	16	HMBi on nitrogen metabolism at 29 DIM
Davidson	2003	Holstein	2		RCB	75	RUP sources on nitrogen metabolism
DeFraen	2002	Holstein	3		3 × 3 LS	18	Effect of corn steep liquor on nutrient metabolism
Dinn	1998	Holstein	3		3 × 3 LS	18	RPMet and RPLys on nitrogen excretion
Doepel	2007	Holstein	3	Yes	2 × 2 LS	8	Glutamine effects on splanchnic metabolism
Doepel	2010	Holstein	1	Yes	4 × 4 LS	4	Essential AA on mammary metabolism
Doepel	2011	Holstein	1	Yes	3 × 3 LS	6	Arg effects on mammary metabolism
Donkin	1989	Holstein	3		SB	4	Effects of RPMet and RPLys on production
Ellison Henson	1997	Holstein	2		3 × 3 LS	12	RUP supplements on nitrogen metabolism
Erickson	1992	Holstein	3		RCB	40	Effects of fat and nicotinic acid
Guinard	1994a	Holstein	3	Yes	4 × 4 LS	4	Effects of casein infusion on mammary uptake
Guinard	1994b	Holstein	3	Yes	4 × 4 LS	4	Effects of Lys infusion on mammary uptake
Guinard	1995	Holstein	3	Yes	4 × 4 LS	4	Effects of Met infusion on mammary uptake
Graulet	2007	Holstein	3		RCB	24	Folic acid and B ₁₂ supplementation on metabolism
Hammon	2008	Holstein	2		3 × 3 LS	3	Fat supplementation on portal metabolism
Hanigan	2004	Holstein	2	Yes	4 × 4 LS	4	Casein infusion on nitrogen metabolism
Hu	2007a	Holstein	3		6 × 6 LS	6	Cation balance effects on metabolism
Hu	2007b	Mixed	3		RCB	48	Cation balance effects on metabolism
Huhtanen	2001	Ayrshire	3	Yes	Inc. LS	5	His, Leu, and glucose infusion on metabolism
Hurtaud	1998	Holstein	2	Yes	4 × 4 LS	4	Effects of glucose infusion on protein yield
Karunanandaa	1994	Jersey	3		4 × 4 LS	8	Effects of RPMet and RPLys on production
King	1990	Holstein	3		3 × 3 LS	6	Protein sources on nitrogen metabolism
Kleinschmit	2006	Holstein	2		3 × 3 LS	12	Evaluation of dried distillers grains on AA utilization
Kleinschmit	2007	Holstein	2		4 × 4 LS	8	Evaluation of dried distillers grains plus soluble on AA utilization

Continued

Table 1 (Continued). Characteristics of studies included in the developmental database

First author	Year	Breed	Parity	Infusion	Study design ¹	Cows sampled	Study objective was to determine: ²
Korhonen	2000	Ayrshire	2	Yes	5 × 5 LS	5	Effect of level of His on milk protein production
Korhonen	2002a	Ayrshire	3	Yes	5 × 5 LS	5	Effect of Ile, Leu, and Val on milk protein production
Korhonen	2002b	Ayrshire	3		4 × 4 LS	4	Effect of protein source on milk protein production
Kung, Jr.	1984	Holstein	2		RCB	94	RUP effects on plasma AA
LaCount	1996a	Holstein	3	Yes	4 × 4 LS	4	Effects of abomasal infusion of L-carnitine
LaCount	1996b	Holstein	3		5 × 5 LS	20	Effects of feeding L-carnitine
Lanham	1992	Holstein	2		RCB	40	Cottonseed and niacin effects on milk protein
Larsen	2009	Holstein	2	Yes	S plot	12	Glucose infusion on AA metabolism at 4 DIM
		Holstein	2	Yes	S plot	12	Glucose infusion on AA metabolism at 15 DIM
		Holstein	2	Yes	S plot	12	Glucose infusion on AA metabolism at 29 DIM
Lee	2012a	Holstein	2		RCB	24	Effect of RPMet and RPLys on low MP diets
Lee	2012b	Holstein	2		RCB	36	Effect of RPMet, RPLys, and RPHis
Lemosquet	2009	Holstein	3	Yes	4 × 4 LS	4	Effect of AA infusion on glucose metabolism
Mackle	1999	Holstein	2	Yes	4 × 4 LS	4	Effect of casein and branched AA on milk protein
Martineau	2007a	Holstein	3		3 × 3 LS	6	Forage preservation on metabolism
Martineau	2007b	Holstein	1		3 × 3 LS	6	Effects of ionophores on protein and glucose
Miettinen	1996	Ayrshire	3	Yes	4 × 4 LS	4	Infused VFA effects on milk yield
Mjoun	2010a	Holstein	3		RCB	36	Dried distillers grain with solubles on AA use
Mjoun	2010b	Holstein	2		RCB	40	Levels of distillers grain with solubles on AA use
Moorby	2002	Holstein	1		RCB	40	Level of concentrate effects on nitrogen balance
Mulrooney	2009	Holstein	2		4 × 4 LS	12	Replace distiller with solubles with canola meal
Nagel	1992	Holstein	3		RCB	22	All 3 studies look at the effects of formic acid
		Holstein	3		S plot	22	or formaldehyde treatment of alfalfa silage with
		Holstein	3		3 × 3 LS	12	and without fishmeal
Nichols	1998	Holstein	3		4 × 4 LS	12	RPMet and RPLys with dried distillers grain and milk
Noftsgger	2005	Holstein	2		4 × 4 LS	8	Evaluation of 3 RPMet sources
Oba	2010	Holstein	3		4 × 4 LS	4	Suitability of triticale distillers dried grain for dairy cows
Overton	1996	Holstein	2		RCB	24	Evaluation of RPMet product
Overton	1998	Holstein	3		S plot	44	Effect of carbohydrate on RPMet response
Palmquist	1982	Jersey	2		4 × 4 LS	4	Both studies looked at AA availability of distillers
		Holstein	2		4 × 4 LS	8	dried grain and performance
Piepenbrink	1996	Holstein	3		5 × 5 LS	10	Performance of cows fed low protein and RPMet
Pisulewski	1996	Holstein	3	Yes	5 × 5 LS	5	Response to increasing doses of infused Met
Preynat	2008	Holstein	3		RCB	24	Effect of folic acid and vitamin B ₁₂ with RPMet
Preynat	2009	Holstein	3		RCB	60	Effect of RPMet and folic acid plus vitamin B ₁₂
Raggio	2004	Holstein	3		3 × 3 LS	6	MP supply on splanchnic flux
Rius	2010a	Holstein	1	Yes	2 × 2 LS	6	Infused starch and casein
Rius	2010b	Holstein	2		RCB	40	Milk protein response to starch and AA
Rogers	1987	Holstein	2		Inc. Blk	36	Milk protein response to RPMet and RPLys
Schwab	1992a	Holstein	3	Yes	4 × 4 LS	4	Effect of casein, Met, or Lys at 30 DIM
	1992a	Holstein	3	Yes	4 × 4 LS	4	Effect of casein, Met, or Lys at 70 DIM
	1992a	Holstein	3	Yes	4 × 4 LS	4	Effect of casein, Met, or Lys at 133 DIM
	1992a	Holstein	3	Yes	4 × 4 LS	4	Effect of casein, Met, or Lys at 170 DIM
Schwab	1992b	Holstein	3	Yes	4 × 4 LS	4	Effect of Met and Lys at 30 DIM
	1992b	Holstein	3	Yes	4 × 4 LS	4	Effect of Met and Lys at 70 DIM
	1992b	Holstein	3	Yes	4 × 4 LS	4	Effect of Met and Lys at 133 DIM
	1992b	Holstein	3	Yes	4 × 4 LS	4	Effect of Met and Lys at 270 DIM
Seymour	1990	Holstein	3	Yes	Inc. Blk	64	Effect of casein or Met + Lys infusion on production
St-Pierre	2005	Holstein	2		RCB	64	Effect of HMB and HMBi on milk protein
Tagari	2007	Holstein	2		2 × 2 LS	8	Mammary uptake of AA of cows fed different starch sources

Continued

Table 1 (Continued). Characteristics of studies included in the developmental database

First author	Year	Breed	Parity	Infusion	Study design ¹	Cows sampled	Study objective was to determine: ²
Vanhatalo	1999	Ayrshire	3	Yes	4 × 5 Inc.LS	5	His alone or His + Met and Lys on AA metabolism
Vanhatalo	2009	Ayrshire	3		5 × 5 LS	5	AA metabolism of cows fed grass or red clover silage
Varvikko	1999	Ayrshire	3	Yes	5 × 5 LS	5	Effect of level of Met infusions
		Ayrshire	3	Yes	5 × 5 LS	5	Effect of level of Lys infusions
Vicini	1988	Holstein	3	Yes	3 × 3 LS	2	Effect of Arg infusion on milk production
Wang	2010	Holstein	2		RCB	60	Effects of RPMet and RPLys on production
Weekes	2006	Holstein	2	Yes	6 × 6 LS	6	Effects of deletion of Met, Lys, His, or branched chain AA on production and metabolism
Wickersham	2004	Holstein	2		RCB	54	Effects of corn steep liquor on milk production

¹LS = Latin square; RCB = randomized complete block; SB = switch-back; Inc.LS = incomplete Latin square; S plot = split-plot; Inc. Blk = incomplete block.

²RP = rumen-protected; HMB = 2-hydroxy-4-(methylthio) butanoic acid; HMBi = isopropyl ester of HMB.

quadratic manner (Table 9 and Figure 2). As can be seen in the figure, p[His] is initially increased by increasing MCP percent, but then decreases at a slightly greater rate as the amount of MCP_MP continues to increase. Forcing the intercept through 0 (data not shown) reveals that p[Val] decreases the most with a slope of −3.10, whereas p[Phe] decreases the least with a slope of 0.61.

Best Mixed Equations

All included cow factors (BW, DIM, parity, milk yield, and MPY) and ration factors [NE_l/kg, NDF, NFC, ether extract, and ash (all % of DM)] as well as MTP_MP and MDP_MP were added to regression equations that included EAAduo to determine if factors other than EAAduo influenced p[EAA]. Resultant equations are presented in Table 10. No cow factor except milk yield or MPY ever entered the equations. Ration factors entered all the associative equations (assuming MDP_MP and MTP_MP as ration factors) except for Met from protein and Arg, which were not improved beyond duodenal flow. The effects of NDF and NFC could both be expressed as having negative associations between duodenal flow of EAA and resultant blood levels. Energy concentration (NE_l/kg) of the diet had no effect on the association of EAAduo with p[EAA]. Milk production was a significant negative factor for association of duodenal Lys, Met from DL-Met, and Val with the respective p[EAA], whereas MPY was negatively associated with plasma Ile, Met from protein sources, and Phe. Adding the total significant terms decreased the RMSE for associations of p[EAA] for His, Lys, Met from DL-Met, Thr, and Val. The RMSE for Met from protein and Phe remained unchanged.

Evaluation of Associative Equations

The regression equations derived from the developmental database were used to predict p[EAA] using terms for EAAduo, EAA_MP, or all factors, including MTP_MP and MDP_MP. The predicted p[EAA] were compared against observed values in the validation database with results presented in Table 11. In general, all associations provided reasonable estimates of p[EAA] with Lys displaying the lowest adjR² and highest RMSE as % of the mean and Met from DL-Met sources providing the highest adjR² and lowest RMSE as % of mean. Quadratic equations generally reduced the RMSE slightly or not at all with resultant small changes in adjR² and RMSE. Expressing duodenal protein flow as EAA_MP or adding ration or cow factors, or both, likewise had small effects both on RMSE or adjR² for predictions of p[EAA] for the validation data-

Table 2. Characteristics of studies included in the validation database

First author	Year	Blood source	Breed	Parity	Infusion	Design ¹	Cows sampled	Study objective was to determine:
Abu-Ghazaleh	2001	Serum	Holstein	3		4 × 4 LS	12	Effects of replacing soybean meal with fish meal
Broderick	1974	Plasma	Mixed	3		5 × 5 LS	5	If AA build up in plasma when requirement is met
Casper 1	1987	Serum	Holstein	2		RCB	23	Effects of whole soybean and soybean meal with Met
Casper 2	1988	Serum	Holstein	2		RCB	28	Effects of added Met on high barley diets
Casper 3	1990	Serum	Holstein	2		RCB	28	Effects of added NFC on high fat rations
Choi	2002	Plasma	Ayrshire	2		5 × 5 LS	5	Compare N flow in cows fed differing a-fraction proteins ²
Choung	1993	Plasma	Holstein	3	Yes	4 × 4 LS	4	Effects of abomasal infusions of casein
Clark	1977	Plasma	Holstein	2	Yes	4 × 4 LS	4	Effects of high quality protein and glucose
Coomer	1993	Plasma	Holstein	3		RCB	24	Effects of added NFC for cows fed high fat rations
Drackley	1986	Serum	Holstein	2		RCB	30	The AA flow to mammary gland of cows fed soybean or sunflower protein
Fathi Nasri	2007	Plasma	Holstein	3		RCB	18	Performance of cows fed soybean meal, raw, or roasted whole soybeans
Garnsworthy	2008	Plasma	Holstein	3		RCB	32	Effect of high or low Leu on ovarian function
Hadrova	2012	Plasma	Holstein	2	Yes	SB	3	If His could be a limiting AA
Hadsell	1988	Serum	Holstein	2		RCB	30	The value of chickpeas as protein source for cows
Haque	2013	Plasma	Holstein	3	Yes	4 × 4 LS	4	Effects of imbalance of AA
Harouna	1997	Serum	Holstein	2		RCB	30	The optimum dietary fat and RUP for lactating cows
Krizova	2008	Plasma	Holstein	2		SB	4	If Leu could be a limiting AA
Krober	2000	Plasma	Brown Swiss	3		4 × 4 LS	4	Effects of Lys, Met, or Lys + Met on milk protein yield
Liu	2000	Serum	Holstein	3		RCB	40	Value of corn distillers grain to a blend of other proteins
Lapierre	2009	Plasma	Holstein	3	Yes	SB	6	If a deficiency of Lys affects protein metabolism
Maiga	1997	Serum	Holstein	2		RCB	40	Evaluate diets for milk protein yield when feeding high fat
Miettinen	1997	Plasma	Ayrshire	3	Yes	4 × 4 LS	4	Effects of casein on production of cows fed silages that differed in fermentation characteristics
Pacheco-Rios	1998	Plasma	Holstein	2		2 × 2 LS	4	Effect of feed restriction on AA metabolism
Papas	1984	Plasma	Holstein	3		RCB	30	Plasma Met levels to determine effectiveness of RPMet
Piepenbrink	1998	Serum	Holstein	2		5 × 5 LS	10	Best system to predict milk and protein yield
Plaizier	2001	Plasma	Holstein	3	Yes	4 × 4 LS	4	Effects of glutamine on milk protein yield
Raggio	2006	Plasma	Holstein	3	Yes	3 × 4 YS	3	Effect of casein and propionic acid on kinetics of glucose and Leu
Richter	2010	Plasma	Holstein	3	Yes	4 × 4 LS	4	Possibility of Leu as third limiting AA
Schei 1	2007	Plasma	Norway Red	2	Yes	4 × 4 LS	8	Effects of starch, glucose, or AA infused ruminally or abomasally in early lactation
Schei 2	2007	Plasma	Norway Red	2	Yes	4 × 4 LS	8	Effects of starch, glucose, or AA infused ruminally or abomasally in late lactation
Spires	1975	Plasma	Holstein	2	Yes	SB	5	N utilization when given casein infusion
Yang	1986	Serum	Holstein	2		RCB	24	Other limiting AA when rumen RPMet was fed

¹LS = Latin square; RCB = randomized complete block; SB = switch-back; YS = Youden square.²a-fraction = NPN and some true protein that is assumed to be instantly degradable (NRC, 2001)

Table 3. Description of cow and ration factors for developmental and validation databases

Item	Developmental database				Validation database			
	Mean	SD	Minimum	Maximum	Mean	SD	Minimum	Maximum
Cow-associated factors								
DIM	125	61	4	321	113	54	54	260
BW, kg	603.1	59.9	410	756	595	50.3	498	733
Milk, kg/d	30.6	6.4	14.8	47.7	30.3	6.5	13.9	40.8
Milk fat %	3.73	0.5	2.37	5.47	3.74	0.7	2.65	5.6
True milk protein, %	2.96	0.28	2.46	4.38	2.98	0.22	2.55	3.83
True milk protein yield, g/d	908	174	491	1,495	896	183	411	1,275
DMI, kg/d	20.3	3.4	8.5	29.4	20.1	3.5	12.3	28.4
Ration-associated factors								
NE _l , ¹ Mcal/kg	1.56	0.11	1.17	1.77	1.58	0.08	1.37	1.78
CP, % of DM	16.4	1.9	9.3	24.7	16.2	2.1	9.0	22.4
ADF, % of DM	19.9	4.6	13.0	65.1	19.1	3.2	14.9	30.4
NDF, % of DM	33.6	5.0	22.0	53.3	32.9	5.5	24.6	52.4
NFC, % of DM	38.4	5.5	22.8	50.5	40.0	6.5	9.1	50.2
Ether extract, % of DM	3.92	1.26	2.16	8.82	4.0	2.25	1.78	23.4
Ash, % of DM	7.66	1.38	4.64	12.11	7.02	1.25	4.86	9.98
MP, g/d	2,182	468	705	3,818	2,168	457	1,257	3,565
Microbial CP, g/d	1,818	320	673	2,791	1,789	278	1,210	2,382
RDP, g/d	2,167	468	770	3,464	2,130	488	850	3,030
RUP, g/d	1,183	111	300	3,330	1,161	395	470	2,370
MDP_MP ²	67.7	8.2	34.4	84.2	67.4	9.0	40.5	84.9

¹Calculated according to NRC (2001).²MDP_MP = microbial digestible CP as a percent of total MP.**Table 4.** Descriptive statistics for duodenal flow and plasma concentration of essential AA in developmental and validation databases

Item	Developmental database					Validation database				
	Mean	SD	CV	Minimum	Maximum	Mean	SD	CV	Minimum	Maximum
EAAduo, ¹ g/d										
Arg	132	32	23.8	43	235	100	29	28.7	57	205
His	62	14	22.8	20	106	63	14	22.9	34	86
Ile	141	29	20.3	46	254	139	25	18.2	84	184
Leu	232	55	23.6	71	466	231	52	22.4	128	330
Lys	180	35	19.6	60	294	182	34	18.7	100	236
Met, protein	53	12	22.7	18	89	53	11	21.3	30	80
Met, DL-Met	64	12	18.4	38	103	66	8	12.4	51	91
Phe	140	29	20.9	45	239	140	29	20.4	81	189
Thr	136	27	20.0	46	223	134	26	19.2	83	180
Val	157	33	20.9	51	259	156	30	19.4	93	204
p[EAA], ² μ M										
Arg	80.8	9.4	11.6	31.2	142.6	71.0	17.3	24.4	37	160
His	45.3	7.0	15.5	14.2	67.7	49.4	17.7	35.8	15	97
Ile	111.8	13.8	12.4	59.2	164.7	115.4	27.3	23.7	45	193
Leu	147.6	23.8	16.1	36.9	226.4	146.8	48.0	32.7	59	256
Lys	79.1	10.0	12.6	47.3	111.7	84.3	18.5	21.9	45	133
Met, protein ³	21.5	8.1	37.7	3.6	70.0	24.0	7.4	30.8	12	90
Met, DL-Met ⁴	35.1	14.2	40.5	13.4	84.5	32.1	10.9	34.0	20	93
Phe	47.9	5.2	10.9	17.8	69.3	48.4	10.2	21.1	27	79
Thr	96.5	10.0	10.3	57.0	164.0	100.3	21.8	21.7	27	159
Val	234.5	32.5	13.9	101.3	391.3	233.3	63.5	27.2	66	439

¹EAAduo = estimated daily duodenal flow of essential AA.²p[EAA] = plasma (and serum except for Arg) concentration of essential AA.³Met, protein = Met derived from intact dietary protein.⁴Met, DL-Met = Met derived from DL-Met sources.

Table 5. Relationships between plasma AA concentration (μM) and duodenal AA flow (AAduo, g/d) for the developmental database

p[AA]	Model type	N	adjR ²	Mean ¹	RMSE ²	RMSE, % mean ³	Intercept, % mean	Best equation
Arg	Linear	366	0.904	80.8	9.0	11.1	62.7	50.64 + (0.220 × Argduo)
	Quadratic		NS ⁴					
His	Linear	403	0.800	45.3	7.6	16.8	46.4	21.02 + (0.392 × Hisduo)
	Quadratic		NS					
Ile	Linear	419	0.769	111.8	14.2	12.7	48.4	54.05 + (0.414 × Ileduo)
	Quadratic		0.783	111.8	13.8	12.3		−8.28 + (1.283 × Ileduo) − (0.003 × Ileduosqr ⁵)
Leu	Linear	417	0.811	147.6	24.4	16.5	25.6	37.79 + (0.483 × Leuduo)
	Quadratic		NS					
Lys	Linear	418	0.824	79.1	9.8	12.4	38.3	30.32 + (0.268 × Lysduo)
	Quadratic		0.829	79.1	9.7	12.2		−9.42 + (0.730 × Lysduo) − (0.001 × Lysduosqr)
Met, protein ⁶	Linear	320	0.877	21.3	3.3	15.5	49.5	10.54 + (0.21 × Metduo)
	Quadratic		0.885	21.3	3.2	15.0		−2.74 + (0.72 × Metduo) − (0.005 × Metduosqr)
Met, DL-Met ⁷	Linear	98	0.904	35.2	5.6	15.9	2.0	0.69 + (0.53 × Metduo)
	Quadratic		NS					
Phe	Linear	414	0.858	48.0	5.4	11.3	55.6	26.58 + (0.153 × Pheduo)
	Quadratic		NS					
Thr	Linear	410	0.906	96.5	11.0	11.4	57.1	55.06 + (0.300 × Thrduo)
	Quadratic		0.912	96.5	10.6	11.0	−7.2	−6.98 + (1.252 × Thrduo) − (0.003 × Thrduosqr)
Val	Linear	420	0.844	234.5	31.1	13.3	15.6	36.49 + (1.264 × Valduo)
	Quadratic		0.851	234.5	30.4	13.0		−101.82 + (3.060 × Valduo) − (0.006 × Valduosqr)

¹Mean = mean plasma AA concentration (μM).²RMSE = root mean square error.³RMSE, % mean = root mean square error as a percent of the mean for linear equations only.⁴NS = quadratic term not significant.⁵EAAduosqr = EAAduo × EAAduo.⁶Met, protein = Met derived from intact dietary protein or infused L-Met.⁷Met, DL-Met = Met was partially supplied from DL-Met, either as rumen protected-Met or infused.

Table 6. Relationships between plasma AA concentration (μM) and duodenal AA flow as percent of MP (AA_MP) for the developmental database

p[AA]	Model type	N	adjR ²	Mean ¹	RMSE ²	RMSE, % mean ³	Intercept, % mean	Best equation
Arg	Linear	366	0.908	80.8	8.8	10.9	23.3	$18.85 + (12.64 \times \text{Arg_MP})$
	Quadratic		0.913	80.8	8.6	10.6		$223.31 - (71.12 \times \text{Arg_MP}) + (8.52 \times \text{Arg_MPsqr}^4)$
His	Linear	403	0.804	45.3	7.6	16.7	3.7	$1.66 + (19.22 \times \text{His_MP})$
	Quadratic		NS ⁵					
Ile	Linear	417	NS					
	Quadratic		NS					
Leu	Linear	418	0.770	147.6	27.0	18.3	55.6	$82.00 + (8.02 \times \text{Leu_MP})$
	Quadratic		NS					
Lys	Linear	320	0.812	79.1	10.1	12.8	14.7	$11.63 + (10.14 \times \text{Lys_MP})$
	Quadratic		0.834	79.1	9.5	12.1		$127.56 - (27.78 \times \text{Lys_MP}) + (3.06 \times \text{Lys_MPsqr})$
Met, protein ⁶	Linear	98	0.874	21.3	3.4	16.0		$-10.44 + (16.76 \times \text{Met_MP})$
	Quadratic		NS					
Met, DL-Met ⁷	Linear	414	0.921	35.3	5.0	14.2	-28.3	$-9.99 + (18.08 \times \text{Met_MP})$
	Quadratic		0.922	35.3	5.0	14.2		$-62.03 + (55.69 \times \text{Met_MP}) - (6.46 \times \text{Met_MPsqr})$
Phe	Linear	420	0.842	47.9	5.7		18.1	$8.67 + (7.63 \times \text{Phe_MP})$
	Quadratic		NS					
Thr	Linear	420	0.907	96.5	10.9	11.3	-15.7	$-15.15 + (22.16 \times \text{Thr_MP})$
	Quadratic		NS					
Val	Linear	420	0.791	234.5	35.9	15.3	-13.5	$-31.70 + (46.44 \times \text{Val_MP})$
	Quadratic		0.797	234.5	35.4	15.1	399.3	$936.19 - (287.95 \times \text{Val_MP}) + (28.82 \times \text{Val_MPsqr})$

¹Mean = mean plasma AA concentration (μM).

²RMSE = root mean square error.

³RMSE, % mean = root mean square error as a percent of the mean.

⁴EAA_MPsqr = EAA_MP $\times$ EAA_MP.

⁵NS = no significant equation or quadratic term not significant.

⁶Met, protein = Met derived from intact dietary protein or infused L-Met.

⁷Met, DL-Met = Met was partially supplied from DL-Met, either as rumen protected-Met or infused.

Table 7. Correlations between MP supply, duodenal flow (g/d), and plasma concentration (μM) of individual essential AA

AA	Duodenal flow		Plasma concentration	
	R ²	P-value	R ²	P-value
Arg	0.832	<0.001	0.922	0.006
His	0.826	<0.001	0.787	<0.001
Ile	0.902	<0.001	0.763	<0.001
Leu	0.853	<0.001	0.808	<0.001
Lys	0.838	<0.001	0.772	0.001
Met, protein ¹	0.877	<0.001	0.804	0.001
Met, DL-Met ²	0.340	<0.001	0.087	0.002
Phe	0.945	<0.001	0.840	<0.001
Thr	0.966	<0.001	0.900	<0.001
Val	0.958	<0.001	0.824	<0.001

¹Met, protein = Met derived from intact dietary protein.

²Met, DL-Met = Met derived from DL-Met sources.

base. In summary, the most efficient overall estimate of p[EAA] remained the equation with estimated EAAduo with a linear relationship.

Effects of Increasing Duodenal Flow of Individual AA

Preliminary regression equations indicated that EAA composition of the MP may have an effect on the p[EAA] of individual AA. However, the high correlation between all EAAduo and individual EAAduo (i.e., high correlation between MP and AA flow) made clear interpretation of any such relationships impossible. For this reason it was decided to compare the effects of an increase in a single EAAduo on the corresponding p[EAA] as well as other AA. The effects of adding Met, Lys, or His singly, as well as the effect of adding Met + Lys were contrasted to the effects of infused casein, an instance where all AA are increased.

When only Met was added either by infusion or as RPMet (all as DL-Met; mean = 19 g/d), Met was the only EAA whose predicted flow was altered ($P < 0.001$; Table 12). This was accompanied by a significant increase in p[Met] as well as a decrease ($P \leq 0.03$) in plasma concentrations of all EAA except Arg and Lys. Although feed intake and milk yield were unchanged, milk true protein % (MTPPct) and MPY tended to increase ($P = 0.07$ for each) by Met addition. When Lys was supplemented (mean = 30 g/d), Lysduo was the only EAA estimated to increase ($P = 0.003$). This addition resulted in higher p[Lys] as well as lower p[His] ($P = 0.004$) and a tendency for p[Phe] to be reduced also ($P = 0.06$; Table 12). No production variables were affected by Lys supplementation in this database. Adding His (10g/d) resulted in predicted higher ($P < 0.01$) Hisduo and higher ($P = 0.01$) p[His]. No other p[EAA] were affected, but increased His supply resulted in both

greater milk production ($P < 0.001$) and MPY ($P = 0.02$), with a tendency ($P = 0.06$) to increase DMI (Table 12).

Given the results above, it was expected that adding Met + Lys (estimated difference in duodenal flow was +18 g of Lys and +7 g of Met) should increase both Lysduo and Metduo, as well as increase plasma Lys and Met, and should result in decreases in other plasma EAA, particularly His. However, except for greater plasma concentration of Lys and Met, no other p[EAA] changes were detected. The addition of both Lys and Met resulted in a greater milk true protein percent, without a difference in MPY (Table 12). When casein was infused into the duodenum, the predicted duodenal flows of all EAA were increased ($P < 0.001$) and all plasma AA increased ($P \leq 0.02$) with the exception of Arg. This resulted in greater ($P \leq 0.04$) MPY and MTPPct.

DISCUSSION

When interpreting these data, it is necessary to keep in mind that duodenal flows of EAA are predicted values, although model predicted values have been shown to be reasonably accurate over a wide range of diet types (Pacheco et al., 2012). It is acknowledged that variation in ingredients and ingredient quality as well as errors in prediction of amount and EAA composition of microbial protein may well lead to errors in prediction of EAAduo in individual studies. The effect of these potential variations on the development of equations should have been reduced by the inclusion of study as a random term in all models (St-Pierre, 2001). Also, because of the nature of the experimental data, many of the reported studies are Latin square designs that may introduce transient or short-term changes in p[EAA], or through mobilization of body protein, no changes in p[EAA] at all. These changes, or lack of changes, may not be seen with longer experimental times. It becomes a matter of conjecture as to when dietary changes become stable. However, without these Latin square studies, the data available become severely limiting.

Given these limitations, little evidence was observed from this study that p[EAA] displayed any break point that would be indicative of fulfilling an EAA requirement as p[EAA] were mainly linearly related to their respective EAAduo. Although the RMSE was lower for quadratic equations, the adjR² was only very slightly enhanced (<0.02) and RMSE as a percent of the mean only slightly decreased ($<1.0\%$) as presented in Table 5. Even when quadratic effects were significant, curvilinear change was introduced only at high levels of duodenal flow (Figure 3a). If more restrictive elimination of samples had been employed (e.g., ± 2.5 SD),

Table 8. Relationships between plasma AA concentration (μM) and percent of MP excreted as MPY (MTP_MP)

p[AA]	adjR ²	Mean ¹	Model	RMSE ²	RMSE, % mean ³	Equation
Arg	NS ⁴	80.8	Linear			
	NS		Quadratic			
His	NS	45.3	Linear			
	NS		Quadratic			
Ile	0.757	111.8	Linear	14.5	13.0	$167.90 - (1.31 \times \text{MTP_MP})^5$
	0.763		Quadratic	14.3	12.8	$208.10 - (2.99 \times \text{MTP_MP}) + (0.017 \times \text{MTP_MPsqr})^6$
Leu	0.806	147.6	Linear	24.8	16.8	$260.41 - (2.60 \times \text{MTP_MP})$
	0.821		Quadratic	23.9	16.2	$380.62 - (7.56 \times \text{MTP_MP}) + (0.048 \times \text{MTP_MPsqr})$
Lys	0.765	79.1	Linear	11.3	14.3	$91.80 - (0.31 \times \text{MTP_MP})$
	NS		Quadratic			
Met, protein ⁷	0.873	21.3	Linear	3.4	16.0	$32.80 - (0.27 \times \text{MTP_MP})$
	0.877		Quadratic	3.4	15.8	$42.18 - (0.65 \times \text{MTP_MP}) + (0.004 \times \text{MTP_MPsqr})$
Met, DL-Met ⁸	NS	35.2	Linear			
	NS		Quadratic			
Phe	0.852	47.9	Linear	5.5	11.5	$68.17 - (0.47 \times \text{MTP_MP})$
	0.854		Quadratic	5.5	11.5	$82.79 - (1.08 \times \text{MTP_MP}) + (0.006 \times \text{MTP_MPsqr})$
Thr	0.901	96.5	Linear	11.2	11.6	$118.96 - (0.55 \times \text{MTP_MP})$
	0.905		Quadratic	11	11.4	$79.00 + (1.09 \times \text{MTP_MP}) - (0.016 \times \text{MTP_MPsqr})$
Val	0.813	234.5	Linear	33.9	14.5	$391.10 - (3.66 \times \text{MTP_MP})$
	0.822		Quadratic	33.1	14.1	$532.10 - (9.52 \times \text{MTP_MP}) + (0.058 \times \text{MTP_MPsqr})$

¹Mean = mean plasma AA concentration (μM).

²RMSE = root mean square error.

³RMSE, % mean = root mean square error as a percent of the mean.

⁴NS = no significant equation or quadratic term not significant.

⁵MTP_MP = milk protein yield (MPY) as a percent of MP.

⁶MTP_MP_{sqr} = MTP_MP $\times$ MTP_MP.

⁷Met, protein = Met derived from intact dietary protein.

⁸Met, DL-Met = Met derived from DL-Met sources.

Table 9. Relationships between plasma AA concentration (μM) and microbial digestible CP as a percent of MP (MDP_MP¹)

p[AA]	adjR ²	Mean ¹	Model	RMSE ²	RMSE, % mean ³	Equation
Arg	NS	80.5	Linear			
	NS		Quadratic			
His	0.793	45.3	Linear	7.8	17.2	$73.37 - (0.41 \times \text{MDP_MP})^5$
	0.801	45.3	Quadratic	7.6		$-7.06 + (2.19 \times \text{MDP_MP}) - (0.021 \times \text{MDP_MPsqr})^6$
Ile	0.754	111.8	Linear	14.6	13.1	$188.83 - (1.11 \times \text{MDP_MP})$
	NS		Quadratic			
Leu	0.831	147.6	Linear	23.2	15.7	$344.07 - (2.87 \times \text{MDP_MP})$
	NS		Quadratic			
p[Lys] μM	0.766	79.1	Linear	11.2	14.1	$94.50 - (0.23 \times \text{MDP_MP})$
	NS		Quadratic			
p[Met] μM , Protein ⁷	0.863	21.4	Linear	3.6	16.8	$34.17 - (0.18 \times \text{MDP_MP})$
	NS		Quadratic			
p[Met] μM , DL-Met ⁸	NS	34.1	Linear			
	NS		Quadratic			
p[Phe] μM	0.843	47.9	Linear	5.7	11.9	$75.74 - (0.41 \times \text{MDP_MP})$
	NS		Quadratic			
p[Thr] μM	NS	96.5	Linear			
	NS		Quadratic			
p[Val] μM	0.824	234.5	Linear	32.9	14.0	$480.16 - (3.61 \times \text{MDP_MP})$
	NS		Quadratic			

¹Mean = mean plasma AA concentration (μM).²RMSE = root mean square error.³RMSE, % Mean = root mean square error as a percent of the mean.⁴NS = no significant equation or quadratic term not significant.⁵MDP_MP = microbial digestible protein as a percent of MP.⁶MDP_MP_{sqr} = MDP_MP $\times$ MDP_MP.⁷Met, protein = Met derived from intact dietary protein.⁸Met, DL = Met derived from DL-Met sources.

Figure 1. Regression plot of association of percent milk true protein output as a percent of MP (MTP_MP) with plasma concentration of AA ($p[EAA]$, μM) over the range of observed data.

it is doubtful that curvilinear effects would have been detected. The plateau of $p[Ile]$ occurs at $128.9 \mu M$ with a $Ileduo$ of 204 g (Figure 3a), whereas $p[Thr]$ peaks at $126.2 \mu M$ with 199 g of $Thrduo$, and $p[Val]$ at $288.1 \mu M$ with 250 g of $Valduo$. The other EAA appear linear

over the range of duodenal flow, including Lys, which has a significant quadratic term. Similarly, $p[Met]$ from protein sources plateaued at $23 \mu M$ with 70 g of $Metduo$ (Figure 3b). This plateau appears to mean little as plasma levels from DL-Met sources were considerably

Figure 2. Regression plot of association between increasing microbial digestible protein as a percent of total MP and plasma concentration of AA ($p[EAA]$, μM) over the range of observed data.

Table 10. Mixed term equations relating plasma concentrations of EAA (μM) to duodenal flow of AA (EAduo) with cow or ration factors, or both, with highest adjR² and lowest root mean square error (RMSE) for developmental database

p[AA], μM	adjR ²	Mean ¹	RMSE	RMSE, % mean ²	Equation
Arg		No improvement possible			
His	0.815	45.3	7.5	16.6	$-38.83 + (0.31 \times \text{Hisduo}) + (2.51 \times \text{MDP_MP}^3) - (0.02 \times \text{MDP_MPsqr}^4) - (1.36 \times \text{EE}^5)$
Ile	0.802	111.8	13	11.6	$164.82 + (1.24 \times \text{Ileduo}) - (0.003 \times \text{Ileduosqr}^6) - (1.73 \times \text{NDF}^7) - (1.92 \times \text{NFC}^8) + (4.35 \times \text{ash}^9)$ $- (0.48 \times \text{MDP_MP}) - (37.92 \times \text{MPY}^{10})$
Leu	0.837	147.6	23.1	15.6	$227.27 + (0.28 \times \text{Leuduo}) - (2.09 \times \text{MDP_MP})$
Lys	0.858	79.1	8.8	11.1	$-95.60 + (0.124 \times \text{Lysduo}) - (0.002 \times \text{Lysduosqr}) - (0.27 \times \text{MDP_MP}) - (2.05 \times \text{Milk}^{11}) + (3.36$ $\times \text{MTP_MP}^{12}) - (0.022 \times \text{MTP_MPsqr}^{13})$
Met, protein ¹⁴	0.89	21.4	3.2	15	$3.51 + (1.02 \times \text{Metduo}) - (0.006 \times \text{Metduosqr}) - (1.90 \times \text{MPY})$
Met, DL-Met ¹⁵	0.928	34.8	4.7	13.5	$-74.74 + (0.52 \times \text{Metduo}) - (0.84 \times \text{Milk}) + (1.48 \times \text{NDF}) + (1.33 \times \text{NFC})$
Phe	0.859	47.9	5.4	11.3	$56.58 + (0.15 \times \text{Pheduo}) - (0.22 \times \text{MDP_MP}) - (15.44 \times \text{MPY})$
Thr	0.917	96.5	10.3	10.7	$-75.40 + (1.16 \times \text{Thrduo}) - (0.003 \times \text{Thrduosqr}) + (0.57 \times \text{NFC}) + (1.73 \times \text{MTP_MP}) - (0.015$ $\times \text{MTP_MPsqr})$
Val	0.864	234.5	29.3	12.5	$370.27 + (3.41 \times \text{Valduo}) - (0.007 \times \text{Valduosqr}) - (4.07 \times \text{NDF}) - (3.95 \times \text{NFC}) - (3.22 \times \text{milk})$

¹Mean = mean plasma AA concentration (μM).²RMSE, % Mean = root mean square error as a percent of the mean.³MDP_MP = microbial digestible protein as a percent of MP (g/g).⁴MDP_MPsqr = MDP_MP $\times$ MDP_MP.⁵Ether extract, % of DM.⁶EAAduosqr = EAAduo $\times$ EAAduo.⁷NDF = NDF, % of DM.⁸NFC = NFC, % of DM.⁹Ash = ash, % of DM.¹⁰MPY = milk true protein yield, kg/d.¹¹Milk = milk yield, kg/d.¹²MTP_MP = milk true protein yield as a % of MP (g/g).¹³MTP_MPsqr = MTP_MP $\times$ MTP_MP.¹⁴Met, protein = Met derived from intact dietary protein.¹⁵Met, DL = Met derived from DL-Met sources.

Table 11. Predicted versus observed values for plasma essential AA concentration for equations from the developmental database against data from the validation database

p[AA], μM	Model	Concentration, μM	SE	adjR ²	RMSE	RMSE, % observed mean	Intercept	Slope	Error resolution, %		
									Mean	Regression	Random
Arg	Observed	71.0	17.3								
	Duo Linear ¹	75.7	5.8	0.830	7.2	10.1	−36.32	1.48	6.3	9.5	84.2
	Duo Quadratic ²			NS ³							
	AA_MP Linear ⁴	76.9	4.7	0.808	8.8	12.4	97.90	−0.29	10.1	5.0	84.9
	AA_MP Quadratic ⁵	77.6	2.7	0.808	8.8		125.37	−0.64	12.5	1.9	85.6
His	Mixed term ⁶			NS							
	Observed	49.4	17.7								
	Duo Linear	45.6	5.9	0.845	8.1	16.4	−22.46	0.16	5.6	2.8	91.6
	Duo Quadratic			NS							
	AA_MP Linear	46.2	4.8	0.803	9.1	18.4	−21.17	1.57	4.1	8.4	87.5
Ile	AA_MP Quadratic			NS							
	Mixed term	51.8	6.0	0.829	8.7	17.6	3.50	0.56	2.2	1.0	96.8
	Observed	115.4	27.3								
	Duo Linear	111.9	10.5	0.820	13.7	11.9	0.12	1.07	2.9	0.4	96.7
	Duo Quadratic	109.6	12.3	0.816	13.8	11.9	23.70	0.86	4.7	1.5	93.8
Leu	AA_MP Linear			NS							
	AA_MP Quadratic			NS							
	Mixed term	107.2	17.0	0.810	14.0	12.1	61.82	0.53	9.3	4.5	86.2
	Observed	146.8	48.1								
	Duo Linear	148.4	25.1	0.856	20.9	14.2	−22.95	1.15	0.2	6.9	92.9
Lys	Duo Quadratic			NS							
	AA_MP Linear	150.2	4.7	0.829	23.0	15.7	−77.20	1.50	0.5	7.0	92.5
	AA_MP Quadratic			NS							
	Mixed term	150.8	30.7	0.837	20.5	14.0	28.14	0.79	1.6	2.8	95.6
	Observed	84.3	18.7								
Met-protein	Duo Linear	79.3	9.1	0.796	9.8	11.6	−1.01	1.09	6.1	10.6	83.3
	Duo Quadratic	89.0	13.2	0.790	9.95	11.7	18.93	0.76	4.8	22.1	73.2
	AA_MP Linear	80.5	5.8	0.672	12.2	14.5	65.61	0.25	3.4	10.5	86.1
	AA_MP Quadratic	81.0	8.4	0.671	12.2	14.5	72.69	0.16	2.3	18.1	79.6
	Mixed term	64.4	4.6	0.711	11.5	13.6	7.88	1.22	54.6	0.0	45.4
Met-DL	Observed	24.0	7.4								
	Duo Linear	24.8	5.2	0.918	2.54	10.6	9.60	0.59	1.0	0.21	78.0
	Duo Quadratic	21.3	2.9	0.915	2.59	10.8	6.47	0.53	12.3	2.2	85.5
	AA_MP Linear	22.7	3.9	0.908	2.70	11.3	12.28	0.53	2.5	13.9	83.6
	AA_MP Quadratic	22.9	4.3	0.911	2.65	11.0	11.66	0.55	1.9	17.0	81.1
Phe	Mixed term	19.2	3.8	0.915	2.58	10.8	12.52	0.61	69.7	1.9	28.4
	Observed	32.1	18.8								
	Duo Linear	35.1	3.0	0.827	5.8	17.9	−16.66	1.31	6.9	7.0	86.1
	Duo Quadratic			NS							
	AA_MP Linear	32.7	3.4	0.913	4.2	13.1	−41.98	2.21	0.4	5.2	94.4
	AA_MP Quadratic	33.2	4.8	0.915	4.2	13.1	−22.42	1.58	1.4	0.4	98.2
	Mixed term	34.5	5.2	0.817	5.8	17.9	−12.91	1.25	6.0	0.0	94
	Observed	48.4	10.2								
	Duo Linear	47.6	4.3	0.885	4.01	8.3	45.68	0.06	0.6	7.4	92.0
	Duo Quadratic			NS							
	AA_MP Linear	48.2	3.0	0.886	4.02	8.4	45.90	0.06	0.0	2.1	98.0
	AA_MP Quadratic			NS							
	Mixed term	48.9	4.2	0.885	4.04	8.3	42.15	0.14	0.3	4.9	94.8

Continued

Table 11 (Continued). Predicted versus observed values for plasma essential AA concentration for equations from the developmental database against data from the validation database

p[AA], μM	Model	Concentration, μM			adjR ²	RMSE	RMSE, % observed mean	Error resolution, %			
		SE	Intercept	Slope				Mean	Regression	Random	
Thr	Observed	100.3	21.8								
	Duo Linear	95.0	7.8		0.860	9.5	9.5	4.9	8.6	86.5	
	Duo Quadratic	104.8	12.2		0.859	9.6	9.5	3.2	21.9	74.9	
	AA_MP Linear	95.0	6.0		0.856	9.7	9.6	5.8	1.4	92.8	
Val	AA_MP Quadratic				NS						
	Mixed term	89.8	11.3		0.856	9.7	9.7	14.8	24.0	61.2	
	Observed	233.3	63.5								
	Duo Linear	232.0	38.5		0.855	28.1	12.1	0.1	1.8	98.1	
	Duo Quadratic	223.0	38.3		0.854	28.2	12.1	3.4	1.2	95.4	
	AA_MP Linear	235.7	13.4		0.787	34.1	14.6	0.1	18.9	81.0	
	AA_MP Quadratic	239.0	8.7		0.782	34.3	14.7	0.8	12.5	86.5	
	Mixed term	234.9	45.7		0.848	28.7	12.3	0.1	3.9	96.0	

¹Linear equation of duodenal AA flow (AAduo).²Quadratic equation of duodenal AA flow (AA_MP and AA_MP²).³NS = no significant equation or quadratic term not significant.⁴Linear equation of duodenal AA expressed as % of MP (AA_MP).⁵Quadratic equation of duodenal AA expressed as % of MP (AA_MP and AA_MP²).⁶Equation derived from inclusion of all significant AAduo, ration, and cow traits.

higher. Interestingly, almost all the studies that were eliminated were either infusion or high doses of RMPet, suggesting that it is possible to obtain unphysiological plasma concentrations under these experimental conditions. At least most the high concentrations were probably achieved through accumulation of D-Met in the plasma, as D-Met has a half-life more than 6 times longer than L-Met (Lapierre et al., 2012a). Additionally, even after these studies were removed, upper limit to the concentration of p[Met] from DL-Met sources, at least under the conditions of the studies included in the database, appeared to be quite high. Overall, as EAAduo increased, the p[EAA] concentration of corresponding EAA increased. However, a biological minimum limit of p[EAA] does seem to exist that the cow maintains under restricted supply as indicated by the intercept term in the linear equations.

Circulating AA concentrations are the result of EAAduo, digestibility, and EAA utilization. Removal of EAA increases with increased supply, either through increased hepatic removal (Raggio et al., 2004), peripheral tissue (as muscle) removal (Dalbach et al., 2011), and removal by the mammary gland (Raggio et al., 2006). Additionally, removal of EAA from plasma may also be the result of a greater use of plasma EAA for non-EAA production for milk protein in both dairy cattle (Lapierre et al., 2012b) and swine (Trottier et al., 1997). So, even expressing supply relative to milk true protein output, thereby expressing a relative excess over the major protein output, did not improve the prediction of p[EAA] (Table 8). All of these observations tend to suggest a rather tight control of p[EAA], increasing steadily with increased amount of EAA reaching the duodenum.

The decreasing p[EAA] with increasing percentage of MDP may suggest that the EAA content of microbial protein is not as well balanced as has been suggested (Patton, 2009). Alternatively, it may be that the AA content of microbial protein or an 80% digestibility for AA of microbial origin as used in the AminoCow model (Evonik Industries, 2012) may not accurately reflect the true availability of these AA. However, the fact that MDP_MP was found to be negatively associated with p[EAA] in several mixed equations suggests that increasing MDP_MP does indeed lead to lower p[EAA]. Even with this potential deficiency, the finding of a curvilinear relationship of p[His] to increasing MCP does suggest that the His may become more deeply deficient than other EAA, but only if MCP represents >70% of total MP. Also interesting is the relatively constant level of p[Lys] as MCP increases, which seems to confirm the proposed relatively high content of Lys in MCP (Clark et al., 1992).

Table 12. Effect of addition of individual EAA to duodenal flow on production variables and plasma EAA concentration

Variable	Met (n = 34)				Lys (n = 11)				His (n = 8)				Met + Lys (n = 41)				Casein (n = 21)			
	Control	Treated	SE	P	Control	Treated	SE	P	Control	Treated	SE	P	Control	Treated	SE	P	Control	Treated	SE	P
Production																				
DMI, kg/d	19.8	20	0.7	0.39	19.2	19.2	1.2	0.88	17.9	18.2	1.4	0.06	23.5	23.5	1.4	0.77	17.4	17.6	1.0	0.60
Milk, kg/d	31.6	31.5	1.4	0.76	28.6	29.0	2.5	0.35	26.2	27.6	2.47	<0.001	25.6	25.8	2.5	0.17	24.6	25.5	2.1	0.16
Milk fat, %	3.61	3.7	0.11	0.13	3.90	3.84	0.14	0.56	4.39	4.04	0.22	0.12	3.48	3.5	0.12	0.39	3.98	3.90	0.25	0.24
Milk True Prot, ¹ %	2.81	2.90	0.05	0.07	2.90	2.97	0.070	0.22	3.08	3.12	0.120	0.20	2.64	2.71	0.19	0.024	3.23	3.30	0.12	0.04
Milk True Prot, kg	0.894	0.912	0.04	0.07	0.934	0.862	0.078	0.18	0.774	0.835	0.086	0.02	0.926	0.949	0.04	0.12	0.738	0.785	0.06	0.02
MP, g/d	2055	2094	74	0.053	2090	2099	156	0.69	1778	1815	141	0.10	2134	2145	934	0.68	1809	2128	144	<0.001
Duodenal																				
Arg, g/d	125	126	6	0.28	124	123	12	0.82	103	104	8	0.13	118	118	8.0	0.90	114	129	10	<0.001
His, g/d	59	59	3	0.45	56	57	5	0.54	50	59	5	<0.01	72	73	7.0	0.68	52	64	5	<0.001
Ile, g/d	133	134	5	0.14	137	136	10	0.38	117	119	8	0.59	155	152	9.0	0.52	122	141	10	<0.001
Leu, g/d	217	220	8	0.22	223	218	18	0.11	188	193	16	0.09	231	235	14.0	0.17	198	230	18	<0.001
Lys, g/d	174	178	7	0.14	165	189	11	0.003	163	165	16	0.62	151	169	12.0	<0.001	151	183	12	<0.001
Met, g/d	49	66	2	<0.001	53	53	5	0.83	53	54	8	0.09	68	75	7.0	<0.001	43	55	4	<0.001
Phe, g/d	133	135	5	0.21	139	137	12	0.39	114	116	8	0.12	140	139	6.0	0.64	118	139	9	<0.001
Thr, g/d	129	130	5	0.24	130	129	9.0	0.72	112	114	7	0.13	120	120	10.0	0.94	115	133	8	<0.001
Val, g/d	148	150	5	0.16	152	151	10	0.80	130	133	9	0.20	139	139	13.0	0.90	134	159	12	<0.001
Plasma (AA)																				
Arg, μM	84.7	85.3	8.6	0.76	73.7	78.3	6.7	0.23	74.0	74.7	5.7	0.85	81.1	83.8	8.3	0.20	75.1	81.5	4.2	0.11
His, μM	46.1	41.2	3.2	<0.001	43.9	37.9	5.3	0.004	20.5	50.0	6.0	0.01	52.2	49.6	4.4	0.18	35.90	45.80	3.2	<0.001
Ile, μM	116.0	110.3	4.3	0.03	123.8	132.6	12.3	0.52	106.9	111.8	8.8	0.16	111.2	112.70	4.2	0.68	105.90	131.7	9.5	<0.001
Leu, μM	161.5	149.1	11.6	<0.001	149.9	137.6	21.5	0.32	123.0	113.9	1.8	0.13	133.1	142.5	11.7	0.22	127.3	170.5	15.8	<0.001
Lys, μM	81.4	80.2	5.2	0.41	63.5	93.1	8.4	0.002	74.0	79.7	5.3	0.15	64.8	77.9	6.2	<0.001	67.9	89.1	5.8	<0.001
Met, μM	24.4	34.2	2.9	<0.001	26.1	23.6	4.2	0.42	34.1	33.9	10.3	0.86	28.4	34.9	2.7	0.003	19.6	22.6	1.2	<0.001
Phe, μM	51.3	48.8	2.4	0.01	53.6	50.1	6.4	0.06	47.6	44.4	6.6	0.20	54.4	53.2	3.1	0.38	47.3	52.1	4.7	0.018
Thr, μM	94.6	90.3	6.4	0.04	115.7	110.5	14.2	0.22	104.2	114.9	6.2	0.13	107.7	104.8	8.6	0.20	93.9	10.3	7.2	<0.001
Val, μM	239.3	222.8	15.7	<0.001	227.5	243.1	27.7	0.13	217.6	215.7	31.2	0.78	196.2	194.2	21.4	0.62	212.5	280.1	24.8	<0.001

¹Milk true protein = milk CP – NPN.

Figure 3. (a) Regression plot of association of duodenal flow of major quantities of EAA with plasma AA concentration (p[EAA], μM) over the range of observed data. (b) Regression plot of association of duodenal flow of His, and Met from 2 sources with plasma AA concentration (p[EAA], μM) over the range of observed data.

Little evidence exists that breed or stage of lactation has an influence on p[EAA], although breed effects on milk protein percent are well known (NRC, 2001) and cows are in negative protein balance early in lactation (Doepel et al., 2002). This suggests EAA-sparing mechanisms, EAA mobilization, or both can buffer the effect of reduced duodenal supply on p[EAA]. Also, DMI, milk production, milk protein yield, and energy intake had very little effect on p[EAA] once AAduo was accounted for. Prior studies have associated DMI with increased milk yield, whereas increased milk production and energy intake have been associated with increases in milk protein yield (Hristov et al., 2004). However,

these effects do not seem to be reflected in p[EAA]. Using developmental equations to predict p[EAA] in the validation database gave consistent agreement with the observed values, as well as with the $\text{adj}R^2$ and RMSE from the developmental database (Table 11). In all cases these equations were useful for predicting p[EAA] if not with great precision. Although expressing duodenal flow as a percent of MP appeared to slightly improve association between duodenal flow and p[Arg], p[Leu], and p[Lys] in the development database, adding these terms to the equations actually lowered both the accuracy and precision of predicting p[EAA] compared with the EAAduo (Table 11) for all EAA in the validation

database. Thus, for predicting p[EAA], use of EAAduo is a better alternative than using EAA_MP.

The fact that MTP_MP was significant only for Lys and Phe in the mixed equation suggests that p[EAA] may be more reflective of duodenal supply than for milk protein output. Further, other than for Leu and Val (Figure 3a), p[EAA] appear to be rather tightly held over a rather wide range of duodenal flow as observed in this database. The observation that p[Val] displayed a slope >1 in relation to Valduo, but a slope decrease of 3.6 as MTP_MP increased is intriguing, although it remains unexplained. Several lines of reasoning, including the intercept of the linear EAAduo equations and the nadir of the branched-chain AA with increasing MTP_MP, leads to the inevitable conclusion that each p[EAA] is regulated in a unique manner specific to that EAA or that group of AA. Indeed, recent studies have shown that an imbalance in one branched-chain AA can affect the concentrations of the other branched-chain AA (Doelman et al., 2014), perhaps because they share the same dehydrogenase for their catabolism.

The response of single EAA addition on the plasma concentrations of other EAA is of interest. When His or Met+Lys was added singly, an increase in plasma concentration of only the added EAA was observed. But addition of either Lys or Met alone caused significant changes in several p[EAA]. Moreover, Met appeared to be much more potent in this respect, causing a decrease in the concentration of all EAA except Arg and Lys, whereas addition of Lys only decreased only His and Phe. When infusing His, Lys, or Met directly into the venous plasma supply, Fisher (1972) found similar changes. The predicted flow of Met and Lys were slightly greater in the single addition than in the addition of Lys + Met (24 vs. 18 g of Lys and 17 vs. 7 g of Met for single addition vs. Lys + Met addition). This suggests the possibility that quantity of the addition might have an effect on the p[EAA] response. However, when tested across the whole database, no association was found between flows of Lys and Met, expressed either as duodenal flow or as percent of MP (data not shown) and p[EAA]. This suggests that factors beyond the influence of the amount Lys or Met influenced the plasma response to increases in these EAA. Other explanations could be that mass of Met or Lys added to the duodenal flow prevented the absorption of other AA (Baumrucker, 1985). It could also be that the Lys or Met stimulated MPY, which in turn demanded more EAA from plasma. It could also be that Lys or Met increased tissue synthesis requiring more EAA from plasma. In any case, this observation indicates that His has the potential to become limiting when limitation of Met or Lys supply is relieved. Although the lactating

goat appears to have a great ability to increase the efficiency of His use in support of lactation, a supply shortage still negatively affected milk protein yield (Bequette et al., 2000).

Interestingly, when both Lys and Met are added in the single addition database, the only effects appeared as increments in p[Lys] and p[Met]. Although it appears that EAAduo can cause changes in the plasma concentrations of several EAA, these changes do not seem to be consistent. These observations taken together suggest the existence of an optimum balance of duodenal AA and that this optimum balance is close to the EAA distribution of casein. However, the optimum EAA balance and amount relative to needs cannot be determined with plasma data.

At first it appears that calculated minimum p[EAA] (as indicated by the intercept term of linear equations for association of EAAduo with p[EAA]) and the calculated maximum could provide estimates of minimum and maximum EAA calculations. However, estimates of p[EAA] minimums and maximums are not consistent. For example, p[Thr] minimum was estimated as 55.1 μM and the maximum at 126.2 μM using the association between EAAduo and p[EAA], but the minimum was 79.0 μM and the maximum 97.6 μM when associating MPY_MP with p[EAA], both taken from the same database. This appears to be further evidence that EAA requirements cannot be described from plasma concentrations.

The results of additions of Hisduo are most interesting in comparison with the other EAAduo. Flows of all EAAduo except Ileduo were increased when Hisduo was increased. This was due to an increase in DMI and resulted in more milk and milk protein, but no increase in milk protein percent. It is not known why Hisduo should cause an increase in DMI in lactating cows when it is known that His is a potent inhibitor of feed intake in rats either through formation of histamine (Kasaoka et al., 2004) or through conversion to histidine peptides (Olszewski et al., 2003). Increased Hisduo through rumen protected His (Lee et al., 2012; Giallongo et al., 2014) or through abomasal infusion (Lapierre et al., 2014; Ouellet et al., 2014) increased DMI, whereas both Weekes et al. (2006) and Stahel et al. (2014) reported a drop in DMI when His was deficient. With only 8 comparisons in the present study, it will take more controlled experiments, preferably with other ruminants and nonlactating cattle, to ferret out whether Hisduo causes an increase in feed consumption. No matter how these increases in duodenal flow came about, they resulted only in higher p[His] as other p[EAA] were unchanged due to His addition. This may be due to an unchanged partial efficiency of AA use associated with

increased DMI and MPY relative to a relatively large supply of His.

Dramatic changes in p[EAA] reported in monogastric species (Forslund et al., 2000; Burke et al., 2012) do not appear in this database for dairy cattle. This may be because of the more or less constant flow of nutrients supplied to the ruminant (Nocek and Braund, 1985) and the relatively small range of dietary protein tested in dairy rations generally and in the current database (Table 3). Why primiparous cows have a greater p[Arg] than older cows is unclear. It might be supposed that because primiparous cows have significantly less milk production, and there appears to be a high demand for Arg for milk (Lapierre et al., 2012b), this may only reflect the relative demand for Arg. However, it is not clear why other EAA did not show similar trends. Demand of EAA requirement for growth and a correspondingly lower DMI and microbial synthesis would seem to exacerbate the EAA deficiencies. Although the lack of breed effects on p[EAA] might be expected, the lack of DIM effects are puzzling. The range of milk production and feed intake across all stages of lactation, as well as the use of study as a random effect, may have combined to mask any association with DIM. In the relatively large number of studies with cows in early lactation, a time when cows are known to be in negative energy and protein balance (Doepel et al., 2002), it was expected to find that p[EAA] were diminished in early lactation. This was not observed. Even when DIM was the only term in the model besides study as random, DIM never had a significant effect on any p[EAA]. This observation confirms that of Foldager et al. (1980) who found no effect on plasma AA due to stage of lactation, although differences have been found between lactating and dry cows (Doepel et al., 2009) as well as transition cows (Meijer et al., 1995). The study of Meijer et al. (1995) also leads to the conclusion that as lactation progresses, the p[EAA] are rather tightly held in spite of initially increasing and then decreasing DMI. In humans p[EAA] are tightly held, particularly as the dietary flow diminishes (Milsom et al., 1979); this may be similar in lactating cattle.

Overall, explanations for the changes in p[EAA] beyond EAAduo are difficult and must remain speculative. Although some of the EAA appear to be used as energy sources by gut tissue (Raggio et al., 2004) and use of AA for gluconeogenesis is high in some metabolic states (Bell, 1995), use of AA for gluconeogenesis is ordinarily low depending on the balance between somatotropin and insulin (Lobley, 1992).

Because p[EAA] did not indicate EAA requirements, nor were changes in p[EAA] evident for cow factors and ration components, differences observed for breed and stage of lactation effects on milk protein yield observed

in other studies might be reflected either in plasma flow through the mammary gland or in differential extraction of individual p[EAA].

CONCLUSIONS

This meta-analysis study indicates that p[EAA] can most efficiently be modeled as linear functions of AAduo. Further, no evidence was found of break points or that p[EAA] is in any way related to EAA requirements for lactation, at least under the conditions of studies used in the current study. Additions of Lys and Met alone, but not together, perturb p[EAA]. Breed differences and stage of lactation effects on milk protein production appear related to either EAA extraction or plasma flow through the udder as they do not appear to be related to p[EAA].

ACKNOWLEDGMENTS

The authors thank J. R. Patton (NittanyCow Software Services, Mifflinburg, PA) for help with editing the manuscript as well as Evonik AG for partial funding.

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Appendix A: Studies Used in the Developmental Data Set (Table 1).

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Appendix B: Studies Used in the Validation Data Set (Table 2).

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