Post-ruminal supplies of glucose and casein, but not acetate, stimulate milk protein synthesis in dairy cows through differential effects on mammary metabolism

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ABSTRACT

Amino acids and glucose have been shown to regulate protein synthesis in the mammary gland through their effects on cellular signaling pathways. Acetate might also have an effect on protein synthesis via the AMP-activated kinase signaling pathway, because it is the main energy source for the mammary secretory cell. Thus, the objective of this experiment was to evaluate the effects of casein and energy-yielding nutrients (acetate and glucose), and their combination, on performance and mammary metabolism. Six multiparous Holstein cows, averaging 49 kg of milk/d, were used in a 6 × 6 Latin square design with 14-d periods. Cows were fed to 100% National Research Council requirements for metabolizable protein (MP) and energy (ME) for 9 d, after which they were feed-restricted for 5 d to 85% of their individual ad libitum intake and then abomasally infused with 1 of 6 treatments. Treatments were acetate (A), glucose (G), each at 5% of ad libitum ME intake, casein (C) at 15% of ad libitum MP intake, A + C, G + C, or a saline solution (negative control). Casein infused alone increased milk protein yield numerically, with 25% recovery of the infused casein in milk protein. Glucose infused alone increased milk and milk protein yield and promoted the highest efficiency of nitrogen utilization (37%), with an efficiency of MP use for milk protein of 58%. We discovered no effect of treatment on mammary plasma flow, and the increase in milk protein yield with glucose infusion was brought about by greater mammary AA clearance rate. Infusion of casein and glucose together further increased milk protein yield in an additive fashion, and 47% of the infused casein was recovered in milk protein. Acetate infused alone had no effect on milk protein yield but increased milk fat yield numerically, suggesting that

the greater amount of acetate taken up by the mammary gland was used for milk fat synthesis. Infusion of acetate and casein together yielded responses similar to those of casein alone. In conclusion, glucose has a major effect on stimulating milk protein synthesis, and the mammary gland has the ability to increase its supply of nutrients to match its synthetic capacity.

Key words: amino acids, abomasal infusions, energy, nitrogen efficiency

INTRODUCTION

Milk protein synthesis is stimulated by both glucose and EAA supplies to the mammary gland (Hanigan et al., 1998a; Lemosquet et al., 2010). The effect of energy from dietary carbohydrates on milk protein yield is typically explained by increased EAA supply from increased microbial protein outflow due to higher availability of fermentable carbohydrates (Broderick, 2003). However, intravenous infusion of insulin (Mackle et al., 1999) and post-ruminal provision of glucose (Rulquin et al., 2004) and starch (Rius et al., 2010a) have also been found to increase milk protein yield, suggesting a direct effect of carbohydrates on stimulating milk protein synthesis.

The mechanisms by which milk protein synthesis is affected by post-absorptive supplies of both protein and energy from carbohydrates include substrate and regulatory effects at the tissue level. Most current nutritional models approach the relationship between substrates and products within the mammary gland as fixed and proportional. In other words, the models predict component synthesis based on the supply of their main substrate. However, this relationship is not so simple and straightforward. The supply of AA in the abomasum or duodenum has been found to increase lactose yield (Lemosquet et al., 2009a; Doepel and Lapierre, 2010; Galindo et al., 2011), and the supply of glucose post-ruinally or propionate in the rumen

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increase milk protein yield (Vik-Mo et al., 1974; Rigout et al., 2003; Rulquin et al., 2004; Raggio et al., 2006; Toerien et al., 2010). Substrates act as building blocks for their respective milk components but can also function as energy providers upon oxidation or contribute to the pool of carbon used to synthesize other milk components.

Nutrients can also function, directly or indirectly, through hormones, as signaling molecules altering transduction pathways that regulate the rates of protein synthesis. It has been demonstrated in vitro that EAA, glucose, and insulin can stimulate milk protein synthesis independently (Appuhamy et al., 2011, 2012; Arriola Apelo et al., 2014; Yoder et al., 2020), challenging the limiting nutrient theory incorporated in every nutrient requirement system for lactating dairy cows. Substrate-independent stimulation of milk protein synthesis could increase post-absorptive AA efficiency (Mackle et al., 1999; Castro et al., 2016) and reduce manure N content. These independent responses must be understood so that the most effective and efficient mix of AA and energy supply can be provided.

Although the effect of glucose or glucogenic nutrients on milk protein yield has been the focus of in vivo studies (Lemosquet et al., 2010; Rius et al., 2010b), the effect of acetate specifically on milk protein synthesis has received little attention. Acetate is the main energy source for mammary cells (Hanigan and Baldwin, 1994) and has the potential to stimulate the mammalian target of rapamycin complex 1 (**mTORC1**) signaling through AMP-activated kinase (**AMPK**; Appuhamy et al., 2014). In fact, intravenous infusions of acetate tended to increase milk protein yield in early-lactation goats fed a protein-restricted diet (80% of requirements), compared with a saline infusion (Safayi and Nielsen, 2013). Likewise, Maas et al. (1995) observed a trend for increased milk protein production when acetate was infused arterially. However, others have not observed responses in milk protein yield with ruminal acetate infusions (Shepherd and Combs, 1998).

Therefore, the hypothesis of this study was that milk protein synthesis is stimulated independently by acetate, glucose, and casein. The objectives of the experiment were to evaluate production responses, especially milk protein yield, to the individual nutrients and to their combination, and to link these responses to mammary metabolism.

MATERIALS AND METHODS

Experimental Procedure

Animal care and experimental procedures were approved by the Animal Care and Use Committee of the

College of Agriculture and Life Science of the University of Wisconsin-Madison. Six multiparous Holstein cows fitted with permanent 10-cm rumen cannulas (Bar Diamond Inc., Parma, ID), averaging (mean $\pm$ SD) at the beginning of the trial 133 ± 28 DIM, 49.8 ± 5.6 kg of milk/d, and 715 ± 45 kg of BW were used in a Latin square design with 14-d periods. Cows were housed in tiestalls with free access to water. All cows were injected with bST (500 mg of Posilac; Elanco Animal Health, Greenfield, IN) every other week, on the fifth day of each experimental period. Cows were randomly assigned to 1 of 6 treatment sequences in a 6×6 Latin square balanced for carryover effects. All cows were fed a common diet throughout the trial, and treatments were infused into the abomasum. Each 14-d experimental period included 9 d of feed offered ad libitum, followed by 5 d of restricted intake (85% of ad libitum intake) in combination with the continuous infusions (22 h/d, stopping only for milking). Feed was restricted during the infusion periods to ensure that the infusions did not cause a feed intake reduction and to ensure that the animals consumed most or all of each meal when offered, thus stabilizing absorption patterns. The amount of 15% was chosen because the combination of the energy contained in the infused nutrients provided approximately 10% of the energy supply, and thus feed offered plus infusions represented, at most, 95% of cows' ad libitum intake. In this manner, the treatments examined the response to the nutrients within the normal nutrient supply range rather than on top of the normal supply, as a response may not occur if animals are at their maximum production potential.

The nutrient composition of the main ingredients of the TMR is shown in Table 1, and the dietary ingredient composition and analyzed dietary nutrient composition are shown in Table 2. The diet was formulated to meet 100% of the NRC (2001) ME requirements and 93.5% of the MP requirements. During the first 9 d of each period, cows were fed ad libitum, once daily at about 0800 h, allowing for 5 to 10% orts. During the last 5 d, feed offered to each cow was restricted to 85% of her respective ad libitum intake (averaged from the previous 6 d of the adaptation period) and provided in 6 equal-sized meals at 0000, 0400, 0800, 1200, 1600, and 2000 h, to minimize postprandial variation of blood metabolite concentrations.

The infusion solutions were formulated based on each cow's ad libitum energy and protein intake for each period and consisted of (1) acetate (**A**) infused in an amount equivalent to 5% of ad libitum ME intake; (2) glucose (**G**) infused in an amount equivalent to 5% of ad libitum ME intake; (3) casein (**C**) infused in an amount equivalent to 15% of ad libitum MP intake; (4) A + C (**AC**); (5) G + C (**GC**); and (6) saline solution (**S**)

Table 1. Nutrient composition of principal dietary ingredients¹

Item	AS	CS	WCS	Conc.
DM, %	34.2	39.1	93.3	90.6
CP, % of DM	20.0	5.7	24.3	21.1
NDF, % of DM	49.5	44.5	46.5	14.8
ADF, % of DM	38.3	24.4	31.8	7.8
NDICP, ² % of DM	2.9	0.9	3.3	3.1
ADICP, ³ % of DM	0.93	0.13	1.53	0.65
Ash, % of DM	10.8	4.1	4.6	10.8
AA, % of CP				
Ala	9.6	7.1	3.7	4.9
Arg	1.5	2.2	10.3	6.4
Asp	8.7	6.4	8.7	9.2
Glu	4.1	11.5	17.9	17.6
His	1.2	1.8	2.5	2.6
Ile	3.9	3.2	2.9	4.1
Leu	6.1	8.3	5.4	8.2
Lys	3.4	2.9	4.2	5.4
Met	1.2	1.6	1.4	1.6
Phe	3.8	4.0	5.0	4.7
Pro	4.2	6.4	3.6	6.3
Ser	2.6	3.8	4.1	4.7
Thr	2.6	3.5	3.1	3.9
Val	5.1	4.4	4.1	4.9

¹AS = alfalfa silage; CS = corn silage; WCS = whole cottonseed, with lint; Conc. = concentrate mix containing (DM basis) 49.5% dry ground corn grain, 14.0% solvent-extracted soybean meal, 15.6% canola meal, 4.8% expeller soybean meal, 5.6% soybean hulls, 3.8% energy booster, 2.3% limestone, 0.8% sodium bicarbonate, 0.4% potassium carbonate, 1.0% calcium monophosphate, 0.6% magnesium oxide, 0.4% potassium magnesium sulfate, 0.7% trace mineral premix + selenium, 0.4% vitamin ADE premix.

²NDICP = neutral detergent insoluble CP.

³ADICP = acid detergent insoluble CP.

as a negative control. Metabolizable energy contents of acetate and glucose used in the calculations were 209 and 673 kcal/mol, respectively. The casein source was sodium caseinate, which contained 98.6% casein, considered 95% digestible (Hanigan et al., 2004). Acetate solutions were prepared from equal proportions (molar basis) of sodium acetate and acetic acid. Sodium chloride was used to equalize the Na content (mean of 196 g/d) of all infusion solutions. The mean nutrient composition of the solutions is shown in Table 3. Solutions were prepared daily with warm tap water to the final volume of 12 L/d and continuously infused into the abomasum via the rumen cannula, using a peristaltic pump as described by Gressley et al. (2006). Acetate solutions had a pH of 4.7 and thus had to be infused separately from the casein, using a dual infusion line to avoid casein precipitation in the container and lines.

Amounts of feed offered and refused were recorded daily. Weekly samples of the silages, whole cottonseeds, concentrate mix, and TMR were taken, and DM contents were determined by oven-drying at 60°C for 48 h. The DM values for TMR were used to calculate DMI. Samples of the ingredients from wk 2 of each period were ground to pass a 1-mm screen (Wiley mill, Arthur H. Thomas, Philadelphia, PA) and analyzed for DM

Table 2. Composition of experimental diet

Item	% of DM
Ingredient	
Alfalfa silage	15.0
Corn silage	35.0
Dry ground corn grain	23.7
SSBM ¹	6.7
Canola meal	7.5
SBM, expeller ²	2.3
Soyhulls	2.7
Cottonseed, whole with lint	2.2
Prilled fatty acids ³	1.8
Calcium carbonate	1.1
Sodium bicarbonate	0.40
Potassium carbonate	0.20
Calcium phosphate	0.50
Magnesium oxide	0.27
K and Mg sulfate	0.20
Trace mineral premix + Se ⁴	0.34
Vitamin ADE ⁵	0.20
Nutrient composition	
CP	15.6
NDF	31.1
ADF	18.7
EE ⁶	5.3
NFC ⁷	39.7
Starch ⁸	28.0
Ash	8.3

¹SSBM = solvent-extracted soybean meal.

²Soybean meal; SoyPlus (Landus Cooperative, Ames, IA).

³Energy Booster 100 (Milk Specialties, Eden Prairie, MN).

⁴Provided (per kg of diet DM): 1 mg of Co, 5 mg of Cu, 0.3 mg of I, 12.3 mg of Fe, 0.01 mg of Mg, 0.2 mg of Se, 19 mg of Zn.

⁵Provided (per kg of diet DM): 203,478 IU of vitamin A, 67,826 IU of vitamin D₃, 678 IU of vitamin E.

⁶EE = ether extract, calculated using NRC (2001) table values for % fat of each ingredient.

⁷NFC = 100 - % of NDF - [% of CP - (6.25 × % of NDIN)/100] - % of fat - % of ash.

⁸Calculated from diet formulation, assuming 1.2, 30, 72, 1.5, 1, 1.5, 1.5, and 0% starch for alfalfa silage, corn silage, dry ground corn grain, solvent-extracted soybean meal, canola meal, expeller soybean meal, soybean hulls, and cottonseed whole with lint, respectively.

at 105°C (method 967.03; AOAC, 1990), total N (Leco 2000 N Analyzer; Leco Instruments, Inc., St. Joseph, MI), ash, and OM (AOAC, 1980), and sequentially for NDF, ADF, and ADIN (Van Soest, et al., 1991) using

Table 3. Mean infused nutrients (g/d) for each treatment¹

Item	S	A	G	C	AC	GC
Acetate	0	913	0	0	935	0
Glucose	0	0	817	0	0	830
Casein	0	0	0	404	423	404
NaCl ²	494	48	494	479	21	479

¹S = abomasal infusion of saline solution; A = abomasal infusion of acetate, at a daily rate of 5% of ad libitum ME intake; G = abomasal infusion of glucose, at a daily rate of 5% of ad libitum ME intake; C = abomasal infusion of casein, at a daily rate of 15% of ad libitum MP intake; AC = abomasal infusion of A + C; GC = infusion of G + C.

²Included in the solutions to equalize the Na supply across all treatments (196 g/d).

heat-stable amylase and sodium sulfite (Hintz et al., 1996), and for NDIN omitting α -amylase and Na_2SO_3 during extraction (Licitra et al., 1996).

Cows were milked twice daily at 0530 and 1730 h and milk weights recorded. Milk samples were collected from both a.m. and p.m. milkings on d 8 to 13 of each experimental period, preserved with 2-bromo-2-nitropropane-1,3-diol, and analyzed for fat, true protein, lactose, SNF, and MUN content by infrared spectroscopy (AgSource Laboratory, Verona, WI) using a Foss FT6000 [Foss North America Inc., Eden Prairie, MN; AOAC (1990) method no. 972.16]. Daily milk composition was computed as weighted means based on the a.m. and p.m. samples and milk yields. Net energy for lactation secreted in milk was calculated as described by Tyrrell and Reid (1965):

$$\text{NE}_{\text{L-milk}}, \text{Mcal/d} = \text{milk, kg/d} \\ \times [((0.0929 \times \text{fat, \%}) + (0.0563 \times \text{true protein, \%}) \\ + (0.0395 \times \text{lactose, \%}))].$$

Energy-corrected milk yield (kg/d) was calculated by dividing $\text{NE}_{\text{L-milk}}$ by a constant energy content of milk of 0.749 Mcal/kg (Krause et al., 2003). The efficiency of N use (**ENU**) was calculated from milk true protein:

$$\text{ENU, g/g} = (\text{milk true protein, g/d} \div 6.38) / \\ (\text{N intake} + \text{N infused, g/d}).$$

Blood samples were taken on d 13 of each period into heparinized test tubes, from a coccygeal vessel and the subcutaneous abdominal vein, at approximately 0630 h (immediately after a.m. milking), 1000, 1330, and 1700 h (immediately before p.m. milking). The composition of coccygeal blood was assumed to be equivalent to that of arterial blood (Emery et al., 1965). Blood was refrigerated until centrifugation ($1,500 \times g$, 4°C , 15 min), and the resulting plasma was stored at -20°C until analysis. Samples of arterial plasma were composited by cow within period for plasma urea nitrogen (**PUN**) analysis (Broderick and Clayton, 1997). The remaining analyses were run on individual plasma samples. Plasma was analyzed for glucose (Karkalas, 1985), BHB (kit no. 2440, Stanbio Laboratory, Boerne, TX), and acetate (kit no. EOAC-100, BioAssay Systems, Hayward, CA). Plasma AA concentration was analyzed by isotope dilution (Calder et al., 1999) using a gas chromatograph coupled with a mass spectrometer (Shimadzu Corp, single quadrupole GCMS-QP2010; Kyoto, Japan).

Mammary plasma flow was estimated according to the Fick principle, using Phe + Tyr, assuming a 3.5%

contribution from blood-borne proteins (Cant et al., 1993):

$$\text{Plasma flow (L/h)} = (\text{milk Phe} + \text{Tyr, } \mu\text{mol/h} \\ \times 0.965) \div (\text{arterial Phe} + \text{Tyr} - \text{venous Phe} \\ + \text{Tyr, } \mu\text{mol/L}),$$

where milk Phe + Tyr was the milk protein yield from the sampling period multiplied by the milk AA composition reported by Lapierre et al. (2012). Milk AA composition was not analyzed because we did not expect the treatments to alter it.

Net fluxes of metabolites across the mammary gland (mammary net flux, $\mu\text{mol/h}$) were calculated for each cow within period as follows:

$$\text{Mammary net flux, } \mu\text{mol/h} = \\ (\text{arterial} - \text{venous, } \mu\text{mol/L}) \times \text{plasma flow, L/h},$$

where arterial – venous was the mean metabolite concentration for the sampling period.

Ratios of mammary uptake to milk output were calculated for each AA thus:

$$\text{Uptake:Output, } \mu\text{mol}/\mu\text{mol} = \text{mammary AA uptake,} \\ \mu\text{mol/h} \div (\text{milk AA secretion, } \mu\text{mol/h}).$$

For milk AA, a 3.5% contribution from blood-borne proteins (Cant et al., 1993) was taken into account by multiplying the AA secretion ($\mu\text{mol/h}$) by 0.965.

Clearance rate represents the apparent affinity of the mammary gland for plasma metabolites. These were computed for each metabolite by rearrangement of the equation described by Hanigan et al. (1998b):

$$\text{Clearance rate, L/h} = \text{mammary uptake,} \\ \mu\text{mol/h} \div \text{venous, } \mu\text{mol/L}.$$

For all the AA-related variables, AA were evaluated individually, and the sum of all AA, all EAA, and all NEAA were calculated on a nitrogen basis.

Statistical Analysis

All data were analyzed using the Mixed procedure of SAS 9.4 (SAS Institute Inc., Cary, NC; 2013). The effects of treatment on performance and PUN were assessed for a Latin square design with cow $\times$ period as the experimental unit. The model included the fixed effects of treatment, period, and their interaction, and the random effect of cow. Degrees of freedom were

calculated using the Kenward-Roger option. Interactions were removed from the model when they were not significant ($P > 0.15$), but all main effects were left in the model regardless of significance. One cow went off feed during data collection in period 3, and her data for that period were discarded. Another cow presented with severe clinical mastitis during the last period and was removed from the trial. Both cows were on the AC treatment, leaving 4 observations for the AC treatment and 6 for the other treatments.

The model used for plasma metabolite concentrations (except PUN) included treatment, period, time of collection, and interaction of treatment $\times$ time of collection as fixed effects, and cow as a random effect. The interaction of treatment $\times$ time was not significant ($P > 0.15$) for any variable and was removed from the model. The covariance structure used for the repeated measures was chosen based on the Akaike information criterion. Data were considered outliers and excluded from the data set when a Student's residual higher than 3 or lower than -3 was detected. Least squares means with the highest standard error of the mean are reported for all data. Significance was declared at $P \leq 0.05$, and Tukey-adjusted P -values were used to compare least squares means.

RESULTS

Animal Performance and Plasma Metabolites

Energy, protein, and EAA balances estimated using the NRC model (NRC, 2001) with the observed inputs (treatment means for milk yields, milk composition, BW, and DMI) are presented in Table 4. Despite the 15% feed restriction during collection periods, the estimated deficiencies (amount supplied vs. amount required) vary from 4.6 to 14.6% for energy and from 5.8 to 16.9% for protein, according to the milk production response to each treatment. When energy and protein supplies from infusion solutions were considered, the total supplies of energy (Mcal/d) were 95, 101, 93, 93, 100, and 96% of requirements for treatments S, A, G, C, AC, and GC, respectively. Likewise, total MP supplies (g/d) were 93, 94, 83, 106, 110, and 98% of requirements for treatments S, A, G, C, AC, and GC, respectively. Total digestible EAA supply for the treatments with casein infusion were computed using the actual average digestible casein inputs for each treatment and casein EAA composition as reported by Lapierre et al. (2012). None of the treatments achieved ideal Met content (% of MP) or Lys to Met ratios (2.3% and 3:1, respectively; NRC, 2001).

Performance data are shown in Table 5. Acetate alone did not promote any changes in milk yield, lac-

tose yield and content, or milk protein yield or content relative to the negative control ($P > 0.05$), but increased fat content compared with S, G, C, and GC ($P < 0.05$). Fat content also was greater for AC than for the 2 treatments with glucose infusions ($P < 0.05$). Glucose, infused alone or in combination with casein, increased milk yield by 14% and milk protein yield by 13% relative to S and A ($P < 0.05$); the effects of AC were intermediate and did not differ from the other treatments ($P > 0.05$). Cows receiving C produced less milk than cows infused with G ($P < 0.05$), but we found no difference in milk protein yield between these 2 treatments. Concentration of MUN was greater for AC than for all other treatments ($P < 0.05$) with a 45% increase relative to the lowest value for G. Glucose infusion alone decreased MUN relative to C, AC, and S ($P < 0.05$), which reflected the greater nitrogen utilization efficiency for the G treatment ($P < 0.05$) relative to the other treatments.

Treatment did not affect PUN ($P > 0.10$), although the numeric changes mimicked MUN concentrations for the extreme values, especially the 44% increase for AC relative to G (Table 6). Arterial glucose concentration was increased by C relative to G and S ($P < 0.05$); the other treatments showed intermediate results. Conversely, C reduced BHB arterial concentration compared with A, AC, and S ($P < 0.05$), whereas treatment A increased BHB relative to G and GC ($P < 0.05$; Table 6). Plasma acetate was increased by 34% in the 2 treatments in which acetate was infused (A and AC), relative to the other 4 treatments ($P < 0.05$).

Arterial concentrations of most of the AA were affected by treatment (Table 6). The infusion of GC increased arterial concentration of total AA nitrogen (AA-N) relative to the 3 treatments that did not receive any casein infusion (A, G, and S; $P < 0.05$); C and AC were intermediate but still higher than A ($P < 0.05$). Likewise, arterial concentration of EAA-N was higher for the casein infusions: AC was higher than A, G, and S ($P < 0.05$), whereas C and GC were higher than G ($P < 0.05$). Conversely, arterial concentration of NEAA-N was not elevated for AC, which was smaller than GC ($P < 0.05$) but still higher than A ($P < 0.05$). In fact, A had lower NEAA-N arterial concentration than all the other treatments ($P < 0.05$), except for AC.

Among the individual AA, Lys, His, Met, Pro, and Tyr followed the same pattern of response as total AA, and their arterial concentrations were increased by casein infusions relative to A, G, and S ($P < 0.05$ for all comparisons except C and AC vs. S for His and Met, C vs. S for Lys, C and AC vs. G for Met, and AC vs. S for Tyr). Interestingly, even though arterial concentrations of EAA-N were smallest for glucose in-

Table 4. NRC (2001) estimates of energy, protein, and amino acid supplies and balances based on actual inputs and amounts of nutrients infused¹

Item	S	A	G	C	AC	GC
Dietary MP supply, ¹ g/d	2,292	2,350	2,248	2,294	2,418	2,282
Dietary MP balance, ¹ g/d	-178	-145	-456	-263	-175	-452
Casein infused, g/d	0	0	0	404	423	403
Total MP balance, g/d	-178	-145	-456	141	248	-49
Diet NE _L supply, ¹ Mcal/d	35.6	36.2	35.0	35.5	37.0	35.4
Diet NE _L balance, ¹ Mcal/d	-1.7	-2.8	-6.0	-2.7	-3.3	-4.9
Acetate infused, Mcal/d	0	3.2	0	0	3.3	0
Glucose infused, Mcal/d	0	0	3.1	0	0	3.1
Casein infused, ² Mcal/d	0	0	0	2.0	2.1	2.0
Total NE _L balance, Mcal/d	-1.7	0.4	-2.9	-0.7	2.1	0.2
EAA supply, g/d						
Arg	111	113	109	127	133	126
His	50	51	49	62	65	62
Ile	111	113	109	144	138	132
Leu	198	202	194	237	249	238
Lys	150	153	147	183	192	184
Met	42	43	41	54	57	55
Phe	95	97	93	117	123	118
Thr	111	113	109	129	135	129
Val	126	128	123	150	157	150
Lys, % of MP	6.54	6.51	6.54	6.78	7.74	6.85
Met, % of MP	1.83	1.83	1.82	2.00	2.00	2.05
Lys:Met	3.57	3.56	3.59	3.39	3.87	3.34

¹NRC (2001) estimates using ingredient analyzed nutrient composition and treatment means for DMI, milk yield and composition, and BW. S = abomasal infusion of saline solution; A = abomasal infusion of acetate, at a daily rate of 5% of ad libitum ME intake; G = abomasal infusion of glucose, at a daily rate of 5% of ad libitum ME intake; C = abomasal infusion of casein, at a daily rate of 15% of ad libitum MP intake; AC = abomasal infusion of A + C; GC = infusion of G + C.

²Casein energy value calculated based on 5 kcal/g.

fusions, some NEAA were increased on this treatment to the same level as C and AC (Ala, Asn, and Ser, $P < 0.05$), but Gly arterial concentration was higher for G than for C, AC, and A ($P < 0.05$). Furthermore, glucose infusion alone or in combination with casein increased arterial concentrations of Thr and Gln to the highest levels among the treatments. Acetate infused alone reduced the arterial concentration of most AA, whereas the negative control overall was in between

the 2 energy-only infusions and the 3 treatments that contained casein.

Mammary Metabolism

Mammary plasma flow was not affected by treatment ($P = 0.78$, Table 7). However, clearance rates of glucose and many AA were altered in response to the infusions (Table 7). Glucose clearance rate was greater for A than

Table 5. Effects of abomasal infusions of acetate, glucose, casein, and combinations on cow performance¹

Item	S	A	G	C	AC	GC	SEM	P-value
DMI, ² kg/d	21.4	21.9	21.0	21.4	22.5	21.3	1.5	0.88
Milk yield, kg/d	36.4 ^c	37.1 ^c	42.8 ^a	37.8 ^{bc}	39.2 ^{abc}	42.3 ^{ab}	2.4	0.03
ECM, kg/d	34.4	36.9	39.2	36.7	38.9	38.5	2.5	0.31
Fat, %	3.85 ^{bc}	4.25 ^a	3.66 ^c	3.83 ^{bc}	4.19 ^{ab}	3.56 ^c	0.26	<0.01
Fat yield, kg/d	1.41	1.58	1.55	1.42	1.65	1.51	0.14	0.32
Protein, %	3.12	3.08	3.04	3.16	3.05	3.11	0.08	0.27
Protein yield, kg/d	1.13 ^b	1.14 ^b	1.30 ^a	1.23 ^{ab}	1.21 ^{ab}	1.32 ^a	0.09	0.05
Lactose, %	4.41	4.48	4.50	4.46	4.52	4.46	0.14	0.57
Lactose yield, kg/d	1.60	1.67	1.93	1.68	1.77	1.88	0.11	0.06
MUN, mg/dL	11.4 ^b	11.5 ^b	10.4 ^b	12.3 ^b	15.8 ^a	11.6 ^b	1.1	0.02
ENU, ³ %	32.7 ^b	32.3 ^b	38.8 ^a	32.4 ^b	27.2 ^c	32.9 ^b	2.4	<0.01

^{a-c}Means within a row followed by different letters differ at $P < 0.05$.

¹S = abomasal infusion of saline solution; A = abomasal infusion of acetate, at a daily rate of 5% of ad libitum ME intake; G = abomasal infusion of glucose, at a daily rate of 5% of ad libitum ME intake; C = abomasal infusion of casein, at a daily rate of 15% of ad libitum MP intake; AC = abomasal infusion of A + C; GC = infusion of G + C.

²DMI without accounting for the infusates.

³ENU = efficiency of nitrogen use (g/g): (milk true protein, g/d ÷ 6.38)/(N intake + N infused, g/d).

Table 6. Effects of abomasal infusions of acetate, glucose, casein, and combinations on arterial concentration of urea N (PUN), glucose, acetate, BHB, and AA¹

Item	S	A	G	C	AC	GC	SEM	P-value
PUN, ² mg/dL	9.7	11.1	8.8	11.0	12.7	10.8	1.1	0.21
Glucose, mM	3.24 ^b	3.39 ^{ab}	3.28 ^b	3.56 ^a	3.45 ^{ab}	3.34 ^{ab}	0.11	0.10
Acetate, mM	0.85 ^b	1.08 ^a	0.82 ^b	0.81 ^b	1.12 ^a	0.80 ^b	0.10	0.01
BHB, mM	0.73 ^{abc}	0.81 ^a	0.64 ^{bcd}	0.56 ^d	0.77 ^{ab}	0.63 ^{cd}	0.06	<0.01
AA, μ M								
Ala	222 ^b	217 ^b	233 ^{ab}	235 ^{ab}	233 ^{ab}	255 ^a	17	0.05
Asn	40.9 ^{bc}	36.5 ^c	44.8 ^b	41.4 ^{bc}	40.1 ^{bc}	51.2 ^a	3.6	<0.01
Asp	20.0	19.9	20.1	20.9	17.5	21.1	1.6	0.56
Cys	172	152	165	179	161	173	11	0.16
Gln	251 ^{ab}	209 ^c	279 ^a	269 ^{ab}	235 ^{bc}	277 ^a	17	<0.01
Glu	83.3	86.4	81.5	83.9	90.9	91.3	6.5	0.67
Gly	303 ^{abc}	261 ^c	339 ^a	267 ^{bc}	263 ^{bc}	322 ^{ab}	30	0.06
His	66.6 ^{bc}	56.0 ^{cd}	53.7 ^d	74.1 ^{ab}	73.3 ^{ab}	79.4 ^a	6.6	<0.01
Ile	175 ^{bc}	202 ^{ab}	156 ^c	197 ^{ab}	229 ^a	180 ^{bc}	17	0.01
Leu	143 ^{cd}	153 ^{bc}	128 ^d	164 ^{ab}	178 ^a	148 ^{bc}	12	<0.01
Lys	112 ^{bc}	108 ^c	102 ^c	123 ^{ab}	132 ^a	129 ^a	7	<0.01
Met	19.0 ^{bc}	17.1 ^c	18.0 ^{bc}	20.4 ^{ab}	19.2 ^{abc}	22.4 ^a	1.4	0.02
Phe	59.0	60.5	56.3	63.2	61.3	60.4	3.0	0.35
Pro	89.5 ^b	77.6 ^b	89.1 ^b	124.0 ^a	122.3 ^a	137.8 ^a	12.4	<0.01
Ser	89.4 ^{bc}	78.9 ^c	97.5 ^{ab}	93.2 ^{ab}	87.5 ^{bc}	104.8 ^a	8.3	0.02
Thr	92.4 ^{bc}	80.6 ^c	103.8 ^{ab}	92.5 ^{bc}	88.1 ^{bc}	116.3 ^a	9.9	0.05
Trp	45.6	49.4	46.4	46.6	52.0	49.2	3.1	0.55
Tyr	48.7 ^b	49.0 ^b	49.1 ^b	59.1 ^a	57.4 ^{ab}	59.7 ^a	3.9	0.02
Val	240 ^c	254 ^{bc}	215 ^c	290 ^{ab}	318 ^a	255 ^{bc}	26	<0.01
Total AA-N ³	2,872 ^{bc}	2,687 ^c	2,855 ^{bc}	3,070 ^{ab}	3,076 ^{ab}	3,201 ^a	123	0.01
EAA-N ³	1,246 ^{bc}	1,251 ^{bc}	1,135 ^c	1,388 ^{ab}	1,488 ^a	1,378 ^{ab}	82	0.01
NEAA-N ³	1,623 ^b	1,436 ^c	1,720 ^{ab}	1,682 ^{ab}	1,587 ^{bc}	1,822 ^a	97	<0.01

^{a-d}Means within a row followed by different letters differ at $P < 0.05$.

¹S = abomasal infusion of saline solution; A = abomasal infusion of acetate, at a daily rate of 5% of ad libitum ME intake; G = abomasal infusion of glucose, at a daily rate of 5% of ad libitum ME intake; C = abomasal infusion of casein, at a daily rate of 15% of ad libitum MP intake; AC = abomasal infusion of A + C; GC = infusion of G + C.

²PUN = plasma urea nitrogen.

³Total AA-N = sum of all analyzed AA, on N basis; EAA-N = sum of His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val, on N basis; NEAA-N = sum of Ala, Asn, Asp, Asn, Cys, Glu, Gln, Gly, Pro, Ser, and Tyr, on N basis.

for G, C, and S ($P < 0.05$), and it was greater for GC than for C ($P < 0.05$). Clearance rates of acetate, BHB, total AA-N, and NEAA-N were not affected by treatment ($P > 0.10$). Conversely, treatment G increased the EAA-N clearance rate relative to A, C, AC, and S ($P < 0.05$), and GC was intermediate. Likewise, G increased clearance rates for His (greater than all the other treatments; $P < 0.05$), Ile, and Leu (relative to all the other treatments except GC; $P < 0.05$), Met (relative to C and GC; $P < 0.05$), Lys (relative to C, AC, and S; $P < 0.05$), Val (relative to A, C, and AC; $P < 0.05$), Pro (relative to C, AC, and GC; $P < 0.05$), and Tyr (relative to C, AC, and S; $P < 0.05$). The clearance rate of Met was also increased by the infusion of acetate alone, compared with C and GC ($P < 0.05$). Moreover, the Asp clearance rate was greater for GC than for A and C ($P < 0.05$). Overall, C and AC had the smallest clearance rates for most AA.

Mammary uptake (Table 8) is a consequence of extra-cellular concentrations and clearance rate, the former being affected by blood flow and clearance. Mammary glucose uptake was greater for A than G, C, AC, and

S ($P < 0.05$), and greater for GC than C and S ($P < 0.05$). Mammary uptake of BHB and acetate were not affected by treatment ($P > 0.10$), even though acetate uptake for treatment C was numerically decreased by 37% relative to the average of the 2 acetate infusions and BHB uptake was numerically decreased by 30% relative to the average of all the other treatments. Mammary uptake of total AA-N was not affected by treatment ($P = 0.13$), although the numeric differences responded in a manner similar to the response of milk protein yield from the milking directly after blood sampling. Treatment GC increased mammary uptake of EAA-N compared with A, C, and S ($P < 0.05$). On the other hand, uptake of NEAA-N was increased by G relative to A, C, and AC ($P < 0.05$). Mammary uptakes of Lys and Ile were increased by GC compared with A, G, C, and S ($P < 0.05$), and AC was intermediate. Threonine mammary uptake was greater for GC than for A, C, and S ($P < 0.05$). Among the NEAA, GC increased mammary uptake of Pro (relative to A, C, and S; $P < 0.05$), Asn (relative to all the other treatments but G; $P < 0.05$), and Ala (relative to A and AC; $P < 0.05$).

Table 7. Effects of abomasal infusions of acetate, glucose, casein, and combinations on mammary plasma flow (MPF) and clearance rates of glucose, acetate, BHB, and AA (L/h)¹

Item	S	A	G	C	AC	GC	SEM	P-value
MPF	694	730	721	665	728	713	65	0.78
Glucose	162 ^{bc}	224 ^a	172 ^{bc}	136 ^c	184 ^{abc}	200 ^{ab}	23	<0.01
Acetate	1,147	1,207	1,441	975	1,335	1,327	268	0.52
BHB	421	333	444	345	361	465	81	0.49
Ala	154 ^{ab}	125 ^{bc}	198 ^a	127 ^{bc}	89 ^c	158 ^{ab}	25	0.02
Asn	321	350	344	291	315	308	31	0.28
Asp	387 ^{ab}	290 ^b	391 ^{ab}	312 ^b	353 ^{ab}	420 ^a	46	0.12
Cys	33	24	49	23	13	22	12	0.18
Gln	242	279	231	204	201	204	38	0.25
Glu	409	512	478	427	468	449	64	0.59
Gly	21	11	29	3	-3	5	15	0.53
His	324 ^b	359 ^b	611 ^a	259 ^b	260 ^b	297 ^b	103	0.04
Ile	341 ^{bc}	290 ^c	440 ^a	318 ^c	276 ^c	433 ^{ab}	58	0.01
Leu	306 ^{bc}	275 ^c	385 ^a	282 ^c	258 ^c	371 ^{ab}	48	0.01
Lys	936 ^b	1,043 ^{ab}	1,264 ^a	915 ^b	861 ^b	1,000 ^{ab}	135	0.09
Met	894 ^{ab}	1,095 ^a	1,127 ^a	752 ^b	925 ^{ab}	823 ^b	120	0.03
Phe	529	489	657	517	551	622	79	0.22
Pro	153 ^{ab}	162 ^{ab}	178 ^a	126 ^b	124 ^b	132 ^b	21	0.08
Ser	333	289	307	306	291	295	48	0.69
Thr	263	334	267	281	294	243	42	0.42
Trp	136	127	153	118	92	145	24	0.36
Tyr	468 ^b	485 ^{ab}	534 ^a	372 ^d	361 ^{cd}	420 ^{ab}	52	0.02
Val	182 ^{abc}	166 ^{bc}	221 ^a	153 ^{bc}	140 ^c	191 ^{ab}	30	0.02
Total AA-N ²	236	238	261	213	203	237	21	0.12
EAA-N ²	350 ^b	338 ^b	436 ^a	315 ^b	301 ^b	375 ^{ab}	46	0.03
NEAA-N ²	170	173	178	147	139	154	17	0.19

^{a-d}Means within a row followed by different letters differ at $P < 0.05$.

¹S = abomasal infusion of saline solution; A = abomasal infusion of acetate, at a daily rate of 5% of ad libitum ME intake; G = abomasal infusion of glucose, at a daily rate of 5% of ad libitum ME intake; C = abomasal infusion of casein, at a daily rate of 15% of ad libitum MP intake; AC = abomasal infusion of A + C; GC = infusion of G + C.

²Total AA-N = sum of all analyzed AA, on N basis; EAA-N = sum of His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val, on N basis; NEAA-N = sum of Ala, Asn, Asp, Asn, Cys, Glu, Gln, Gly, Pro, Ser, and Tyr, on N basis.

Treatment G also increased Ala, Asn, and Ser mammary uptake compared with A, C, and AC ($P < 0.05$). Mammary uptake of Gly was only different from 0 ($P < 0.05$) for G and S.

Milk protein, milk fat, and lactose yields from the milking immediately after blood sampling are presented in Table 8 to allow comparison with the mammary uptake of the main precursors of milk components. The differences among treatments for milk protein and lactose yields were not as great at this milking as they were for the average of the whole sampling period; thus, treatment effect did not reach significance ($P = 0.15$ for protein; $P = 0.29$ for lactose). On the other hand, fat yield at this milking was higher for AC than for C and S ($P < 0.05$) and higher for A than for C ($P < 0.05$).

Ratios of mammary AA uptake to milk AA output are presented in Table 9. This ratio for total AA-N represents the nitrogen balance across the mammary gland and should be close to unity when all the AA are considered and assuming that contributions from peptides are minimal and estimates of blood flow are unbiased. Total AA-N ratios were greater for G and S than for A and AC ($P < 0.05$). In addition, total AA-N ratios were less than 1 ($P < 0.10$) for all treatments

except G and S. Infusion of AC increased the EAA-N ratio relative to A, G, and C ($P < 0.05$); GC and S were greater than A and G ($P < 0.05$); and G was smaller than all the other treatments except A ($P < 0.05$). Conversely, G increased the NEAA-N uptake-to-output ratio compared with all the other treatments except S ($P < 0.05$), whereas AC had the smallest NEAA-N ratio (smaller than G and S, $P < 0.05$). The ratio of mammary uptake to milk output was greater than 1 ($P < 0.10$) for all treatments for EAA-N and less than 1 ($P < 0.05$) for all treatments for NEAA-N. For individual AA, infusions affected only Ala, Asn, Lys, and Ser ($P < 0.10$). The Ala ratio was greater for G than for A, C, and AC ($P < 0.05$), and greater for GC and S than for AC ($P < 0.05$). The Asn ratio was greater for G than for A, C, and AC ($P < 0.05$), and GC was greater than C and AC ($P < 0.05$). The Ser uptake-to-output ratio was greater for G, C, GC, and S than for A and AC ($P < 0.05$). Finally, the Lys ratio was greater for AC than for A and G ($P < 0.05$) and greater for GC and S than for G ($P < 0.05$). For all 6 treatments, the ratio of mammary uptake to milk output was less than 1 ($P < 0.10$) for Asn, Asp, Glu, Leu, Met, Pro, Ser, Thr, and Tyr. On the other hand, the ratio was greater than 1

Table 8. Effects of abomasal infusions of acetate, glucose, casein, and combinations on mammary uptake of glucose, acetate, BHB, and AA (mmol/h) and milk component yields (g) for the milking immediately after blood sampling¹

Item	S	A	G	C	AC	GC	SEM	P-value
Glucose	424 ^c	566 ^a	451 ^{bc}	411 ^c	450 ^{bc}	521 ^{ab}	52	0.03
Acetate	361	460	372	307	500	356	64	0.12
BHB	185	174	177	124	170	171	31	0.45
Ala	28.0 ^{abc}	23.2 ^c	36.4 ^a	24.2 ^{bc}	21.1 ^c	33.3 ^{ab}	5.2	0.03
Asn	8.8 ^{bc}	8.5 ^c	10.4 ^{ab}	8.4 ^c	8.8 ^{bc}	11.1 ^a	1.1	0.02
Asp	5.0	4.9	5.2	4.5	4.0	5.6	0.8	0.48
Cys	5.6	3.4	7.5	4.0	2.2	3.6	1.8	0.19
Gln	44.5	39.7	48.1	41.7	36.5	43.3	5.8	0.57
Glu	21.4	25.3	23.0	21.6	25.0	24.5	2.9	0.63
Gly	6.9	2.6	8.2	0.9	-0.3	2.4	4.8	0.62
His	14.3	12.6	14.3	13.6	13.6	16.6	1.7	0.40
Ile	38.6 ^b	39.2 ^b	40.9 ^b	41.1 ^b	40.3 ^{ab}	46.9 ^a	3.4	0.09
Leu	29.3	29.2	30.7	31.6	30.6	34.9	2.5	0.14
Lys	45.8 ^b	44.7 ^b	45.7 ^b	46.2 ^b	50.2 ^{ab}	53.7 ^a	3.6	0.07
Met	7.2	7.2	7.7	7.2	7.6	8.5	0.7	0.24
Phe	17.4	17.3	18.8	18.1	18.3	20.6	1.5	0.14
Pro	11.0 ^b	9.7 ^c	12.6 ^{ab}	12.3 ^b	12.3 ^{ab}	14.7 ^a	1.5	0.03
Ser	18.6 ^{ab}	15.0 ^c	20.7 ^a	19.3 ^b	15.9 ^b	21.2 ^{ab}	1.8	0.01
Thr	17.6 ^b	16.9 ^c	19.8 ^{ab}	18.0 ^b	18.2 ^{ab}	20.6 ^a	1.5	0.04
Trp	5.1	5.3	5.6	4.8	4.1	5.8	0.9	0.67
Tyr	13.4	13.7	14.7	14.1	14.0	15.9	1.2	0.20
Val	32.4	31.9	34.3	34.0	32.6	36.7	2.9	0.45
Total AA-N ²	503	474	543	494	485	567	42	0.13
EAA-N ²	287 ^b	280 ^b	298 ^{ab}	293 ^b	299 ^{ab}	337 ^a	23	0.10
NEAA-N ²	217 ^{ab}	194 ^b	245 ^a	201 ^b	185 ^b	230 ^{ab}	23	0.10
Milk components ³								
Milk protein	565	569	615	591	596	673	48	0.15
Milk fat	662 ^{bc}	749 ^{ab}	706 ^{abc}	616 ^c	808 ^a	757 ^{ab}	59	0.10
Lactose	796	834	917	855	884	962	68	0.29

^{a-c}Means within a row followed by different letters differ at $P < 0.05$.

¹S = abomasal infusion of saline solution; A = abomasal infusion of acetate, at a daily rate of 5% of ad libitum ME intake; G = abomasal infusion of glucose, at a daily rate of 5% of ad libitum ME intake; C = abomasal infusion of casein, at a daily rate of 15% of ad libitum MP intake; AC = abomasal infusion of A + C; GC = infusion of G + C.

²Total AA-N = sum of all analyzed AA, on N basis; EAA-N = sum of His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val, on N basis; NEAA-N = sum of Ala, Asn, Asp, Asn, Cys, Glu, Gln, Gly, Pro, Ser, and Tyr, on N basis.

³Milk components produced in the milking immediately after blood sampling.

($P < 0.10$) for all treatments for His, Ile, Lys, Phe, and Val. For Trp, the ratio was greater than 1 ($P < 0.10$) for A, G, GC, and S, and not different from 1 ($P > 0.10$) for C and AC.

DISCUSSION

Effects of Casein

Increases in milk protein yield with post-ruminal casein infusion have been reported in many studies (Broderick et al., 1970; Clark et al., 1977; Whitelaw et al., 1986; Vanhatalo et al., 2003; Raggio et al., 2006). Although we did not observe an effect of casein infusion on milk protein yield in the present study, the numerical increase of 100 g/d represented a 25% recovery of infused casein protein in milk protein relative to the saline control (g of milk protein over control divided by g of casein infused). This was very close to the average recovery of 21% reported by Hanigan et al. (1998a). Both Whitelaw et al. (1986) and Doepel et al. (2004)

observed declining efficiency of MP utilization for milk protein synthesis as supply approached requirement. A maximal marginal efficiency of MP use of about 45% was reported by both groups when MP supply was well below requirements. Cows on treatment C were predicted to be 10% MP deficient before infusion, and the observed 25% marginal efficiency is consistent with earlier observations but clearly much lower than the 67% marginal efficiency used in the NRC (2001) model.

One possibility for the lack of a greater response to casein infusion could be that energy supply to the animal, and thus to the mammary cells, was restricted. Protein synthesis is an energy-dependent process (Lobley, 1990) known to be regulated by energy supply (Appuhamy et al., 2014). Lack of response in milk protein yield to abomasal casein infusion was previously observed when cows were 30% feed restricted (Rius et al., 2010a). Provision of glucose with casein in the present trial yielded a marginal recovery in milk protein of 47% after adjusting for the 10 g/d difference in basal MP intake between GC and S, resulting in

Table 9. Effects of abomasal infusions of acetate, glucose, casein, and combinations on the ratio of mammary AA uptake to milk AA output¹

Item	S	A	G	C	AC	GC	SEM	P-value
Ala	1.47 ^{ab*}	1.21 ^{bc}	1.76 ^{ca*}	1.22 ^{bc}	0.93 ^c	1.43 ^{ab*}	0.19	0.02
Asn	0.56 ^{abc*}	0.53 ^{bc*}	0.61 ^{a*}	0.51 ^{c*}	0.52 ^{c*}	0.60 ^{ab*}	0.04	0.04
Asp	0.37 [*]	0.28 [*]	0.35 [*]	0.33 [*]	0.31 [*]	0.35 [*]	0.05	0.53
Cys	1.78 [†]	1.06	2.32 [*]	1.18	0.72	0.95	0.56	0.17
Gln	1.38 [*]	1.25 [*]	1.38 [*]	1.23 [*]	1.09	1.11	0.13	0.25
Glu	0.50 [*]	0.59 [*]	0.50 [*]	0.49 [*]	0.59 [*]	0.50 [*]	0.05	0.31
Gly	0.52 [†]	0.22 [*]	0.69	0.07 [*]	0.03 [*]	0.07 [*]	0.35	0.46
His	1.60 [*]	1.40 [*]	1.46 [*]	1.46 [*]	1.51 [*]	1.56 [*]	0.13	0.77
Ile	1.69 [*]	1.71 [*]	1.65 [*]	1.74 [*]	1.67 [*]	1.74 [*]	0.07	0.55
Leu	0.77 [*]	0.76 [*]	0.75 [*]	0.80 [*]	0.77 [*]	0.78 [*]	0.03	0.34
Lys	1.59 ^{*ab}	1.54 ^{*bc}	1.46 ^{*c}	1.55 ^{*abc}	1.66 ^{*a}	1.58 ^{*ab}	0.05	0.03
Met	0.73 [*]	0.73 [*]	0.72 [*]	0.69 [*]	0.73 [*]	0.73 [*]	0.01	0.16
Phe	1.15 [*]	1.14 [*]	1.14 [*]	1.15 [*]	1.15 [*]	1.15 [*]	0.01	0.72
Pro	0.25 [*]	0.22 [*]	0.26 [*]	0.27 [*]	0.26 [*]	0.27 [*]	0.02	0.15
Ser	0.61 ^{a*}	0.49 ^{b*}	0.62 ^{a*}	0.59 ^{a*}	0.50 ^{b*}	0.58 ^{a*}	0.03	<0.01
Thr	0.94 [*]	0.90 [*]	0.93 [*]	0.92 [*]	0.90 [*]	0.92 [*]	0.03	0.82
Trp	1.38 [*]	1.40 [*]	1.40 [*]	1.19	1.13	1.36 [*]	0.21	0.83
Tyr	0.86 [*]	0.87 [*]	0.86 [*]	0.86 [*]	0.85 [*]	0.86 [*]	0.01	0.56
Val	1.14 [*]	1.11 [*]	1.11 [*]	1.15 [*]	1.19 [*]	1.09 [*]	0.05	0.57
Total AA-N ²	1.00 ^a	0.94 ^{b*}	1.00 ^a	0.95 ^{ab*}	0.91 ^{b*}	0.95 ^{ab*}	0.03	0.04
EAA-N ²	1.28 ^{ab*}	1.24 ^{cd*}	1.22 ^{d*}	1.25 ^{bc*}	1.31 ^{a*}	1.27 ^{ab*}	0.02	<0.01
NEAA-N ²	0.78 ^{ab*}	0.70 ^{bc*}	0.82 ^{a*}	0.70 ^{bc*}	0.63 ^{c*}	0.69 ^{bc*}	0.05	0.03

^{a-d}Means within a row followed by different letters differ at $P < 0.05$.

¹S = abomasal infusion of saline solution; A = abomasal infusion of acetate, at a daily rate of 5% of ad libitum ME intake; G = abomasal infusion of glucose, at a daily rate of 5% of ad libitum ME intake; C = abomasal infusion of casein, at a daily rate of 15% of ad libitum MP intake; AC = abomasal infusion of A + C; GC = infusion of G + C.

²Total AA-N = sum of all analyzed AA, on N basis; EAA-N = sum of His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val, on N basis; NEAA-N = sum of Ala, Asn, Asp, Asn, Cys, Glu, Gln, Gly, Pro, Ser, and Tyr, on N basis.

*Different from 1.0 ($P < 0.05$).

†Tendency to be different from 1.0 ($P < 0.10$).

184 g/d greater milk protein yield on GC. Dietary MP supply was similar (2,294 g/d for C vs. 2,282 g/d for GC), and amounts of casein infused were identical for C (404 g/d) and GC (403 g/d). Thus, efficiency of conversion of MP into milk protein should have been similar (Doepel et al., 2004). The almost 90% increase in MP efficiency upon glucose infusion (47% for GC vs. 25% for G) suggests that energy could indeed have limited the response on C. However, when casein was infused with acetate (AC), recovery of the extra protein in milk protein relative to saline (i.e., 423 g infused plus 126 g from diet MP supply) was only 13.5% (45% decrease vs. C). Although acetate is an important energy source for the mammary cells of ruminant animals (Hanigan and Baldwin, 1994), its infusion should have stimulated response to casein infusion had ATP been limiting protein synthesis.

Compared with the negative control, casein infusion increased arterial glucose concentrations but slightly decreased mammary glucose clearance rate and plasma flow, resulting in a numerically smaller mammary glucose uptake. Mammary uptake of acetate and BHB, both sources of energy to the mammary gland, were also numerically smaller for C, suggesting that the energy demand of mammary cells was not limited by

the availability of energy substrates. Increased arterial glucose concentration with casein infusions has been previously reported, with a concomitant increase in net liver glucose flux (Galindo et al., 2011). Increased glucose availability with casein infusion is often associated with increased mammary glucose uptake for lactose and milk yield (Lemosquet et al., 2009a; Galindo et al., 2011). However, glucose uptake did not change, and, accordingly, no increase in lactose or milk yield was observed in the present study for C compared with S.

Another possibility for the limited response to infusion of casein alone could be the limitation of specific EAA, such as Met, because its supply was lower than recommended levels (NRC, 2001). However, this seems unlikely, because all 3 casein treatments increased arterial concentration of most EAA relative to saline. Additionally, AA clearance rates and mammary plasma flow were numerically smaller, resulting in only numeric increases in EAA uptake on C relative to S. Because the mammary gland has the ability to control local blood flow and clearance rates to match nutrient supply with synthetic demand (Bequette et al., 2000; Cant et al., 2003), the numeric decline in clearance rates for all of EAA suggests that EAA supply increased more than EAA use for milk protein synthesis (Hanigan et al.,

2000; Hundal and Taylor, 2009). If 1 or more EAA had been severely limiting, a large increase in clearance rates would have been expected for those AA on C relative to S (Bequette et al., 2000).

In the absence of a fully integrated post-absorptive model that represents AA transporter regulation, it is not possible to predict the AA limiting production, but the relative change in clearance rates may be an indication of which AA were more limiting. We calculated the percent change in clearance rates for the EAA between treatments S and C and found that the largest relative changes in clearance rates were for His (20%), Met (16%), Trp, (13%), Tyr (21%), and Val (16%). These changes are all modest compared with the 30-fold changes observed by Bequette et al. (2000) when a severe His deficiency was imposed. Thus, it seems that the low marginal response to C simply reflects the response surface given static values for other inputs such as energy substrates and hormonal signals.

Post-ruminal infusions of EAA have increased mammary AA uptake relative to either water or isonitrogenous infusions of urea, Pro, Glu, and Ser, on diets that were both protein deficient (Doepel and Lapierre, 2010; Haque et al., 2015) and protein sufficient (Raggio et al., 2006; Haque et al., 2015). However, in these studies, increased mammary AA uptake was accompanied by increased milk protein output. When no response in milk protein yield was observed to casein infusion, mammary AA uptake was also not increased (Rius et al., 2010a). The numeric difference in milk protein yield between C and S in the milking immediately following blood sampling was approximately 55 g/d (26 g in 11.3 h of milking interval), which should have been associated with greater mammary AA uptake. However, plasma Arg was not measured in the present study, which contributes 4 g of nitrogen per mole of AA taken up, and mammary Arg uptake often increases with casein infusion (Doepel and Lapierre, 2010; Rius et al., 2010a; Haque et al., 2015). In fact, ratios of mammary uptake to milk output for total AA-N were less than 1 for C but not for S, suggesting that Arg might have contributed more nitrogen on treatment C than S. Ratios of mammary uptake to milk output were not different between C and S for any AA, suggesting no change in intramammary metabolism.

The ratios between mammary AA uptake and milk AA output observed in the present study were close to 1 for total AA-N for all treatments, suggesting a net 0 nitrogen balance across the gland, which is consistent with previous work (Hanigan et al., 2001). However, Met and Leu ratios were less than 1 for all treatments. A deficiency of Met has previously been noted when calculating blood flows using the Fick principle unless

Met is used for the calculation, but Leu uptake is generally found to be more than output in milk (Lapierre et al., 2005). This could be a result of underestimating mammary blood flow or AA contribution from peptides, which was not measured.

In summary, the response of milk protein to casein infusion alone was less than would be predicted by the NRC (2001) model even though the cows were MP deficient according to that model. However, the marginal response was consistent with prior work and with the diminishing-returns response curve of Doepel et al. (2004). The inefficiency of infused casein use was reflected by numeric increases in PUN compared with saline, and AA were likely used for gluconeogenesis given the observed increases in arterial glucose concentration.

Effects of Glucose

Infusion of glucose had the greatest effects on milk and milk protein yield. When infused alone, glucose increased milk protein yield by 170 g/d, lactose by 330 g/d, and milk yield by 6.4 kg/d relative to the negative control. This is an impressive response, considering that the cows were in negative MP balance according to NRC (2001). In fact, the efficiency of conversion of NRC-estimated MP to milk protein was 58% with glucose infusion, compared with 49.5% for saline. This resulted in an ENU of 36.7% for the glucose infusion, whereas the other treatments ranged from 30.1 to 33.1%. When glucose was infused along with casein, the response was greater than for separate infusions of casein and glucose, suggesting a partial additive effect. However, due to the extra supply of protein provided by GC, the efficiency of NRC-estimated MP use for milk protein synthesis remained at 49%.

The response to glucose was not surprising, considering several studies have shown positive effects of dietary starch (Broderick, 2003; Rius et al., 2010a; Cantalapiedra-Hijar et al., 2014), post-ruminal glucose supply (Hurtaud et al., 2000; Rigout et al., 2003; Rulquin et al., 2004; Lemosquet et al., 2009a), and ruminal propionate supply (Rigout et al., 2003; Raggio et al., 2006; Lemosquet et al., 2009b) on milk protein yield. However, determining what caused the response is challenging. Infusion of glucose alone did not increase arterial glucose concentration. In contrast, several of the studies reporting increased milk protein with glucogenic nutrients also reported higher arterial glucose concentration (Lemosquet et al., 2009a,b; Rius et al., 2010a). Another common response to supplying glucogenic nutrients is increased mammary plasma flow (Rulquin et al., 2004; Raggio et al., 2006; Rius et al., 2010a). In the present experiment, mammary

plasma flow was not affected by treatment; however, mammary clearance rate for most AA was highest on the glucose treatment, which allowed for increased mammary AA uptake to account for the extra protein synthesized. The decreased arterial concentrations of most AA observed in this trial accompanying glucose infusions was also reported by Mackle et al. (1999) in a hyperinsulinemic-euglycemic clamp study. The exceptions were the glucogenic AA Asn, Gln, Gly, and Ser, which increased in concentration, suggesting these AA were spared from gluconeogenesis.

Glucose infusion increased clearance rates of all EAA except Thr, which is consistent with stimulation of milk protein synthesis causing reduced intracellular EAA supplies and increased EAA transport activity (Hanigan et al., 2000). The combination of glucose and casein ablated the clearance rate responses, with the exceptions of Ile, Leu, and Phe. However, the increased arterial concentrations allowed the meeting of the increased AA demands by mass action supply alone. Exceptions were Ile, Leu, and Phe, which had arterial concentrations very similar to those on S and, hence, required continued elevation of the clearance rate to meet tissue demands.

Despite the numerical increase in lactose yield with both glucose treatments (G and GC, average 305 g/d compared with saline), glucose uptake increased only on GC. Recent studies have suggested that AA can contribute to galactose synthesis (Lapierre et al., 2013; Maxin et al., 2013). However, that hypothesis is not supported by the work of Forsberg et al. (1985), who detected no label in lactose when ^{14}C -labeled lactate was included in the media of galactogenic mammary tissue slices—that is, the label entering the lower portion of the glycolytic pathway is not transferred to the upper portions of the pathway. It is more likely that glucose oxidized in the pentose phosphate pathway to produce NADPH was reduced, leaving more glucose available for lactose synthesis. The loss in NADPH production with this shift could be replaced through the oxidation of more acetate in the cytosolic isocitrate dehydrogenase pathway (Hanigan and Baldwin, 1994). Several studies have reported that, as the rate of whole-body glucose appearance increases, the ratio of lactose to whole-body glucose appearance decreases (Rigout et al., 2003; Lemosquet et al., 2004, 2009a). This may be a consequence of glucose partitioning away from the mammary gland toward other tissues, due to greater action of insulin with increased glucose, or changes in glucose utilization within the mammary cell (Lemosquet et al., 2009a). Blood lactate concentration was not measured in the present study, and lactate can also be used by the mammary gland as an energy source, sparing glucose to for lactose synthesis.

Effects of Acetate

As mentioned earlier, mTORC1 coordinates nutritional and endocrinal signals to regulate protein translation in the mammary cell. Besides protein kinase B (AKT), mTORC1 is under the regulation of AMPK, which is able to sense cellular energy status. When cellular energy levels are low, AMPK is activated by phosphorylation and inhibits processes that consume ATP, such as protein synthesis, which is affected via inhibition of mTORC1, as demonstrated by Burgos et al. (2013) in bovine mammary epithelial cells. Glucose and lactate are the main energy precursors in the mammary cells of monogastric mammals (Larson, 1985). In ruminants, however, due to lower arterial concentrations of glucose and higher concentrations of acetate compared with non-ruminants, a need exists to spare glucose for synthesis of lactose and NADPH, making acetate of greater importance as an energy substrate in ruminant mammary cells (Bickerstaffe et al., 1974). This suggests that acetate could potentially stimulate milk protein synthesis via AMPK inhibition, resulting from higher cellular energy status. Indeed, Safayi and Nielsen (2013) reported a tendency ($P = 0.11$) for increased milk protein yield in response to intravenous infusion of acetate in goats in early lactation. Furthermore, Rook and Balch (1961) observed increased milk protein yield with ruminal infusion of 960 g/d of acetate over several days.

In the present study, however, acetate exhibited no effect on yield of milk or milk protein and responded to feed restriction in a manner similar to the negative control. When infused along with casein, effects were similar to those from casein alone, supporting the observation that acetate itself had no effect. The difference between the present results and those reported by Safayi and Nielsen (2013) might be due to site of acetate infusion (abomasal vs. intravenous), which promoted greater differences in arterial acetate concentrations in acetate infusions relative to the saline control (45% vs. 30% in the present study). Another possibility is that goats responded to acetate infusion with increased milk protein yield only in early lactation (21 DIM), when the mammary gland appears to be more responsive and feed intake often is insufficient to meet the nutritional requirements for milk production. Supporting the lack of an effect, Castro et al. (2016) observed very low responses to acetate when modeling results from a mammary tissue slice system.

Numerically greater mammary acetate uptake for the 2 acetate infusions (treatments A and AC, $P < 0.12$), with no changes in acetate clearance rates, suggested that acetate uptake was a direct consequence of arterial acetate concentration, as demonstrated also

by Purdie et al. (2008). Even though more acetate was taken up by the mammary glands with acetate infusions, it appears to have been used primarily for milk fat synthesis, rather than for energy production, as suggested by the numeric increases in milk fat yield in both acetate infusions. This observation was further supported by the greater mammary glucose uptake with the acetate infusion alone, which would be needed to supply glycerol and NADPH for de novo fatty acid synthesis (Chaiyabutr et al., 2008). In contrast, Purdie et al. (2008) did not report increased milk fat yield with 10-h close arterial acetate infusion, despite 123% increase in arterial glucose concentration and 128% increase in mammary acetate uptake.

Despite no increase in milk protein yield and an MP supply similar to that of the negative control, arterial concentrations of NEAA-N were reduced in A relative to S. The reason for the reduced arterial NEAA concentrations in the present study was not greater mammary uptake. No changes in mammary AA metabolism were expected, because no changes were observed in milk protein yield relative to saline, and indeed the 2 acetate treatments behaved similarly.

CONCLUSIONS

Abomasal infusion of casein and glucose for 5 d in cows that were 15% feed-restricted increased milk protein yield by 100 and 165 g/d, respectively. Recovery of infused casein was 25%, and efficiency of MP utilization for milk protein yield (milk protein yield/predicted total MP supply) was 46% with the casein infusion. The increase in milk protein yield with glucose infusion was brought about by an increase in mammary clearance rate for all EAA except Thr, overcoming the decreased arterial concentrations of most AA. In addition, glucose infusion increased utilization of circulating NEAA for milk protein synthesis (increased ratio of mammary uptake to milk output), reducing the use of EAA for synthesis of NEAA (decreased uptake-to-output ratio) and therefore reducing intramammary AA catabolism. These shifts in mammary gland metabolism in response to glucose infusion resulted in an efficiency of MP utilization for milk protein synthesis of 58%. The combination of glucose and casein infusions further increased milk protein yield (184 g/d) and almost doubled the recovery of infused casein to 47%. Acetate infusion alone had no effect on milk protein yield but numerically increased milk fat yield, suggesting that the greater amount of acetate taken up by the mammary gland was used for milk fat synthesis rather than ATP production. Infusion of acetate and casein together promoted responses similar to those of casein alone. In conclusion, glucose stimulated milk

protein synthesis, and the mammary gland responded by increasing clearance of EAA to match its synthetic capacity. Additionally, these results emphasize the limitations of the NRC (2001) model in predicting the independent metabolic responses to glucose and casein, because it implicitly assumes that a single nutrient is limiting milk protein synthesis at any point in time, whereas the system exhibits a multi-substrate response surface with multiple nutrients potentially limiting production at any time.

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