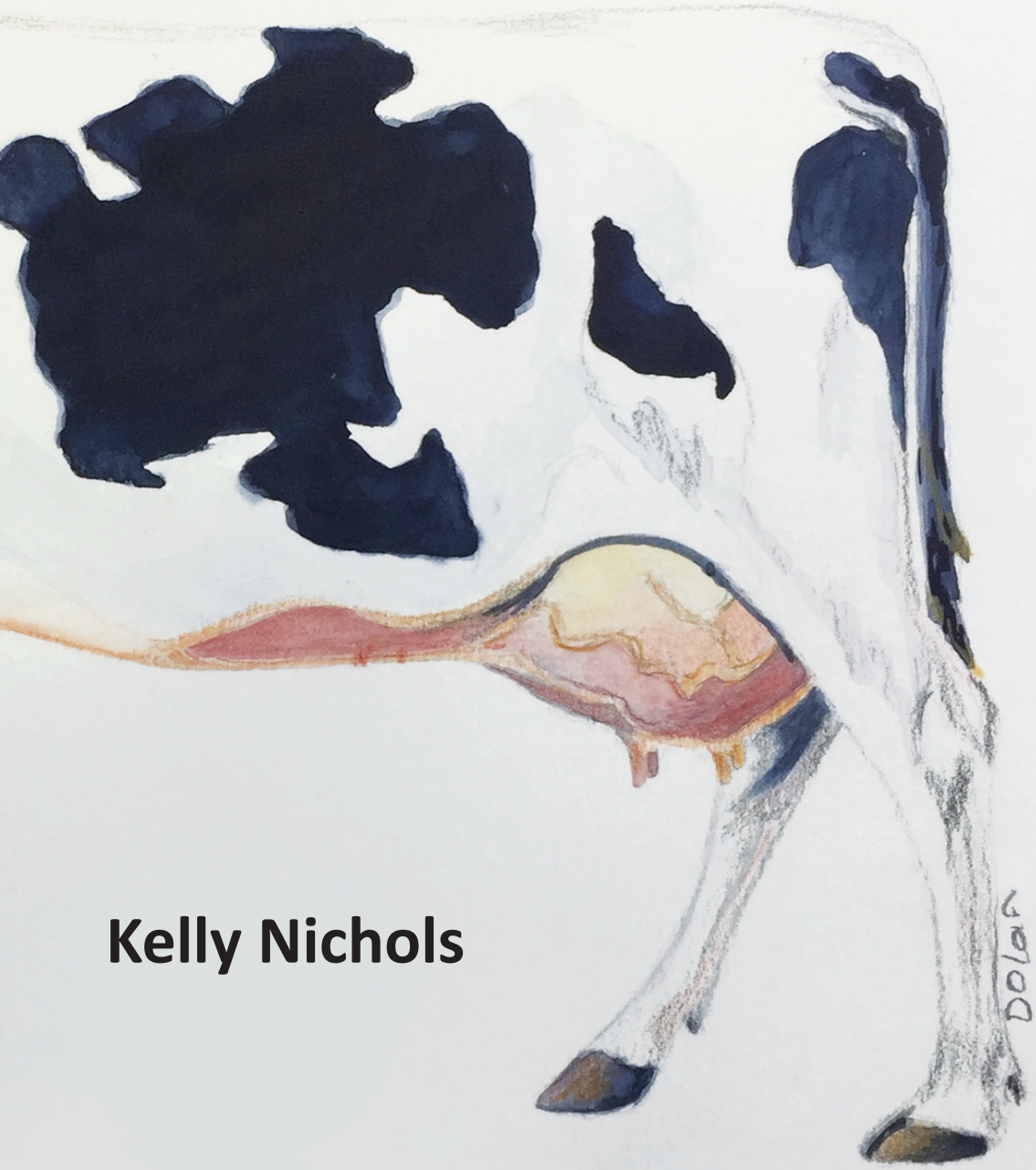
Whole-body and mammary gland metabolism in dairy cattle

Impact of postabsorptive energetic substrates and amino acid profiles



Kelly Nichols

Propositions

1. Supplemented energy type (glucogenic versus lipogenic) matters with regard to improving milk nitrogen efficiency at low and high metabolizable protein levels.
   (this thesis)

2. The essential amino acid profile of metabolizable protein is important when aiming to achieve positive impacts on milk nitrogen efficiency.
   (this thesis)

3. Thinking and writing scientifically is not easier if English is one's native language.

4. Labelling plant-based beverages as 'milk' is fallacious and poses a significant health risk to growing children.

5. During animal experiments, having a plan 'B' is good, but having a plan 'B' through 'Z' is necessary.

6. Sustainable Development Goal 4 set forth by the United Nations, *ensure inclusive and equitable quality education and promote lifelong learning opportunities for all*, will be the most impactful with regard to meeting the goals of the 2030 Agenda for Sustainable Development.

7. Exposing youth to the responsibilities of animal husbandry is an undervalued practice for instilling the characteristics of responsibility, commitment, and compassion in later life.

Propositions belonging to the thesis, entitled

Whole-body and mammary gland metabolism in dairy cattle – Impact of postabsorptive energetic substrates and amino acid profiles

Kelly Nichols
Wageningen, 12 April 2019

Whole-body and mammary gland metabolism in dairy cattle

Impact of postabsorptive energetic substrates and amino acid profiles

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Kelly Nichols

Thesis

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Chapter 1

General Introduction

Importance of Dairy Production: Implications for Food and Environmental Security

Milk protein produced from ruminants can be impactful with regard to the synthesis of healthy human-edible protein from non-human edible biomass (de Vries and De Boer et al., 2010; Pereira, 2014; Thorning et al., 2016). Milk can be classified as a 'good source' of protein based on its digestible indispensable amino acid (AA) score, and scores higher than proteins derived from soya beans, peas, rice, or wheat (Marinangeli and House, 2017; Mathai et al., 2017). In this way, dairy products contribute to the nutritive value of human diets. Global demand for meat and milk is expected to steadily increase in the coming decades (Mottet et al., 2017; OECD/FAO, 2015). Therefore, intensification of animal production is necessary to ensure future food security, but increased levels of productivity must be met while minimizing adverse effects on land, water, air, and biodiversity (FAO, 2018). Dairy production can also be impactful with respect to increasing efficiency in the agriculture sector. Ruminants augment the human food supply by deriving energy and protein substrates from human-inedible biomass and converting them into energy- and protein-rich human-edible food. In this way, they are more efficient compared with non-ruminants on the basis of human-edible inputs to outputs (Dijkstra et al., 2013a). Regarding the dairy sector, production of 1 kg milk protein can result in a lower environmental impact than 1 kg beef, pork, chicken, or egg protein, depending on sustainability targets (de Vries and de Boer et al., 2010; van Zanten et al., 2016). Environmental pollutants and greenhouse gas emissions are trade-offs with respect to the positive impacts of ruminant production systems on human food supply. The dairy industry contributes to nitrogen (N) pollution, which negatively impacts air and water quality, and in turn affects the health of humans and ecosystems (Townsend et al., 2003; de Klein et al., 2010; Dijkstra et al., 2013b). Improvements in gross N efficiency on dairy farms can allow substantial reductions in N excretion through manure. Efficient transfer of feed N into milk is economically important to dairy farmers. Dietary protein sources, the main source of N to the animal, are often the most expensive component of dairy cattle diets. Furthermore, there is legislative pressure in several intensively farmed areas, such as the Netherlands, to minimize N emissions from agricultural sources (EU Directive 91/676/EEC; Van Grinsven et al., 2013).

In order to ensure a sustainable source of human-edible protein from milk, the modern dairy cow must be fed in such a way that she can effectively consume, digest, absorb, and partition energy and protein towards production of high-quality milk. Achieving this requires precise nutrition and feed management at the farm and animal level. The following introduction focuses on aspects of energy and protein nutrition in lactating dairy cows related to milk production and N efficiency at the whole body, mammary gland, and

mammary cell level, and concludes with gaps in knowledge leading to the hypotheses challenged in this thesis.

Energy and Protein Nutrition in Lactating Dairy Cattle

Ruminants play a key role in converting human-inedible feedstuffs into high-quality human edible food. Given the losses occurring during digestive processes, the theoretical maximum milk N efficiency (feed N into milk N) for a dairy cow producing 40 kg/d of fat- and protein-corrected milk with 31.5 g/kg true protein is 43% (Dijkstra et al., 2013c). In practice, milk N efficiency is highly variable (10-40%; Hristov et al., 2004; Calsamiglia et al., 2010), where on average the capture of dietary N in milk is approximately half this theoretical value. In lactating dairy cows, the largest portion of N intake not captured in milk is excreted in manure, where urinary N output is more susceptible to changes in N intake compared with fecal N (Huhtanen et al., 2008). Nitrogen in manure gives rise to emission of ammonia, leaching of other pollutants into groundwater, and emission of the greenhouse gas N_2O. Altogether, this excretion contributes to the environmental burden of milk protein production (de Klein et al., 2010; Dijkstra et al., 2013b). Unavoidable N losses within the animal arise from incomplete digestion of microbial protein, synthesis of microbial nucleic acids, and maintenance requirements (Dijkstra et al., 2013c). These routes offer little potential for reduction of N losses. Rather, dietary strategies to reduce N losses should focus on optimizing rumen degradable N, dietary energy level, and absorbed AA profile.

Various dietary factors contribute to the overall efficiency with which N is used in dairy cows. Major research focus has been placed on quantifying the relationship between dietary energy and protein, with the aim to formulate diets with sufficient energy and with an optimal AA profile to support postabsorptive metabolic processes. The source of energy in dairy rations is important with regard to the metabolic effects on the animal, such as regulation on hormonal cascades and metabolite partitioning towards energy or anabolism. Furthermore, in order to make improvements in milk N efficiency and maintain profitable milk production levels, the variable efficiency with which AA are used by dairy cows should be more precisely understood. Increasing total absorptive AA supply does not guarantee positive responses in N efficiency or milk protein production, because as AA supply increases, transfer efficiency of absorbed AA into milk protein usually decreases (Hanigan et al., 1998; Doepel et al., 2004; Nichols et al., 2016) and AA catabolism and N excretion usually increases (Bach et al., 2000; Castillo et al., 2001; Raggio et al., 2004). If extra N intake supplies metabolizable protein (MP) with a desirable EAA profile for milk protein synthesis, milk N efficiency can be improved (Haque et al., 2012; Haque et al., 2015). Additionally, increasing non-AA energy supply to the animal may reduce AA catabolism and enhance efficiency of N capture in milk protein (Rius et al., 2010a,b; Cantalapiedra-Hijar et al., 2014a).

The content and the type of energy and protein in lactating cow diets are important factors influencing nutrient transfer from feed into milk components. Importantly, metabolic, hormonal, and cellular adaptations to absorbed nutrients delineate clear relationships between dietary energy and protein, and minute changes in metabolism from the whole-body to the cellular level can improve or inhibit efficiency of nutrient use.

Dietary Energy Sources and Postabsorptive Metabolism

With regard to macronutrient metabolism in a lactating dairy cow, energy is derived from carbohydrate, fat, and protein (**Figure 1.1**). Rising demand for sustainably produced milk protein products emphasizes the importance of understanding how energy-yielding feedstuffs affect synthesis of milk and its components. This importance is reflected in several studies investigating substitutions of different forages and concentrate components into dairy cattle rations (e.g. Maxin et al., 2013a; Cantalapiedra-Hijar et al., 2014a; Piantoni et al., 2015; van Hoeij et al., 2017). A small fraction of these studies quantify the effects of different energy substrates on whole-body energy and N balance using calorimetry (van Knegsel et al., 2007a; Reynolds et al., 2018). The next paragraphs discuss glucogenic, aminogenic, and lipogenic nutrients from dietary components with regard to their contribution to absorption of energy-yielding metabolites, with emphasis on their use by and partitioning between postabsorptive tissues.

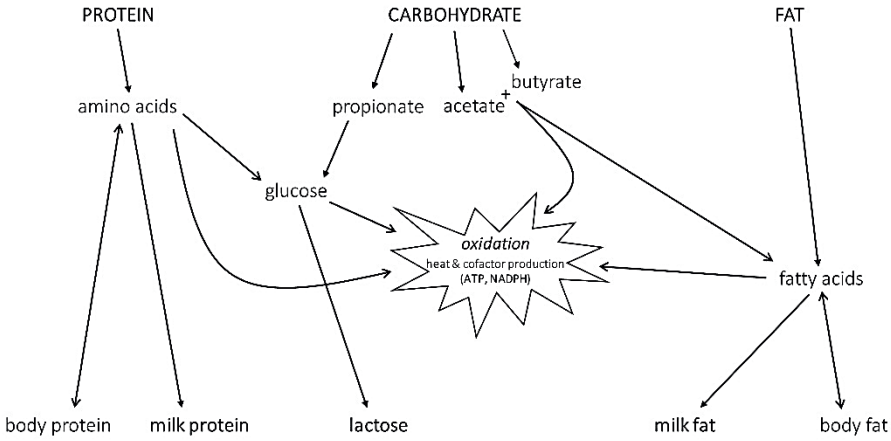

Figure 1.1. Partitioning of macronutrients and their major metabolites for energy and synthetic processes in a lactating dairy cow.

Glucogenic nutrients. Glucogenic nutrients are those derived from dietary carbohydrates that yield glucose through digestion and metabolism, and are particularly important during lactation when glucose use by the mammary gland is high. Net glucose absorption from the gastrointestinal tract of ruminants accounts for less than one third of their whole-body glucose appearance, meaning they rely highly on gluconeogenesis in the fed state (Huntington et al., 2006; Reynolds, 2006). Circulating glucose concentrations are maintained by hepatic gluconeogenesis (85-90% of whole-body glucose appearance; Bergman et al., 1970), with a minor contribution from renal gluconeogenesis. The primary glucose precursor for lactating dairy cows is the volatile FA (VFA) propionate, which accounts for more than half of the input to the gluconeogenic pathway in the liver after its absorption into portal drainage from the rumen (Amaral et al., 1990; Benson et al., 2002; Reynolds et al., 2003). Lactate and glycerol serve as quantitatively minor glucose precursors (≤ 15%; van der Walt et al., 1983; Lozano et al., 2000), and make relatively larger contributions to glucose synthesis in early lactation when dietary starch intake may be greater and when tissue mobilization from adipose and skeletal muscle is higher (Benson et al., 2002; Reynolds et al., 2003). Depending on degradability and quantity, starch that escapes rumen fermentation may be hydrolysed to glucose monomers in the small intestine (Nocek and Tamminga, 1991; Mills et al., 1999), contributing to the minimal portion of intestinally absorbed glucose.

Absorbed AA (except Leu and Lys) can make a net contribution to gluconeogenesis through their metabolism in the tricarboxylic acid (TCA) cycle (Bequette et al., 2006). This contribution can be quantitatively important (≤ 30%) to whole-body glucose flux (Lindsay, 1980; Danfær et al., 1995; Lozano et al., 2000). Lindsay (1980) estimated that 3% of hepatic EAA flux and 10 to 25% of hepatic non-EAA (NEAA) flux would undergo gluconeogenesis, with Ala and Gln contributing to the majority of glucose output. Galindo et al. (2011) suggested that their observed increase in whole-body glucose flux originated from hepatic glucose synthesis from infused AA or casein, while neither Blouin et al. (2002) nor Hanigan et al. (2004a) observed an effect of increased MP supply on hepatic glucose release. Like lactate and glycerol, the contribution of AA to gluconeogenesis may be quantitatively greater in early lactation (Reynolds et al., 2003; Larsen and Kristensen, 2013), but the importance of this contribution is variable and may be affected by MP supply in the postpartum period (Larsen et al., 2015).

The portal-drained viscera (PDV: the gastrointestinal tract, pancreas, spleen, and associated adipose tissue) mainly uses glucose extracted from the arterial supply (El-Kadi et al., 2006; Reynolds, 2006). Net PDV glucose utilization varies with the extent of fermentation in the rumen and glucose absorption from the small intestine, and the extent of glucose use by the rumen epithelium and enterocytes, but may account for approximately 12% of whole-body glucose utilization in lactating dairy cattle (Galindo et al., 2011). In peripheral tissues, glucose uptake by adipose and skeletal muscle is under greater regulation by insulin

compared with tissues where glucose use is obligate (e.g., mammary gland, uterus during pregnancy; Brockman and Laarveld, 1986). Particularly in early and mid-lactation, the mammary gland uses circulating glucose with the highest priority relative to other peripheral tissues, accounting for approximately 77% of whole-body glucose flux (Lemosquet et al., 2009; Galindo et al., 2011; Galindo et al., 2015).

Lipogenic nutrients. Microbial degradation of fibrous organic matter in the rumen yields the lipogenic VFA acetate and butyrate. The ratio of acetate to propionate in the rumen is higher for rations with a greater inclusion level of fibrous forage relative to those with a greater inclusion of starch-rich concentrates. The former would be considered a relatively more 'lipogenic' ration, and the latter would be considered a 'glucogenic' ration. Approximately 70% of acetate produced in the rumen appears in portal blood (Bergman and Wolff, 1971; Kristensen, 2001), and hepatic metabolism usually results in a net release of acetate (Reynolds et al., 2003; Kristensen, 2005). Acetate provides the main substrate for lipogenesis and oxidation in peripheral tissues of ruminants (Bergman, 1990). The majority of butyrate produced in the rumen is metabolised in the rumen wall to ketone bodies, mainly β-hydroxybutyrate (BHB), resulting in a low portal recovery of butyrate (10-25% of ruminal butyrate production; Kristensen et al., 2000). After metabolism by rumen epithelium and other tissues of the gastrointestinal tract, the majority of portal butyrate and BHB (66-80%) is used during first pass through the liver for synthesis of acetate, other FA, and ketone bodies, leaving approximately one third available to peripheral tissues for oxidation or lipogenesis (Bergman and Wolff, 1971; Bergman, 1990; Lozano et al., 2000).

Ingested dietary fat also contributes to absorptive lipogenic energy supply. The lipid content of common forages, including fresh grass, is typically ≤ 4% (NRC, 2001), but feeding FA supplements can be useful to increase the energy density of dairy rations. Contribution of dietary lipids to volatile FA production is minor, as their proportion in the diet is small and only the carbohydrate moiety, not the long-chain FA, is fermented. Fats with a low degree of saturation are susceptible to rumen biohydrogenation reactions, and can negatively impact functionality of rumen microbes and thus fiber digestion and dry matter intake (Pantoja et al., 1996; Allen, 2000). To combat this, rumen-bypass fat sources are commonly added to dairy rations, either in the form of saturated FA assumed to be rumen-inert, or as rumen-protected unsaturated FA which can be absorbed in the small intestine. In this way, rumen-bypass fat sources can increase metabolizable energy intake with minimal effects on digestive processes.

Circulating non-esterified FA (NEFA) or triacyglycerides (TAG) bound in various lipoprotein forms represent FA absorbed from the diet and synthesised from acetate and BHB (together accounting for approximately 50% of FA entry rate), and those appearing from lipolysis in PDV and peripheral tissue adipose depots (Pethick et al., 2005). In lactating dairy cattle, the mammary gland utilizes the majority of circulating acetate, BHB, and FA for milk

fat synthesis. Extra-mammary lipogenesis in ruminants occurs predominantly in adipose tissue, with little de novo lipogenesis occurring in the liver (Chilliard, 1993; Bauchart et al., 1996).

Dietary Protein and Amino Acid Profile

The main components of dietary protein in dairy cattle rations are described in **Textbox 1.1**. In general, the marginal response to extra duodenal AA decreases when dietary protein supply meets or exceeds requirements (Doepel et al., 2004; Arriola Apelo et al., 2014). Most protein evaluation systems still assume a constant efficiency of MP use, which results in overestimation of milk protein yield at high protein intakes and underestimation at low protein intakes (Doepel el al., 2004; Lapierre et al., 2018). Estimated MP requirements are based on efficiencies of major metabolic processes (maintenance, lactation, reproduction). These processes have requirements for AA, not protein; therefore, the AA profile of absorbed MP is important. Individual EAA supplies in dairy rations are predicted as a proportion of MP in most feeding systems (Lapierre et al., 2018), but variable proportions of absorbed EAA are captured in milk protein. This can be attributed to intermediary splanchnic metabolism of AA and effects of arterial concentrations of AA and other metabolites on mammary gland AA extraction and intra-organ metabolism (Lapierre et al., 2006; Cant et al., 2018).

Protein degradation and N appearance in the rumen increases with dietary crude protein (CP) content, but elevated CP intake may not lead to additional AA absorption if rumen-fermentable carbohydrate is not adequately matched to rumen-degradable protein (RDP) to support rumen microbial protein synthesis. The balance between RDP and fermentable energy has been the focus of abundant research with the aim to maximize N capture in the rumen (McCormick et al., 2001; Hristov and Ropp, 2003; Ipharraguerre and Clark, 2005). Microbial biomass is the primary protein source for ruminants, contributing quantitatively most significantly to MP and absorptive N supply (Clark et al., 1992; Tamminga et al., 1994; Sok et al., 2017). High-quality protein sources, or individual AA directly, can be protected against ruminal degradation and fed to increase MP supply and complement the AA profile of microbial protein entering the intestine. Feeding rumen-bypass protein sources is an approach to increase MP with a known AA profile, as an alternative to feeding high CP rations (>16%) with the aim to achieve adequate AA supply for desired milk production levels. Balancing rations with EAA in support of milk protein synthesis has been shown to improve postabsorptive N efficiency and increase milk protein yield (Wright et al., 1998; Bach et al., 2000; Arriola Apelo et al., 2014). Therefore, manipulation of the profile of absorbed EAA can be a nutritional tool to stimulate sequestration of AA in milk protein and minimize catabolism of excess AA.

Textbox 1.1. Composition of protein in ruminant diets

Dietary crude protein (CP) is defined as the nitrogen (N) content of feedstuffs × 6.25, based on the assumption that the average N content of feedstuffs is 16 g per 100 g of protein. Calculated CP content includes both protein and non-protein N. Metabolizable protein (MP) is defined as the true protein that is digested postruminally and the component amino acids (AA) absorbed by the small intestine (NRC, 2001). This consists of ruminally synthesized microbial CP, ruminally undegraded feed, and endogenous proteins secreted into the digesta. As the building blocks for protein synthesis, AA are the required nutrients with respect to dietary protein requirements. When feeding ruminants to optimize N efficiency, the distinction between dietary CP, estimated MP supply to the animal, and the AA composition of absorbed protein becomes important with respect to meeting requirements for maintenance, growth, lactation, and reproduction while minimizing catabolism of excess or underutilized AA.

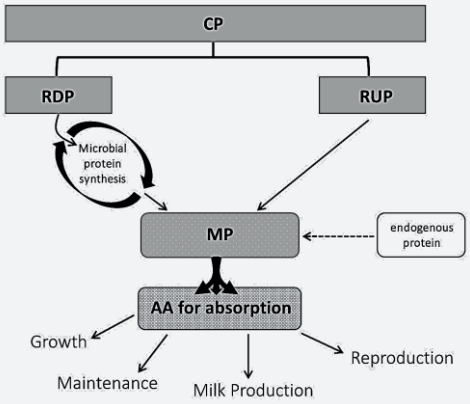

Figure 1.2. Compositional breakdown of dietary protein in ruminant diets. CP = crude protein; RDP = rumen-degradable protein; RUP = rumen-undegradable protein; MP = metabolizable protein; AA = amino acid

Constituents of MP are partly digested in the abomasum and hydrolysed to peptides and AA in the small intestine where they are absorbed. Approximately 64% of microbial CP is assumed to contribute to MP, and the digestibility of RUP sources are estimated based on the digestion coefficients for their feed ingredients (NRC, 2001). Feedstuffs vary widely in their relative proportions of protein and non-protein N, in their rate and extent of protein degradation in the rumen, and in the intestinal digestibility and AA composition of their ruminally undegraded fraction. An ideal pattern of absorbed AA theoretically exists for each of the physiological functions in **Figure 1.2**. Therefore, aiming to supply MP with a desirable AA profile to meet animal requirements (at a given production level and physiological state) is a target for improving the utilization efficiency of dietary protein in ruminants.

Postabsorptive Amino Acid Metabolism – Impact of Amino Acid Profile

Quantity and composition of absorbed AA impacts their metabolism in splanchnic tissues. The PDV removes a significant quantity of absorbed AA on a net basis for synthesis of endogenous export proteins, replenishment of sloughed epithelial cells, and energy generation (Lobley and Lapierre, 2003; Hanigan et al., 2004b). From the luminal AA supply, NEAA, specifically Glu and Gln, are more highly metabolised by the gastrointestinal tract compared with EAA (Lapierre et al., 2000; Berthiaume et al., 2001; El-Kadi et al., 2006). The PDV displays variable but low affinity for EAA (Lapierre et al., 2000; El-Kadi et al., 2006). The branched-chain AA (BCAA; Ile, Leu, Val) are the EAA most highly oxidized by the gastrointestinal tract (Berthiaume et al., 2001; El-Kadi et al., 2006). The PDV predominantly extracts EAA from the arterial supply which have avoided metabolism by peripheral tissues, or which originate from breakdown of endogenous protein pools (MacRae et al., 1997a,b; Hanigan et al., 2004b).

Amino acids entering the liver consist of those in the portal vein recently absorbed by or released from the PDV, and those arriving from peripheral circulation. Removal and metabolism of individual AA by the liver is variable (Bach et al., 2000; Blouin et al., 2002; Raggio et al., 2004), and is affected by portal blood flow rate and concentrations of AA and glucagon (Hanigan et al., 2004a; Crompton et al., 2018). Importantly, the liver regulates whole-body AA homeostasis by removing from arterial circulation those AA not used by peripheral tissues (Lobley and Lapierre, 2003; Hanigan et al., 2004a). Therefore, peripheral tissue AA uptake at first-pass affects the composition and supply of AA to the liver, and thus influences hepatic AA catabolism. Of the EAA, Met and Phe are affected most by hepatic metabolism (Hanigan et al., 2004a; Raggio et al., 2004; Berthiaume et al., 2006). The BCAA and Lys are preferentially metabolized in extra-hepatic tissues, resulting in almost no net hepatic uptake of these AA (Bach et al., 2000; Raggio et al., 2004; Berthiaume et al., 2006). Non-EAA are removed in greater quantities by the liver relative to EAA (Bach et al., 2000; Blouin et al., 2002; Berthiaume et al., 2006), because they are more likely to be in surplus with respect to productive use by the mammary gland (Hanigan et al., 2004a; Larsen et al., 2015).

Since the liver regulates arterial AA concentrations by removing those not used by peripheral tissues, it can be viewed as a responder, not a regulator, of AA supply to the mammary gland. The mammary gland is the greatest net user of EAA in a lactating dairy cow (Lapierre et al., 2012), and has specific nuances regarding AA use (discussed below and in **Textbox 1.2**). Mammary AA transport activity changes to match cellular AA supply with AA need (Cant et al., 2003). Amino acids not sequestered by the gland are released into venous drainage where they are available for uptake by PDV tissues, or enter the liver where they are metabolized (gluconeogenesis, synthesis of hepatic export and constituent proteins), released back into peripheral circulation, or are oxidized and excreted as urea. Within the

range of mammary synthetic capacity, stimulating milk protein synthesis will increase AA sequestration into milk protein and reduce recycling to splanchnic tissues. In this way, providing a postruminal AA profile with the potential to be used optimally by the mammary gland can be impactful with regard to milk N efficiency and the reduction of N excretion.

After accounting for the transfer of absorbed AA into milk protein, Hanigan et al. (2004b) estimated that at least two-thirds of postabsorptive EAA losses could be accounted for by splanchnic tissue use. Because net AA loss from oxidation in the udder is small (Bequette and Backwell, 1997; Bequette et al., 1998), the remaining losses likely occur in other major peripheral tissues such as skeletal muscle and skin (Lobley, 2003). Skeletal muscle is an important labile protein pool from which AA are mobilized when requirements for production and maintenance are not met, particularly in early lactation (Chibisa et al., 2008). The BCAA and Lys are particularly susceptible to extraction by peripheral tissues due to their propensity for extra-hepatic catabolism (Lapierre et al., 2005; Raggio et al., 2006). Furthermore, BCAA may be implicated in adipose tissue metabolism when circulating glucose levels are increased (Nichols et al., 2016; Curtis et al., 2018).

Mammary Gland Metabolism

Dietary intake and postabsorptive interactions influence mammary substrate supply, and in turn impact adaptation of the gland within the postabsorptive system to enable synthesis of milk macronutrients. Arterial metabolite concentrations, endocrine signaling and mammary regulation of blood flow affect mammary sequestration and intra-organ metabolism of substrates for milk protein, fat, and lactose production.

Metabolite uptake. Metabolite sequestration by the gland is altered according to tissue requirements for milk synthesis. Mammary metabolite removal is driven by blood flow rate to the tissue and respective metabolite concentrations in arterial blood (Hanigan et al., 1998; Cant et al., 2016). Net metabolite uptake is a consequence of bidirectional transport across the plasma membranes of mammary epithelial cells from arterial influx. Cellular transport systems for glucose, AA, and FA are dynamic and contribute to the overall tissue net uptake response that can be calculated with arteriovenous differences and accurate measures of blood flow (Shennan and Peaker, 2000). Importantly, net uptake does not represent transport activity, but represents intracellular sequestration of transported substrate via metabolic transformations. Transported substrate that is not sequestered intracellularly will return to venous circulation and not contribute to the arteriovenous concentration difference across the gland (Madsen et al., 2015). Blood flow rate to mammary tissue is altered by changes in vasodilatory systems and intramammary balance of metabolites and ATP (Cant et al., 2003; Cieslar et al., 2014), both of which are affected by changes in hormone signaling and nutritional status. Opposing effects of dietary nutrients on concentrations of

milk precursors and mammary ATP utilization illicit varying effects on observed mammary blood flow rate. For example, insulin and glucose infusions decrease circulating concentrations of energy-generating 2-carbon compounds, and mammary blood flow increases in response (Mackle et al., 2000; Bequette et al., 2001; Curtis et al., 2018). Further, when deficiencies of single AA are imposed, mammary blood flow may increase (Bequette et al., 2000; Cant et al., 2003).

If blood flow to the mammary gland is increased by a single metabolite, delivery of the other metabolites in plasma will also be increased. When considering the relationship between arterial concentration, blood flow, and metabolite removal, the mammary gland kinetic response to greater blood flow is not necessarily similar to its response to greater metabolite concentrations (Hanigan et al., 1998). A change in arterial concentration of a given metabolite or a change in blood flow will not yield equal changes in mammary uptake, unless tissue affinity for that metabolite is invariably high. Mammary clearance rate is the proportion of metabolite uptake relative to its concentration in mammary venous drainage. This parameter describes the affinity for uptake relative to mammary cell supply at a given moment, where mammary cell supply is more closely related to what appears in the venous drainage, representing extracellular concentrations (what was not transported into mammary cells, or what flowed out of mammary cells unsequestered).

Intramammary metabolism. Net uptake represents sequestration of metabolites into mammary cells; therefore, in order for uptake of a particular metabolite to be affected, its intramammary metabolism must be up- or down-regulated. Net uptake of metabolites relative to their output in milk can be used to estimate intramammary synthetic and catabolic pathways. Mammary glands take up glucose, acetate, BHB, FA, and AA from blood to synthesize the major milk components lactose, fat, and protein. Considerable intramammary catabolism and repartitioning of N and carbon can occur between metabolites, and between pathways that generate energy, such as the TCA cycle. Bovine mammary glands are unable to convert gluconeogenic substrates (including lactate, pyruvate, glycerol and glutamate) to glucose due to the virtual absence of glucose-6-phosphatase in bovine mammary tissue (Scott et al., 1976), but synthesis of galactose is possible from hexose phosphate intermediates via the pentose phosphate pathway (Wood et al., 1965).

The majority of mammary gland glucose uptake is used for lactose synthesis, and at least 80% of lactose is derived from plasma glucose (Bickerstaffe et al., 1974). Mammary gland priority for glucose is high relative to other body tissues, but rates of mammary glucose sequestration and secretion of lactose are not always correlated, especially when arterial glucose concentrations are increased (Nielsen et al., 2001; Cant et al. 2002; Rigout et al. 2002). Lactose synthesis is also regulated by mammary mechanisms independent of glucose supply, such as glucose phosphorylation and transport capacity (Xiao and Cant, 2005). In addition to lactose synthesis, glucose is required for synthesis of glycerol and will be oxidized

to yield NADPH for milk fat synthesis (0.12 and 0.19 g glucose per g of milk fat for these processes, respectively; Dijkstra et al., 1996). Intramammary glucose not used for synthesis of lactose and milk fat is oxidized to provide carbon for NEAA synthesis and contributes as an energy source yielding NADPH and ATP (Smith et al., 1983). Reported oxidation of intramammary glucose ranges from 11-15% in mammary tissue in vitro and in vivo (Bickerstaffe et al., 1974; Xiao and Cant, 2005).

Acetate and BHB are the main substrates for milk FA synthesis in ruminant mammary glands (Bauman et al., 1970; Dils, 1983). Acetate is oxidized to generate NADPH and ATP, and makes a quantitatively more significant contribution to oxidative metabolism in the gland than glucose (Bickerstaffe et al., 1974). Fatty acids with chain lengths of <16 carbons, and approximately 50% of 16-carbon FA, are synthesized de novo by the mammary gland where acetyl-CoA acts as a primer, malonyl-CoA elongates the primer via the enzyme FA synthase, and NADPH is used for the reductive steps of this elongation. Fatty acids with chain lengths >16 carbons, and the other 50% of 16-carbon FA, are derived from ingested dietary FA or from those that are mobilized from adipose tissue. Because de novo FA synthesis uses glucose for glycerol and NADPH, alterations in availability of preformed LCFA (≥16 carbons) for mammary sequestration can have an effect on intramammary glucose balance. Reduction in mammary de novo FA synthesis and the associated glucose utilization could contribute glucose for lactose production when diets are supplemented with LCFA (Chilliard, 1993; Hammon et al., 2008).

Amino acids are used by the mammary gland with a certain pattern, described in **Textbox 1.2**. Together, group 1 and group 2 AA encompass all 10 AA that are essential for dairy cattle, with the addition of Tyr that is a NEAA. Group 2 AA in mammary cells that are in excess of their requirement for output in milk true protein are used mainly for de novo synthesis of NEAA and are catabolized for energy. In addition, AA may contribute to galactose synthesis (Lapierre et al., 2013; Maxin et al., 2013b), and ketogenic AA can contribute to milk fat synthesis through their metabolism to acetyl-CoA and other TCA cycle intermediates. Non-EAA are typically taken up by the gland in insufficient amounts relative to their output in milk, and have no stimulatory effect on milk protein synthesis when supplemented postruminally (Metcalf et al., 1996; Doepel and Lapierre, 2010). During insulin stimulation and when protein supply is limited, the gland retains more EAA from arterial influx, reduces intramammary AA catabolism, and reduces AA exit via venous drainage (Mackle et al., 2000; Bequette et al., 2001).

Textbox 1.2. Amino acid groups with respect to mammary gland utilization

Observations from early studies of mammary gland metabolism led to the definition of 2 groups of AA differing with respect to their pattern of mammary gland utilization (Mepham, 1982):

Group 1: His, Met, Phe (+Tyr), and Trp

Group 2 : Arg, Ile, Leu, Lys, Thr, and Val

Group 1 AA are defined by their 1:1 ratio of mammary gland uptake to milk protein output (U:O). Theoretically, transfer of group 1 AA from blood plasma into milk is stoichiometric. Although the U:O ratio is maintained close to unity for this group as a whole, it can vary for the individual EAA (Lapierre et al., 2012). Biological variation resulting in U:O <1 implies that intramammary compensation is occurring or that peptides are taken up by the gland to support protein synthesis, and U:O >1 implies that AA are being metabolized within the gland. Intramammary Phe is metabolized exclusively to Tyr, so consideration of Phe+Tyr is required in order to obtain an accurate representation of intramammary Phe metabolism as a group 1 AA. Mepham (1982) did not include His in either group, but it has been established as a group 1 AA (Lapierre et al., 2012), and is considered as such in current mammary net balance calculations.

Group 2 AA are taken up by the gland in excess of their output in milk protein (U:O >1), but there is variation among the AA in this group (Lapierre et al., 2012). The U:O of Arg is always in the most considerable excess, and averages 2.5 under a range of protein supplies. The mammary gland will extract and use an excess of Ile, Leu, and Val, but excess uptake of these AA appears to be non-obligate when supply is limited, whereas Lys displays some level of obligate catabolism, even at a marginal supply. The U:O of Thr does not show a clear pattern with respect to supply, and in some cases may appear to behave more similarly to group 1 AA than group 2 AA.

Mammary Gland Protein Synthesis

Intracellular pathways affecting milk component synthesis play a significant regulatory role in the efficiency with which dietary nutrients are incorporated into milk (Nielsen et al., 2001; Qiao et al., 2005; Cant et al., 2018). In particular, focusing on the molecular events regulating protein synthesis in response to dietary changes and physiological and hormonal states are necessary to completely understand the interactions involved in the transfer of dietary N into milk N.

Besides their role as metabolic substrates, the function of AA as signalling molecules is important to protein metabolism. This affects the way tissues interact with respect to AA partitioning through hormone cascades, and at the cellular level through cell signaling pathways. Protein synthesis is tightly regulated at the transcriptional and translational levels. The intracellular signaling cascades nucleated by the mechanistic target of rapamycin complex 1 (mTORC1; **Figure 1.3**, **Textbox 1.3**) and the integrated stress response (ISR) network (**Figure 1.4**, **Textbox 1.4**) have been proposed as candidate signaling pathways regulating the effects of nutritional manipulation on milk protein synthesis (Moshel et al., 2006; Burgos et al., 2010; Appuhamy et al., 2011). In mammary cells in vitro and in vivo, these pathways respond to energy status and AA supply to affect translational control of cellular protein synthesis. Both mTORC1 and the ISR network have been implicated in acute regulation of protein synthesis in mammary epithelial cells in vitro (Burgos et al., 2010; Appuhamy et al., 2011; Burgos et al., 2013) and in vivo within 36 h (Rius et al., 2010b; Toerien et al., 2010), but flux through these networks does not always explain the transcriptional and translational response of mammary secretory cells in vivo when milk protein yield is altered during chronic nutritional intervention over several days (Toerien et al., 2010; Doelman et al., 2015; Nichols et al., 2017). As an alternative to upregulated machinery in single cells, protein secretory capacity could also be enhanced through an increase in the number of secretory cells. Activation of differentiation of mammary epithelial progenitor cells into secretory cells in response to long-term nutritional intervention would support persistency of milk protein secretory capacity. Milk secretory cell differentiation requires development of an endoplasmic reticulum (ER; Oka and Topper, 1971), which is the site of synthesis of milk proteins, fat globules, and the subunits of lactose synthase. Biogenesis and function of the ER is regulated by signaling pathways that constitute the unfolded protein response (UPR; **Figure 1.5**, **Textbox 1.5**). It is likely that the temporal response of the lactating mammary gland in the adaptation to fluctuating energy and protein status and secretory load is highly regulated through synchronicity between these cellular machineries, but conclusive evidence of this is lacking.

Textbox 1.3. The mTORC1 regulation of downstream protein synthesis

The intracellular mechanistic target of rapamycin complex 1 (mTORC1) is activated by hormones [insulin, insulin-like growth factor 1 (IGF-1)], intracellular nutrients [amino acids (AA)], and energy status [ATP:AMP ratio]. Phosphorylated adenosine monophosphate (AMPK) activates ATP-generating processes in the cell while inhibiting ATP-consuming functions, such as protein synthesis (Ma and Blenis, 2009). Under anabolic conditions, hormones and AA lift inhibition on mTORC1. At this point, the complex may apply its downstream effects on translation initiation factors and ribosomal kinases to mediate mRNA translation (Saxton and Sabatini, 2017). Phosphorylation of the binding protein of eukaryotic initiation factor 4E (4EBP1) and of a kinase of the ribosomal protein S6 kinase 1 (S6K1) accelerates initiation of mRNA translation. In addition to its well-established role in regulating mRNA translation for protein synthesis, mTORC1 has been implicated in many other signaling networks including the integrated stress response, lipid and nucleotide synthesis, mitochondrial function, protein degradation, and cell apoptosis and proliferation (Morita et al., 2015).

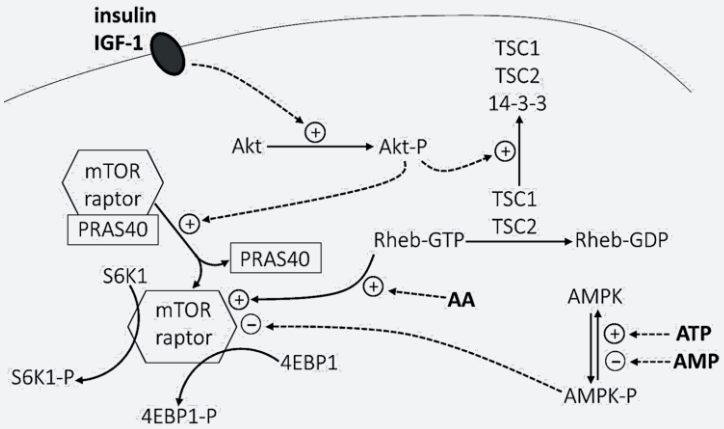

Figure 1.3. Regulation of the mechanistic target of rapamycin (mTOR) complex 1 to initiate downstream mRNA translation (adapted from Cant et al., 2018). Solid arrows represent mass flux and dashed arrows represent effector mechanisms; + and – represent activation and inhibition, respectively; -P represents phosphorylation. 4EBP1 = eukaryotic initiation factor 4E-binding protein 1; 14-3-3 = 14-3-3 protein; AA = amino acid; Akt = protein kinase B; AMP = adenosine monophosphate; AMPK = adenosine monophosphate-activated protein kinase; ATP = adenosine triphosphate; GDP = guanosine diphosphate; GTP = guanosine triphosphate; IGF-1 = insulin-like growth factor 1; PRAS40 = 40-kDa proline-rich Akt substrate; raptor = regulatory associated protein of mTOR; Rheb = Ras homolog enriched in brain; S6K1 = 70-kDa ribosomal protein S6 kinase 1; TSC = tuberous sclerosis complex.

Textbox 1.4. The ISR pathway in protein secretory load

The integrated stress response (ISR) network responds to intracellular stress conditions to reduce cellular anabolic load. The central pathway of this network involves activation of eukaryotic initiation factor 2 (eIF2) through the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) by the eIF2B enzyme. In its GTP-bound state, eIF2 initiates translation and elongation of peptide chains. The α subunit of eIF2 is phosphorylated by 4 kinases. Of these, protein kinase R-like endoplasmic reticulum kinase (PERK) is responsive to ATP-status and endoplasmic reticulum stress, and general control nondepressible 2 kinase (GCN2) is responsive to amino acid deprivation. Phosphorylated eIF2α inhibits the catalytic eIF2Bε subunit, compromising the eIF2-GDP to eIF2-GTP exchange and reducing global protein synthesis (Proud, 2005; Muaddi et al., 2010; Baird and Wek, 2012). Cross-talk between the ISR network and the mTORC1 pathway has been suggested through protein kinase B (Akt) and glycogen synthase kinase-3 (GSK3) to regulate mRNA translation (Rommel et al., 2001; Proud et al., 2005). GSK3 inhibits eIF2B through phosphorylation on the ε subunit to reduce protein translation. Activation of Akt inhibits GSK3, which may link regulation of the ISR network and the mechanistic target of rapamycin complex 1 through insulin and insulin-like growth factor 1 (IGF-1).

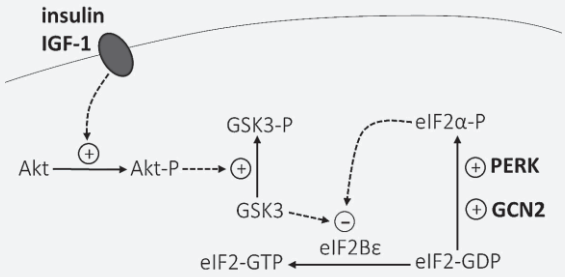

Figure 1.4. Regulation of the integrated stress response pathway (adapted from Cant et al., 2018). Solid arrows represent mass flux and dashed arrows represent effector mechanisms; + and – represent activation and inhibition, respectively; -P represents phosphorylation. Akt = protein kinase B; eIF2 = eukaryotic initiation factor 2; GCN2 = general control nondepressible 2; GDP = guanosine diphosphate; GSK3 = glycogen synthase kinase-3; GTP = guanosine triphosphate; IGF-1 = insulin-like growth factor; PERK = protein kinase R-like endoplasmic reticulum kinase.

Textbox 1.5. The UPR in secretory cell differentiation

The unfolded protein response (UPR) initiates a number of cellular responses to restore ER homeostasis. Signalling cascades of the UPR play a role in defining the phenotype of secretory cells and in their adaptation to stimuli (Reimold et al., 2001; Huh et al., 2010; Davis et al., 2016). Initiation of the UPR occurs by dissociation of the chaperon protein BiP onto 3 ER transmembrane proteins, protein kinase R-like endoplasmic reticulum kinase (PERK), activating transcription factor (ATF) 6, and inositol-requiring enzyme 1 (IRE1), each initiating different UPR arms (Hetz et al., 2015). Phosphorylation of PERK activates the ISR network supressing global protein synthesis and stimulating translation of ATF4 and its targets, C/EBP homologous protein (CHOP) and growth arrest and DNA damage-inducible 34 (GADD34; Walter and Ron, 2011). GADD34 dephosphorylates ISR constituents, counteracting the effects of PERK, to resume protein synthesis once homeostasis of ER function is restored (Ma and Hendershot, 2003). The second arm of the UPR through ATF6 activates transcription of genes for ER-associated proteins, including BiP and X-box binding protein (XBP1). The third UPR arm mediated by phosphorylated IRE1 excises XBP1 mRNA to generate the active spliced form. The product of spliced XBP1 translation is a transcription factor that stimulates expression of proteins involved in differentiation of the secretory phenotype, including rough ER formation and secretory vesicle maturation (Huh et al., 2010).

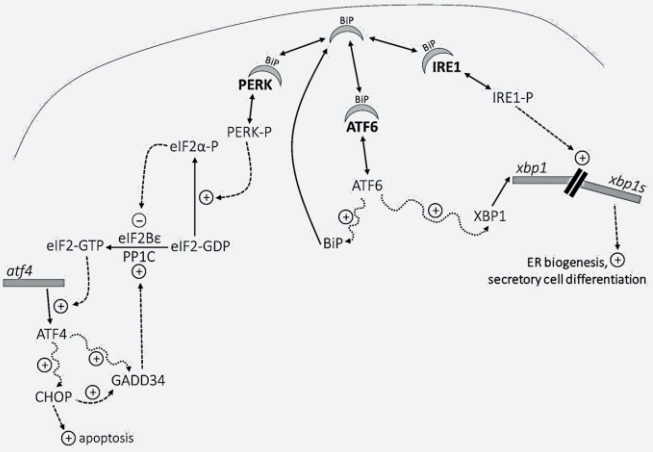

Figure 1.5. Arms of the unfolded protein response contributing to adaptation of secretory capacity and secretory cell differentiation (adapted from Cant et al., 2018). Solid arrows represent mass flux, dashed arrows represent effector mechanisms, wavy dotted lines represent activation of gene transcription, + and – represent activation and inhibition, respectively; -P represents phosphorylation. ATF = activating transcription factor; BiP = binding protein; CHOP = C/EBP homologous protein; eIF = eukaryotic initiation factor; ER = endoplasmic reticulum; GADD34 = growth arrest and DNA damage-inducible 34; IRE1 = inositol-requiring enzyme 1; PERK = protein kinase R-like endoplasmic reticulum kinase; PP1C = protein phosphatase 1C; XBP1 = X-box binding protein-1; xbp1s = XBP1 splice fragment.

Postabsorptive Energy–Protein Interactions

The interactions between energy and protein in postabsorptive metabolism are complex and impactful with regard to efficiency of milk production and dietary N use. Increases in total milk yield observed when dietary CP or MP content is increased are in part because of a concomitant increase in dry matter intake usually observed with higher protein diets (Broderick, 2003; Daniel et al., 2016; Martineau et al., 2016). Increasing postruminal AA supply commonly increases whole-body glucose flux, total milk yield, and lactose yield (Clark et al., 1977; Lemosquet et al., 2009; Galindo et al., 2011). Amino acids could contribute to whole-body glucose flux through glucose-sparing in the PDV or through gluconeogenesis, or through a combination of both. However, abomasal casein infusions did not increase portal glucose absorption (El-Kadi et al., 2006) or portal glucose flux (Galindo et al., 2011). The greater contribution of AA to whole-body glucose appearance is assumed to be mainly though hepatic gluconeogenesis (Galindo et al., 2011). Increasing glucogenic energy at low and high dietary protein levels can improve transfer efficiency of absorbed AA into milk protein by reducing AA catabolism across the gut and splanchnic bed (Hanigan et al., 2004b; Cantalapiedra-Hijar et al., 2014b). Amino acid supply to the liver can be altered by peripheral tissue use, and this largely determines the magnitude of AA catabolism (Lobley and Lapierre, 2003; Raggio et al., 2004). Therefore, regardless of energy type, the AA profile available for the mammary gland plays an essential role in postabsorptive protein metabolism, the partitioning of N between excretion in milk and manure, and retention in body tissues.

Milk protein yield responses to provision of additional energy in the form of glucose, starch, propionate or acetate have been positive in some reports (Rulquin et al., 2004; Raggio et al., 2006; Rius et al., 2010b; Safayi and Nielsen, 2013), but nil in others (Reynolds et al., 2001; Purdie et al., 2008; Nichols et al., 2016). A portion of this difference may arise from differences in metabolite partitioning between the liver and peripheral tissues as a result of endocrine responses to dietary energy source, such as those stimulated by starch compared with fat or fiber. For example, insulin, which can stimulate lipogenesis and redirect acetate, BHB, and some AA towards peripheral tissues (Griinari et al., 1997; Nichols et al., 2016; Curtis et al., 2018), responds to increased circulating glucose, but not to increased FA (Palmquist and Jenkins, 1980; van Knegsel et al., 2007b; van Hoeij et al., 2017). This endocrine response promotes body energy retention which is usually observed with glucogenic diets, whereas lipogenic diets are more likely to promote transfer of dietary energy into milk. Furthermore, mammary blood flow increases in response to decreased circulating concentrations of energy-generating 2-carbon compounds, impacting EAA utilization by the gland (Mackle et al., 2000; Bequette et al., 2001; Curtis et al., 2018). Mammary EAA utilization in response to increased circulating LCFA supply has not been well characterised. At the mammary cell level, protein synthesis regulatory cascades are sensitive to insulin, energy level, and AA (Burgos et al., 2010; Rius et al., 2010b; Appuhamy et al., 2011). It is not solely glucose supply to the

mammary gland that regulates lactose synthesis (Nielsen et al., 2001; Cant et al. 2002; Xiao and Cant, 2005), and signaling pathways responding to EAA may interact in the regulation of protein and lactose synthetic capacity and secretion at the cellular level (Anderson et al., 2007; Nichols et al., 2017) .

Knowledge Gaps

Efforts and innovations in intensive dairy farming continue to advance towards precision feeding, maximizing nutrient use efficiency, and minimizing emissions to the environment. However, inconsistencies and gaps exist in current knowledge which limit our ability to improve the transfer of energy and N from a variety of feedstuffs into milk. With regard to the effects of glucogenic, lipogenic, and aminogenic nutrients on postabsorptive metabolic processes and their impact on N efficiency of lactating dairy cows, the following knowledge gaps have been identified, and will be addressed in the following chapters of this thesis:

- Interactions between lipogenic energy and dietary MP level, and how they differ from the interactions between glucogenic energy and MP level, with respect to milk component production and milk N efficiency.
- Effects of lipogenic energy on mammary gland AA metabolism.
- Quantification of whole-body energy and N balance when MP level is increased by postruminal supplementation of EAA at different levels and in different profiles.
- Effect of postruminal EAA profile on mammary gland metabolite utilization.
- In vivo characterisation of cellular regulation of protein synthesis in the mammary gland in response to nutritional intervention.

RESEARCH OBJECTIVES AND OUTLINE

Based on the knowledge gaps identified above, the objective of this thesis was to investigate effects of postruminal absorption of different energy substrates (lipoogenic, glucogenic, aminogenic) and AA profiles, at the whole-body and mammary gland level, with respect to their application for improving milk N efficiency in dairy cattle. To this end, the studies presented in the following chapters take a sequential approach to elucidating the effects of nutritional intervention on metabolism at the animal, tissue, and cell level, by investigating parameters such as lactation performance and whole-body energy and N balance, net metabolite flux across the mammary gland, and mammary cell regulation of biosynthetic processes.

In **Chapter 2**, the effects of energy supplementation from rumen-protected protein or rumen-inert fat in dairy cow diets on milk production, milk composition, and nutrient digestibility are presented. **Chapter 3** describes the effects of these energy sources on mammary gland metabolite kinetics, and **Chapter 4** discusses their effects on expression of genes associated with mammary gland cellular pathways contributing to energy generation and secretory capacity, using RNA isolated from milk fat. The experiment described in **Chapter 5** used climate respiration chambers to measure energy and N balance of cows abomasally infused with glucose, palm olein, and EAA. Mammary gland metabolite kinetics in response to these infusions are discussed in **Chapter 6**. The experiment described in **Chapter 7** investigated the effect of AA profile within a constant MP supply on energy and N balance by abomasally infusing different EAA profiles into cows housed in climate respiration chambers, and **Chapter 8** describes mammary gland metabolite kinetics in response to the same EAA profiles. Finally, **Chapter 9** offers an integrative discussion of the results presented throughout this thesis.

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Chapter 2

Feed and nitrogen efficiency are affected differently but milk lactose production is stimulated equally when isoenergetic protein and fat is supplemented in lactating dairy cow diets

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ABSTRACT. Fifty-six Holstein-Friesian cows were used in a randomized complete block design to test the effects of supplemental energy from protein (PT) and fat (FT) on lactation performance and nutrient digestibility in a 2 × 2 factorial arrangement. During the control period, cows were adapted for 28 d to a basal total mixed ration consisting of 34% grass silage, 33% corn silage, 5% grass hay, and 28% concentrate on a dry matter (DM) basis. Experimental rations were fed for 28 d immediately following the control period and consisted of (1) low protein, low fat (LP/LF), (2) high protein, low fat (HP/LF), (3) low protein, high fat (LP/HF), or (4) high protein and high fat (HP/HF). To obtain the HP and HF diets, intake of the basal ration was restricted and supplemented isoenergetically (net energy basis) with 2.0 kg/d of rumen-protected protein (soybean + rapeseed, 50:50 mixture on DM basis) and 0.68 kg/d of hydrogenated palm fatty acids (FA) on a DM basis. Milk production and composition, nutrient intake, and apparent digestibility were measured during the final 7 d of the control and experimental periods. No interaction was found between PT and FT on milk production and composition. Yields of milk, fat- and protein-corrected milk, and lactose increased in response to PT and FT and lactose concentration was unaffected by treatment. Milk protein concentration and yield increased in response to PT, and protein yield tended to increase in response to FT. Milk fat concentration and yield increased in response to FT and were unaffected by PT. Milk urea concentration increased and nitrogen efficiency decreased in response to PT. Feed and nitrogen efficiency were highest on the LP/HF diet and both parameters increased in response to FT, whereas milk urea concentration was not affected by FT. Energy from fat increased the concentration and yield of ≥16-carbon FA in milk and decreased the concentration of FA synthesized de novo, but had no effect on their yield. Concentration and yield of de novo-synthesized FA increased in response to PT. Concentration and yield of polyunsaturated FA increased and decreased in response to PT and FT, respectively. Apparent total-tract digestibility of crude fat decreased in response to PT, and FT increased crude protein digestibility. Energy supplementation through rumen-inert hydrogenated palm FA appears to be an efficient feeding strategy to stimulate milk production with regard to feed and nitrogen efficiency compared with supplementing an isoenergetic level of rumen-protected protein.

Key words: rumen-protected protein, hydrogenated palm fatty acid, milk lactose, digestibility

INTRODUCTION

Rising demand for sustainably produced milk protein products (OECD/FAO, 2015) emphasizes the importance of understanding how energy-yielding feedstuffs affect efficient synthesis of milk and its components. Several studies compare the effects of glucogenic substrates with lipogenic substrates (Grum et al., 1996; van Knegsel et al., 2007; Boerman et al., 2015), but comparisons between isoenergetic supplements of aminogenic and lipogenic nutrients are scarce. It is well established that protein degradation in the rumen increases with dietary CP content. If available protein in the rumen exceeds microbial needs, or if availability of AA exceeds postabsorptive requirements, excess NH_3 is produced and excreted as urea (Colmenero and Broderick, 2006; Dijkstra et al., 2013). Energy is required to process and excrete a surplus of N, which increases heat production and decreases retained energy and milk energy (Reed et al., 2017). As an alternative to feeding high-CP diets, balancing rations to supply sufficient levels of EAA in support of milk protein synthesis has been shown to improve postabsorptive N efficiency and increase milk protein yield (Haque et al., 2012; Lee et al., 2012; Arriola Apelo et al., 2014). Thus, great focus has been placed on maximizing dietary protein utilization by increasing postruminal supply of EAA and energy precursors through rumen-protected (RP) products.

Lactose is the main osmotic driver of total milk yield; therefore, the glucogenic capacity of a dietary ingredient can have a profound effect on lactation performance. Glucose supply to the mammary gland can be increased through absorption of glucogenic nutrients and flux through hepatic gluconeogenesis. Ideally, the majority of dietary AA would be used by the mammary gland for milk protein synthesis at first pass, but AA will be used for gluconeogenesis when concomitant supply of glucogenic energy is limiting for productive purposes. The corollary is that, by supplying rapidly available energy precursors, a larger proportion of dietary AA can be used for milk protein synthesis (Rius et al., 2010a,b). However, lactation responses to supplemental glucose availability are not always positive and depend on the nutritional status and production potential of the animal (Cant et al., 2002; Nichols et al., 2016). Saturated fat is energy dense and included in lactating cow diets as a source of nonfermentable energy. Saturated long-chain fatty acids (LCFA) are supplemented into dairy rations with the goal of increasing milk production while minimizing inhibitory effects on functional digestibility (Jenkins, 1993). In contrast, supplementation with unsaturated fatty acids (FA) has been associated with perturbed DMI, altered ruminal biohydrogenation and microbial activity, and depressed milk fat synthesis (Allen, 2000; Baumgard et al., 2001). Saturated LCFA have the potential to provide high amounts of gross energy to the animal, but on a net basis do not directly contribute to glucose precursors necessary for lactose synthesis by the mammary gland. The apparent effect of supplemental fat on DMI and digestibility leads to variation in cow performance across studies (Rabiee et al., 2012). If DMI is not severely decreased and digestibility remains unaffected, the high

energy density of rumen-inert fat supplements increases ME consumption and may improve energetic efficiency through the direct transfer of FA into milk (Hammon et al., 2008; Boerman et al., 2015).

The interaction between AA and glucose or glucose precursors on milk production has been examined in several studies (Raggio et al., 2006; Lemosquet et al., 2009; Nichols et al., 2016). Furthermore, many studies compare the effects of glucogenic or lipogenic nutrients through forage substitution in the diet (Cantalapiedra-Hijar et al., 2014; Boerman et al., 2015; Piantoni et al., 2015) or abomasal infusion (Oldick et al., 1997), but the interaction between protein and fat supplementation has not been extensively investigated. We expected that, at isoenergetic levels, the inherent properties of aminogenic versus lipogenic energy would differently affect whole-body metabolism, which may be reflected in milk production responses. Supplemental protein may yield AA and stimulate protein synthesis in both the mammary gland and extramammary tissues, but as a glucogenic substrate, AA may yield glucose potentially in support of lactose synthesis. In contrast, fat supplementation provides FA that may contribute to milk fat yield but do not directly yield substrates to increase milk protein or lactose synthesis. However, this may be achieved if intramammary glucose is spared through a reduction in de novo FA synthesis. Thus, our objective was to characterize the independent and interactive effects of isoenergetic protein and fat supplementation on milk production and composition and nutrient digestibility, where changes to the energy content of treatment diets were accomplished by supplementation with RP protein and rumen-inert fat.

MATERIALS AND METHODS

Experimental Design and Treatment Diets

All experimental procedures were approved by the Animal Care and Use Committee for Nutreco Nederland B.V. (Amersfoort, the Netherlands) and conducted under the Dutch Law on Animal Experiments. Fifty-six Holstein-Friesian dairy cows (167 ± 87 DIM; 2.8 ± 1.9 lactations; 20 primiparous, 36 multiparous) were used in a randomized complete block design where two 28-d feeding periods (control and experimental) consisted of 21 d of diet adaptation and 7 d of data collection. Supplemental energy from protein (**PT**) or fat (**FT**) was tested in a factorial arrangement. During the control period, cows were fed a basal diet as a TMR meeting NE_L and MP requirements consisting of 34% grass silage, 33% corn silage, 5% grass hay, and 28% concentrate on a DM basis. Cows were blocked by parity, DIM, and DMI of the final 7 d of the control period. Within blocks, cows were randomly assigned to 1 of 4 diets for the experimental period: 1) low protein, low fat (**LP/LF**; 95% MP, 95% NE_L), 2) high protein, low fat (**HP/LF**; 131% MP, 107% NE_L), 3) low protein, high fat (**LP/HF**; 95% MP, 107% NE_L), or 4) high protein and high fat (**HP/HF**; 131% MP, 119% NE_L), where MP and NE_L are expressed relative to animal requirements in the control period. For all treatments, basal diet

intake for individual cows was restricted to 95% of their ad libitum intake recorded during the control period. A 2.0-kg 50:50 mixture (DM basis) of RP soybean meal and rapeseed meal (SoyPass + RaPass; both rumen-protected by xylose-treatment; Borregaard LignoTech, Sarpsborg, Norway) and 0.68 kg rumen-inert hydrogenated LCFA (mainly C16:0 and C18:0; Hidropalm; Norel, Madrid, Spain) on a DM basis were supplemented into the concentrate portion of each basal TMR to obtain the HP and HF diets. Therefore, feed intake restriction was accomplished by limiting only the basal portion of the diet (to minimize variation in basal diet intake across treatments), and additional daily intake on HP and HF diets arose from the addition of the respective supplement. Therefore, differences in DMI between each diet were created by design (**Table 2.1**) to allow isoenergetic intake of protein and fat on diets with HP and HF.

Ingredient and chemical composition of the control and experimental diets are presented in **Tables 2.1 and 2.2**. During the final 10 d of each period, concentrates contained 0.25% titanium dioxide as an inert marker for estimation of apparent total-tract digestibility (ATTD). Cows were housed in a free-stall barn with ad libitum access to water. The TMR were mixed and distributed once daily at 1000 h via electronic intake control boxes (Insentec, Marknesse, the Netherlands). Refused feed was removed 3 times per week and intake control boxes were checked daily for functionality. Feed intake measurements of individual animals were facilitated via electronic ear transponders which allowed cows (n = 14) access to a set of 9 intake control boxes supplying their respective diet. When individual cows reached their set daily feed intake according to their treatment, access to the intake control boxes was denied until the next day at 0400 h when the boxes were re-set for the next 24-h period. Cows were milked twice daily at 530 and 1630 h and milk production was recorded electronically at each milking.

Table 2.1. Formulated TMR component intake and composition of concentrate fed during the control period (CNTL) and during the experimental period for the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or from protein and fat (HP/HF)

Item	CNTL	LP/LF	HP/LF	LP/HF	HP/HF
Ingredients, kg of DM/d					
Grass silage	7.1	6.7	6.7	6.7	6.7
Corn silage	6.9	6.5	6.5	6.5	6.5
Grass hay	1.00	0.98	0.98	0.98	0.98
Concentrate[1]	5.8	5.5	5.5	5.5	5.5
SoyPass + RaPass[2]	-	-	2.0	-	2.0
Hidropalm[3]	-	-	-	0.68	0.68
Total predicted DMI, kg/d	20.8	19.7	21.7	20.5	22.5
Concentrate composition,[4] g/kg of DM					
Soy hulls	328	328	239	292	219
Wheat	264	264	193	235	177
Soybean meal	228	228	166	203	152
Rapeseed meal	100	100	73	89	67
Limestone, 36% Ca	10	10	7	9	7
Magnesium oxide	7	7	5	6	5
Monocalcium phosphate	7	7	5	6	5
Mineral and vitamin premix	5	5	6	5	6
Sodium chloride	5	5	4	5	3
Urea	3	3	-	3	-
SoyPass	43	43	167	38	154
RaPass	-	-	136	-	125
Hidropalm	-	-	-	109	82

[1]Portion of concentrate excluding rumen-protected protein and hydrogenated palm fatty acid supplements. The basal concentrate composition (excluding the addition of SoyPass + RaPass or Hidropalm) was identical for all treatments.

[2]A 50:50 mix (DM basis) of SoyPass (xylose-treated rumen-protected soybean meal) and RaPass (xylose-treated rumen-protected rapeseed meal) manufactured by Borregaard LignoTech, Sarpsborg, Norway.

[3]Hidropalm (hydrogenated palm fatty acids; 85% free fatty acids and 15% triglycerides; 50% C16:0 and 47% C18:0) manufactured by Norel Animal Nutrition (Madrid, Spain).

[4]Titanium dioxide was included at 0.25% of DM to the concentrate fed in the final 10 d of each period.

Table 2.2. Analyzed and calculated chemical composition of ingredients (grass silage, corn silage, grass hay, and concentrates) and complete TMR fed during the control period (CNTL) and during the experimental period for the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or from protein and fat (HP/HF)

	Roughage[1]			Concentrate					TMR[2]				
Item	Grass silage	Corn silage	Grass hay	CNTL	LP/LF	HP/LF	LP/HF	HP/HF	CNTL	LP/LF	HP/LF	LP/HF	HP/HF
Analyzed[3]													
DM, g/kg	539	359	867	889	893	860	907	902	519	522	539	534	553
Gross energy, MJ/kg of DM	18.5	18.6	18.0	17.9	17.9	17.7	20.8	20.0	18.3	18.3	18.2	19.2	19.1
Crude ash	119	35	63	70	80	75	73	75	75	78	76	76	76
CP	187	61	61	239	251	285	221	273	153	157	177	151	176
Crude fat	45	44	25	32	34	24	156	126	40	41	37	77	73
NDF	445	369	607	336	308	338	272	285	398	390	393	376	371
ADF	258	203	338	216	206	201	179	169	232	229	225	220	213
ADL	14	14	34	13	13	26	11	21	15	15	19	14	17
Starch	NA[4]	363	NA	208	182	144	155	119	178	171	159	163	149
Sugar	16	NA	124	60	60	49	52	64	28	28	27	27	34
Calculated[5]													
DVE[6]	80	45	58	152	152	205	131	178	87	87	111	83	105
OEB[7]	32	-40	8	56	56	47	49	40	14	14	14	13	13
NE_L,[8] MJ/kg DM	6.25	6.87	5.45	7.22	7.19	7.57	8.95	8.63	6.68	6.68	6.85	7.23	7.26

[1]Identical roughages were used in the control and experimental periods.

[2]Values for TMR were calculated based on ration composition and analyzed and calculated values obtained for roughages and concentrate.

[3]Values for all components were analyzed by wet chemistry (Wageningen University, Wageningen, the Netherlands) and are presented in g/kg of DM unless otherwise stated.

[4]NA = not analyzed.

[5]Calculated values for grass silage and corn silage were obtained from near-infrared spectroscopy analysis (Eurofins Agro, Wageningen, the Netherlands) and values for grass hay were obtained from the CVB Table Ruminants (CVB, 2008). Concentrate values were estimated from raw material composition from the CVB Table Ruminants (CVB, 2008).

[6]Intestinal digestible protein (CVB, 2008).

[7]Rumen degradable protein balance (CVB, 2008).

[8]NE_L calculated with the VEM system (CVB, 2008).

Sample Collection and Analysis

Samples of TMR, concentrates, grass silage, corn silage, and grass hay were collected once weekly during each period and stored at -20°C until analysis. Feces was collected by rectal grab sampling on d 24, 25, and 26 of each period at 730 and 1330, 930 and 1530, and 1230 and 1830 h, respectively, to account for potential diurnal variation in digestibility over 3 d. At each collection time point, 75-g aliquots of fresh feces were immediately pooled into a composite sample by cow and stored at -20°C until analysis. Milk samples were obtained from each cow 4 times per week at 2 subsequent morning and afternoon milkings via automatic samplers in the milking parlour collecting a fixed volume of milk per kg produced. Samples were pooled into a weekly morning and afternoon sample by individual cow, stored at 4°C, and analysed within 3 d. An additional aliquot of each milk sample was stored separately at -20°C until analysis of milk FA.

Samples of grass silage, corn silage, grass hay, concentrates and feces were analyzed for DM, ash, N, crude fat, starch (except grass silage and grass hay), sugars (except corn silage), NDF, ADF (except feces), ADL (except feces), gross energy, and titanium (concentrates and feces only). After thawing at room temperature, samples were dried at 60°C until a constant weight was reached and ground to pass a 1-mm screen using a Wiley mill (Peppink 100AN, Olst, the Netherlands). Fresh samples of silages and feces were used to determine N concentration. For the determination of NH_3 content, fresh silage samples were deproteinized by the addition of 10% (wt/vol) trichloroacetic acid solution followed by centrifugation (10 min at 14 000 × *g* at room temperature). Subsequently, indophenol blue was formed using the Berthelot reaction with phenol and hypochlorite in an alkaline solution, which was determined spectroscopically at 623 nm. The DM content of air dry samples was gravimetrically determined by drying at 103°C until a constant weight was reached (ISO 6496; ISO, 1999b). Ash was determined after combustion at 550°C (ISO 5984; ISO, 2002a). Crude protein content was calculated as N × 6.25, where N was determined using the Kjeldahl method with $CuSO_4$ as a catalyst (ISO 5983; ISO, 2005). Hydrolysis with HCl and extraction with light petroleum was used to determine crude fat content of samples (ISO 6492; ISO, 1999a). Starch was determined enzymatically (ISO 15914; ISO, 2004). Grass silage, grass hay and concentrates were analyzed for sugars according to Abrahamse et al. (2008). The NDF content of samples was analyzed according to Van Soest et al. (1991) after pre-treatment with α-amylase but without sodium sulfite. Acid detergent fiber and ADL were analyzed in feed samples using methods described by Van Soest et al. (1991) and Robertson and Van Soest (1981) using sulfuric acid, respectively. An adiabatic bomb calorimeter (IKA-C700, Janke and Kunkel, Heitersheim, Germany) was used for determination of GE content (ISO 9831; ISO, 1998). Titanium content was analyzed using sulphuric acid digestion in the presence of Cu at 420°C. The subsequent Cu complex formed by addition of peroxide was determined spectroscopically at 408 nm. Reported values for nutrient content of the TMR were

calculated from ration composition and analyzed values obtained for roughage and concentrates. Reported intestinal digestible protein (DVE; see Table 2.2), rumen degradable protein balance (OEB; see Table 2.2), and NE_L were obtained by near-infrared spectroscopy analysis for corn and grass silage (Eurofins Agro, Wageningen, the Netherlands) and were estimated from CVB tables for grass hay (CVB, 2008). For concentrates, DVE, OEB, and NE_L were calculated based on table values and the composition of raw materials (CVB, 2008), and for each TMR these parameters were calculated from ration composition of all roughage and concentrate ingredients.

Pooled morning and afternoon milk samples were analyzed weekly for fat, protein, lactose and milk urea by mid-infrared spectroscopy (ISO 9622; ISO, 2013; Qlip NV, Zutphen, the Netherlands). Milk FA composition was analyzed by gas chromatography. Frozen milk samples were thawed and 50 mL from each sample was pooled into a composite sample from which milk fat was extracted. Fatty acid methyl esters (FAME) were prepared from fat fractions (ISO 15884; ISO, 2002b) and analyzed (ISO 16958; ISO, 2015) on a gas chromatograph (Thermo Focus GC, Thermo Fisher, Milan, Italy) with a split/splitless injector operated in split injection mode (split ratio 10:1; split flow 25.5 mL/min) at a temperature of 250°C, using a WCOT fused silica capillary column (Agilent CP-Sil 88, Agilent, Santa Clara, CA) with CP-select CB for FAME as stationary phase (100 m × 0.25 mm i.d.) and helium as carrier gas, and fitted to a flame ionization detector (FID; 275°C). The initial temperature was held at 60°C for 5 min, then raised to 165°C at a rate of 15°C/min and held for 1 min, then raised to 225°C at a rate of 2°C/min and held for 20 min. A volume of 1 µL was injected. Peaks were identified and quantified using pure methyl esters (Larodan, Malmö, Sweden; Lipidox, Stockholm, Sweden; Nu-Chek Prep, Elysian, MN). Results of FA were corrected for FID response and expressed as grams per 100 g of total FA. Yields of FA (g/d) were calculated using milk fat yield and individual FA weight proportions (g/100 g FA) to determine yield on a mass basis using the average proportion of FA in milk fat (93.7 g/100 g) derived from individual FA molecular weight and corrected for the glycerol portion of triglycerides and other milk lipid classes according to Glasser et al. (2007).

Statistical Analysis

Milk yield, milk composition and DMI were averaged over the final 7 d of each period. Apparent total-tract digestibility (ATTD) was calculated by the marker ratio technique using titanium dioxide measured in feed and feces samples collected in the final week of each period. Variances in lactation performance, milk FA profile, and digestibility were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) with level of protein and fat and their interaction as fixed effects and parameters measured during the control period used as covariates according to the following model:

$$Y_{ijkl} = \mu + \beta \cdot \mu_l + block_i + PT_j + FT_k + (PT \times FT)_{jk} + \varepsilon_{ijkl},$$

where Y_{ijkl} = observed trait, μ = overall mean, β = control covariate parameter, μ_l = observed trait in the control period of cow l, $block_i$ = random block effect (i = 1 to 14), PT_j = fixed protein effect (j = 1 to 2), FT_k = fixed fat effect (k = 1 to 2), $(PT \times FT)_{jk}$ = interaction between fixed PT and FT effects, and ε_{ijkl} = residual random error term. Differences were considered significant at $P \leq 0.050$ and tendencies at $0.050 < P \leq 0.100$. Multiple comparisons between treatment means were made using the Tukey-Kramer method when a PT × FT interaction was detected at $P \leq 0.100$.

RESULTS AND DISCUSSION

Dietary treatments in this study were designed such that manipulation of supplemental energy from PT and from FT contributed most significantly to the quantity of aminogenic or lipogenic nutrients reaching the small intestine. The proportion of all basal diet ingredients was maintained, individual intake was restricted to 95% of ad libitum level, and RP protein and rumen-inert fat were supplemented in isoenergetic amounts to the restricted basal diet. Therefore, additional daily intake of MP and NEL on HP and HF diets arose only from the addition of the respective supplement. Wright et al. (1998) employed a similar design, where allocation of their basal diet was restricted by 10 or 20% and additional intake was made up of varying amounts of a low-RDP, EAA-balanced concentrate. Our design was unique compared with studies where manipulation of forage or concentrate type changes the profile of glucogenic and lipogenic nutrients available from the diet (Cantalapiedra-Hijar et al., 2014; Boerman et al., 2015; van Hoeij et al., 2017), or where fat-rich concentrates and RUP sources are added as a portion of the total ration DM without feed restriction (Canale et al., 1990; Hoffman et al., 1991; Chan et al., 1997). No significant effects of PT or FT ($P > 0.13$) were found when DMI, milk production, or milk composition from the final 7 d of the control period were tested considering block and treatment of the cows in the experimental period, indicating that cows were distributed evenly across the experimental diets after the control period.

Dry matter intake was designed to differ between treatments due to the addition of PT and FT supplements to the basal TMR through the concentrate (Table 2.1). The LP/HF TMR contained more gross energy and calculated NEL (on a DM basis) compared with HP/LF (Table 2.2). However, as intended, calculated NE_L intake was similar between HP/LF and LP/HF, 147 MJ/d and 141 MJ/d, respectively, and higher (155 MJ/d) when HP and HF were combined, and this pattern is reflected in the significant increase in digestible energy intake in response to PT and FT (**Table 2.3**). Therefore, our objective to increase energy intake through protein and fat supplementation was achieved despite a lower observed DMI than designed for LP/HF and HP/HF diets (Table 2.3). Feeding supplemental fat is associated with variable responses in DMI, but commonly results in no change or a hypophagic effect (Harvatine and

Allen, 2006a), where DMI is affected less as the degree of FA saturation increases (Pantoja et al., 1996). The significant effect of PT on DMI (Table 2.3) is due to our experimental design.

Lactation Performance

The main objective of this work was to study the effects on lactation performance when extra energy comes from aminogenic or lipogenic supplements. The response to PT and FT was independent and additive, as the interaction between these factors had no effect on milk yield or composition (Table 2.3). Cows on HP diets produced 1.9 kg/d more milk compared with LP diets, and cows on HF diets produced 1.6 kg/d more milk compared with LF diets. Milk protein concentration and yield were increased by PT whereas fat concentration and yield were increased by FT. This production response is in agreement with previous studies feeding aminogenic or lipogenic nutrients. Schor and Gagliostro (2001) increased milk and protein yield when pasture-based forage was supplemented with blood meal of low rumen degradability compared with high rumen-degradable soybean meal. Lock et al. (2013) increased milk fat yield and concentration in dairy cattle by replacing soyhulls with a C16:0-rich FA supplement at 2% of DM, and Relling and Reynolds (2007) stimulated 187 g/d of milk fat synthesis when rumen-inert SFA were fed compared with a non-supplemented diet. However, few studies exist that compare the effect of aminogenic and lipogenic nutrients supplemented at an isoenergetic level. Chan et al. (1997) supplemented Met and Lys from a mix of fish, blood, and soybean meal compared with a low-quality protein source from corn gluten meal, with and without supplementation of rumen-inert LCFA at 2.5% of DM, and observed similar lactation responses to our study with regards to milk protein and lactose yield. Canale et al. (1990) supplemented RP Met and Lys, calcium salts of FA, or both, and reported increased milk yield and decreased protein content in response to FA, increased protein content in response to RP AA, and found an interaction for milk fat yield where it increased with FA but only when RP AA were supplemented.

Lactose content was unaffected by PT or FT, but both factors independently increased lactose yield, suggesting postruminal supplementation with AA and saturated LCFA affected mammary glucose utilization through alterations in whole-body glucose availability, local mammary gland glucose sequestration, or intramammary glucose partitioning. The effect of PT on lactose yield is not surprising, as infusions of casein or AA mixtures increase milk protein but also lactose yield (Galindo et al., 2011; Nichols et al., 2016), suggesting AA supplementation affects whole-body glucose partitioning and mammary gland glucose metabolism (Lemosquet et al., 2009; Haque et al., 2012). Contrary to AA, LCFA are not conventionally gluconeogenic, yet the same level of lactose synthesis was maintained on the LP/HF diet compared with the HP/LF diet. Compared with glucogenic diets, lactose yield may be equal to or higher from cows fed lipogenic diets (van Knegsel et al., 2007; Hammon et al., 2008; Lohrenz et al., 2010), but is lower in other studies (Cantalapiedra-Hijar et al., 2014; Boerman et al., 2015; van Hoeij et al., 2017). Differences in response to glucogenic and

lipogenic diets, with regard to lactose yield, could be due to energy partitioning and whole-body glucose availability in different stages of lactation. On a net basis, FA do not directly contribute to glucose precursors necessary for mammary lactose synthesis. Increased lactose yield in response to dietary supplementation with SFA has been attributed to greater mammary supply of LCFA and their direct incorporation into milk fat reducing mammary glucose requirement for de novo FA synthesis, thus sparing glucose for lactose synthesis (Grummer and Carroll, 1991; Chilliard, 1993; Hammon et al., 2008). In agreement, we observed increased incorporation of preformed LCFA into milk when energy was supplemented from fat. Concentration of total preformed (>16 carbons) and mixed (16 carbons) FA increased 4 and 7%, respectively, in response to FT (**Table 2.4**), and the yield of total preformed and mixed FA increased 15 and 17%, respectively, in response to FT (**Table 2.5**). The concentration of total de novo-synthesized FA (<16 carbons) decreased 13% in response to FT. Total yield of de novo-synthesized FA was not affected by FT, as the decline in concentration was offset by the increase in total milk FA yield. If triglycerides compose 98% of milk fat (Jensen and Newberg, 1995), total milk triglyceride production increased 121 g/d and de novo FA synthesis decreased only 12 g/d in response to FT. Although this shift in milk FA profile from de novo-synthesized FA toward LCFA in response to FT may have facilitated some intramammary repartitioning of glucose toward lactose synthesis, we suspect mammary glucose sequestration was stimulated by gluconeogenesis from AA or other endogenous energy precursors increasing arterial glucose supply and by responses in local mammary gland extraction mechanisms in support of lactose synthesis on HF diets.

Individually, concentration of all de novo FA, with the exception of C4:0, decreased in response to FT, whereas yield of C8:0, C10:0, C11:0, C12:0, *iso* C14:0, C15:0, and *iso* C15:0 decreased, yield of C4:0 increased, and yield of the remaining de novo FA were unaffected (Table 2.4 and 2.5). In response to PT, concentration and yield of C4:0 (yield only), C6:0, C8:0, C10:0, C11:0, C12:0, C14:0, and *iso* C14:0 increased as well and concentration and yield of total de novo FA. Other studies have shown higher proportions of de novo FA when glucogenic diets are compared with lipogenic diets (Grum et al., 1996; van Knegsel et al., 2007; Boerman et al., 2015). In our study, supplementation of RP protein may have allowed greater production of short-chain FA through metabolism of AA into α-ketoacids to produce acetyl-CoA and other tricarboxylic acid cycle intermediates. In support, we observed a numerical increase in milk fat yield of 53 g/d on HP compared with LP diets, which agrees with previous reports of stimulated milk fat production when postruminal EAA are administered (Vanhatalo et al., 2003; Nichols et al., 2016). The pattern in concentration and yield of C4:0 across treatments differed from other de novo FA identified in milk fat. The C4:0 FA is produced directly by reduction of BHB or by condensation of acetyl units in a malonyl-CoA-independent pathway, whereas other de novo FA are synthesized via the malonyl-CoA pathway mainly from acetyl-CoA (Palmquist et al., 1993a). Similar to results presented by

others (Cant et al., 1993; van Knegsel et al., 2007; Lock et al., 2013), C4:0 concentration did not respond to PT or FT, but yield increased in response to FT alongside total milk fat yield.

The increase in concentration and yield of C16:0 is largely responsible for the increase in concentration and yield of total mixed FA in response to FT, as concentrations and yield of *iso* C16:0 and C16:1 *trans*-9 decreased or did not change, respectively, in response to FT, and concentration of C16:1 *cis*-9 was unchanged but the yield of this FA increased. Similarly, the response in concentration and yield of total preformed FA in response to FT is driven by individual increases in concentration and yield of both C18:0 and C18:1 *cis*-9, which together make up the largest proportion of reported preformed FA. Concentration and yield of all other preformed FA were unchanged or decreased in response to FT. The rumen-inert fat supplement used in our study supplies almost exclusively C16:0 and C18:0 and contributes very little as substrate for rumen biohydrogenation reactions, which suggests increases in the profile of these SFA in milk was due to their extraction by the mammary gland and incorporation into milk fat. The concentration of total SFA was unaffected by FT because, despite the increase in C16:0 and C18:0 stimulated by FT, all saturated de novo FA (except C4:0) actually decreased in response to FT. Subsequently, SFA yield increased alongside total milk fat yield in response to FT.

Fat supplementation tended to increase milk protein yield by 45 g/d. Depending on the concomitant supply of AA available to the glands, non-AA energy precursors can allow the use of available EAA for milk protein synthesis by sparing them from gluconeogenesis or energy-yielding oxidative processes (Raggio et al., 2006; Rius et al., 2010a). Milk urea concentration was highest and N efficiency was lowest on HP diets, indicating greater AA catabolism when energy was supplemented from an aminogenic source compared with supplementation from a lipogenic source. Milk urea concentration was not affected by FT, but a tendency for a PT × FT interaction on this parameter suggests FT may have depressed AA catabolism when in the presence of PT. Interestingly, although adding fat to the basal diet tended to improve milk N efficiency to a greater extent at the low than at the high PT level, milk urea content was not affected. Milk urea concentration in general may serve as an on-farm indicator to guide nutritional strategies in efforts to reduce emissions of N to the environment, but the relationship between milk urea and N excretion is variable (reviewed by Spek et al., 2013). Our current results with respect to supplementation of PT and FT indicated that milk urea concentration was not necessarily a sound indicator of N efficiency. Increased AA catabolism and lowered N efficiency associated with feeding increased CP levels has been well characterized (Broderick, 2003; Colmenero and Broderick, 2006). However, when low-quality dietary protein is replaced with highly digestible and EAA-balanced RUP (Wright et al., 1998; Noftsger and St-Pierre, 2003), or when energy is supplemented (Rius et al., 2010b), this response is altered in support of lowered milk urea and improved efficiency of N use by the mammary gland. Cantalapiedra-Hijar et al. (2014)

found improved N efficiency on a starch-based, 12.0% CP diet when it stimulated the same level of milk protein yield as a fiber-based, 16.5% CP diet, but resulted in lower urinary N excretion. In the present study, N efficiency was improved on LP/LF compared with HP/LF, or on LP/HF compared with HP/HF. Importantly, our results support the addition of fat as an energy source to improve N utilization by lactating cows at high and low MP levels, although the effect was more pronounced at the low protein level.

Table 2.3. Performance of lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or from protein and fat (HP/HF)[1]

	Treatment[2]					*P*-value[3]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM	PT	FT	PT × FT
DMI, kg/d	19.7	21.4	19.5	21.4	0.28	<0.001	0.758	0.668
DE intake,[4] MJ/d	252	276	268	291	4.8	<0.001	0.001	0.967
Yield								
Milk, kg/d	26.6	28.7	28.4	30.0	0.70	0.012	0.031	0.707
Fat, g/d	1199	1254	1325	1375	33.0	0.102	<0.001	0.931
Protein, g/d	915	1013	974	1041	24.1	<0.001	0.054	0.495
Lactose, g/d	1205	1307	1301	1375	30.8	0.007	0.012	0.656
Composition, %								
Fat	4.52	4.51	4.75	4.64	0.078	0.435	0.022	0.520
Protein	3.48	3.60	3.47	3.51	0.029	0.009	0.111	0.185
Lactose	4.54	4.57	4.58	4.57	0.018	0.453	0.352	0.278
FPCM,[5] kg/d	28.5	30.6	31.0	32.4	0.73	0.019	0.005	0.655
Milk urea, mg/dL	21[a]	28[b]	22[a]	26[b]	0.7	<0.001	0.430	0.059
Feed efficiency[6]	1.44	1.43	1.58	1.51	0.040	0.298	0.004	0.471
Feed energy efficiency,[7] %	34.7	34.2	35.8	34.7	0.98	0.405	0.409	0.791
N efficiency,[8] %	28.9[a]	26.1[b]	32.2[c]	27.1[ab]	0.69	<0.001	0.002	0.071

[a-c]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Data are least squares means from the final week of the experimental period using data from the final week of the control period (CNTL) as covariates for each parameter.

[2]LP/LF = basal TMR fed at 95% of CNTL ad libitum intake with no protein or fat supplement; HP/LF = TMR fed at 95% of CNTL ad libitum intake supplemented daily with 2.0 kg SoyPass + RaPass on a DM basis; LP/HF = TMR fed at 95% of CNTL ad libitum intake supplemented daily with 0.68 kg Hidropalm on a DM basis; HP/HF = TMR fed at 95% of CNTL ad libitum intake supplemented daily with 2.0 kg SoyPass + RaPass and 0.68 kg Hidropalm on a DM basis. For all treatments n = 14.

[3]PT = effect of energy from protein; FT = effect of energy from fat.

[4]Digestible energy intake = gross energy (GE) intake (MJ/d) × apparent total-tract GE digestibility.

[5]Fat- and protein-corrected milk (FPCM) = (0.337+ 0.116 × fat % + 0.06 × protein %) × milk yield (kg/d) (CVB, 2008).

[6]FPCM (kg/d)/DMI (kg/d).

[7][Milk energy (MJ/d)/DE intake (MJ/d)] × 100. Milk energy content is calculated according to the equation of Tyrrell and Reid (1965) based on observed milk fat, protein, and lactose content.

[8][Milk N yield (g/d)/N intake (g/d)] × 100.

Table 2.4. Milk fatty acid (FA) composition of lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or from protein and fat (HP/HF)

	Treatment[1]					*P*-value[2]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM	PT	FT	PT × FT
Concentration, g/100 g of FA								
C4:0	2.93	2.94	2.96	2.95	0.034	0.999	0.629	0.771
C6:0	2.08	2.19	1.94	2.02	0.026	<0.001	<0.001	0.596
C8:0	1.30	1.40	1.11	1.21	0.022	<0.001	<0.001	0.949
C10:0	2.95	3.27	2.38	2.63	0.066	<0.001	<0.001	0.561
C11:0	0.41	0.45	0.33	0.34	0.013	0.040	<0.001	0.367
C12:0	3.49	3.87	2.73	3.06	0.084	<0.001	<0.001	0.753
C14:0	11.8	12.0	10.2	10.7	0.17	0.020	<0.001	0.446
iso C14:0	0.21	0.24	0.15	0.18	0.011	0.008	<0.001	0.813
C14:1 *cis*-9	1.19	1.23	1.03	1.04	0.035	0.452	<0.001	0.796
C15:0	1.15	1.13	0.91	0.96	0.023	0.514	<0.001	0.154
iso C15:0	0.29	0.27	0.24	0.24	0.009	0.265	<0.001	0.331
anteiso C15:0	0.47	0.45	0.40	0.40	0.010	0.459	<0.001	0.296
C16:0	32.4[a]	31.3[b]	34.4[c]	34.2[c]	0.34	0.025	<0.001	0.076
iso C16:0	0.28	0.28	0.24	0.23	0.010	0.771	<0.001	0.766
C16:1 *cis*-9	1.56	1.65	1.65	1.83	0.079	0.105	0.104	0.565
C16:1 *trans*-9	0.44	0.43	0.38	0.39	0.013	0.961	<0.001	0.418
C17:0	0.54	0.54	0.44	0.44	0.010	0.802	<0.001	0.801
iso C17:0	0.68	0.62	0.63	0.62	0.025	0.163	0.335	0.301
C17:1 *cis*-9	0.22	0.24	0.19	0.19	0.008	0.551	<0.001	0.200
C18:0	9.3	9.0	10.9	10.4	0.23	0.082	<0.001	0.713
C18:1 *cis*-9[3]	19.2	18.9	21.0	20.0	0.42	0.081	<0.001	0.389
C18:1 *cis*-12	0.31	0.31	0.27	0.26	0.010	0.569	<0.001	0.403
C18:1 *cis*-13	0.43[ab]	0.44[a]	0.38[bc]	0.33[c]	0.015	0.203	<0.001	0.089
C18:1 *trans*-9	0.02	0.04	0.03	0.03	0.010	0.337	0.591	0.253
C18:1 *trans*-10 + *trans*-11	1.53	1.67	0.98	0.99	0.061	0.213	<0.001	0.273
C18:1 *trans*-15 + C18:1 *cis*-11	0.61	0.66	0.57	0.59	0.024	0.055	0.003	0.310
Total CLA[4]	0.90	0.96	0.69	0.67	0.038	0.675	<0.001	0.289
C18:2n-6	2.11[a]	2.57[b]	1.88[a]	2.00[a]	0.074	<0.001	<0.001	0.024
C18:3n-3	0.49	0.53	0.39	0.44	0.011	<0.001	<0.001	0.924
C18:3n-6	0.23	0.25	0.22	0.22	0.012	0.551	0.135	0.367
C20:0	0.04	0.04	0.03	0.03	0.003	0.739	0.004	0.721
C20:2n-6	0.019	0.022	0.015	0.017	0.0011	0.013	<0.001	0.627
C20:3n-6	0.07	0.09	0.07	0.08	0.005	0.034	0.408	0.376
C20:4n-3	0.03	0.04	0.03	0.04	0.004	0.427	0.607	0.726
C20:4n-6	0.12	0.14	0.11	0.12	0.005	0.003	<0.001	0.470
C20:5n-3	0.050	0.049	0.045	0.044	0.0024	0.540	0.035	0.986
C22:0	0.08	0.08	0.07	0.07	0.002	0.901	<0.001	0.882
C22:5n-3	0.06	0.06	0.05	0.05	0.002	0.105	<0.001	0.200
C24:0	0.033	0.033	0.027	0.027	0.0014	0.917	<0.001	0.993
n-6 to n-3 ratio[5]	4.09	4.56	4.45	4.25	0.158	0.392	0.889	0.035
Summations[6]								
De novo	28.3	29.5	24.4	25.6	0.41	0.003	<0.001	0.953
Preformed	37.1	37.0	39.0	37.7	0.61	0.176	0.020	0.240

Table 2.4 (continued). Milk fatty acid (FA) composition of lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or from protein and fat (HP/HF)

	Treatment[1]					*P*-value[2]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM	PT	FT	PT × FT
Mixed	34.7[a]	33.6[b]	36.6[c]	36.7[c]	0.36	0.079	<0.001	0.042
SFA	70.4	70.1	70.0	70.7	0.48	0.713	0.862	0.290
MUFA	25.5	25.3	26.5	25.7	0.45	0.245	0.124	0.502
PUFA	4.08[a]	4.66[b]	3.49[c]	3.65[c]	0.107	0.001	<0.001	0.058

[a-c]Means within a row with no common superscripts differ ($P < 0.05$).

[1]LP/LF = basal TMR fed at 95% of control period (CNTL) ad libitum intake with no protein or fat supplement; HP/LF = TMR fed at 95% of CNTL ad libitum intake supplemented daily with 2.0 kg SoyPass + RaPass on a DM basis; LP/HF = TMR fed at 95% of CNTL ad libitum intake supplemented daily with 0.68 kg Hidropalm on a DM basis; HP/HF = TMR fed at 95% of CNTL ad libitum intake supplemented daily with 2.0 kg SoyPass + RaPass and 0.68 kg Hidropalm on a DM basis. For all treatments n = 14.

[2]PT = effect of energy from protein; FT = effect of energy from fat.

[3]C18:1 *cis*-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, as these 2 FA could not be separated in the analysis. The proportion of C18:1 *trans*-12 is considered negligible.

[4]Total CLA consists of mainly C18:2 *cis*-9,*trans*-11.

[5]Ratio between the sum of C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, and C20:4n-6 and the sum of C18:3n-3, C20:4n-3, C20:5n-3, and C22:5n-3.

[6]De novo-synthesized FA (<16 carbons) originate from mammary de novo synthesis, preformed FA (>16 carbons) originate from mammary plasma extraction, mixed FA (16 carbons) originate from both de novo-synthesized and preformed FA. Sum of the respective SFA, MUFA, and PUFA reported in this table.

Table 2.5. Milk fatty acid (FA) yield (g/d) of lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or from protein and fat (HP/HF)

	Treatment[1]					*P*-value[2]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM	PT	FT	PT × FT
C4:0	32.9	34.8	36.8	38.3	0.98	0.077	<0.001	0.838
C6:0	23.4	25.8	24.2	26.2	0.62	<0.001	0.287	0.796
C8:0	14.6	16.4	13.9	15.7	0.40	<0.001	0.062	0.955
C10:0	33.0	38.1	29.8	34.2	1.01	<0.001	<0.001	0.718
C11:0	4.64	5.30	4.12	4.40	0.193	0.014	<0.001	0.282
C12:0	39.0	44.8	34.1	39.6	1.28	<0.001	<0.001	0.910
C14:0	131	140	127	139	3.6	0.004	0.408	0.781
iso C14:0	2.26	2.80	1.90	2.26	0.172	0.004	0.004	0.558
C14:1 *cis*-9	13.4	14.4	12.8	13.4	0.52	0.107	0.115	0.765
C15:0	12.8	13.3	11.4	12.3	0.44	0.108	0.006	0.663
iso C15:0	3.29	3.36	2.90	3.11	0.184	0.436	0.077	0.696
anteiso C15:0	5.17	5.20	4.99	5.09	0.166	0.671	0.385	0.826
C16:0	369	374	427	440	11.5	0.344	<0.001	0.679
iso C16:0	3.05	3.26	2.99	3.01	0.160	0.425	0.294	0.514
C16:1 *cis*-9	17.5	19.6	20.4	23.9	1.40	0.034	0.007	0.581
C16:1 *trans*-9	4.90	5.03	4.73	5.04	0.222	0.337	0.715	0.684
C17:0	5.88	6.36	5.51	5.77	0.245	0.144	0.057	0.652
iso C17:0	7.55	7.25	7.82	8.00	0.340	0.858	0.146	0.489
C17:1 *cis*-9	2.49	2.78	2.40	2.43	0.137	0.233	0.119	0.345
C18:0	103	105	136	135	4.2	0.922	<0.001	0.743
C18:1 *cis*-9[3]	211	221	259	257	8.3	0.606	<0.001	0.436
C18:1 *cis*-12	3.38	3.60	3.38	3.29	0.166	0.704	0.350	0.356
C18:1 *cis*-13	4.74	5.09	4.64	4.34	0.271	0.927	0.122	0.240
C18:1 *trans*-9	0.26	0.56	0.38	0.37	0.142	0.295	0.806	0.268
C18:1 *trans*-10 + *trans*-11	16.6	19.5	12.0	12.3	0.85	0.068	<0.001	0.163
C18:1 *trans*-15 + C18:1 *cis*-11	6.69	7.76	7.07	7.58	0.354	0.013	0.739	0.370
Total CLA[4]	9.9	11.3	8.6	8.4	0.61	0.277	0.002	0.173
C18:2n-6	23.7[a]	30.1[b]	23.9[a]	25.5[a]	1.20	0.002	0.072	0.056
C18:3n-3	5.37	6.25	4.82	5.66	0.168	<0.001	0.002	0.903
C18:3n-6	2.45	2.87	2.70	2.81	0.211	0.148	0.593	0.411
C20:0	0.46	0.49	0.40	0.44	0.034	0.351	0.120	0.908
C20:2n-6	0.20	0.26	0.19	0.22	0.015	0.001	0.081	0.441
C20:3n-6	0.81	1.02	0.93	1.02	0.078	0.035	0.432	0.403
C20:4n-3	0.37	0.44	0.41	0.47	0.062	0.265	0.603	0.941
C20:4n-6	1.34	1.65	1.31	1.49	0.066	<0.001	0.126	0.270
C20:5n-3	0.54	0.58	0.57	0.58	0.040	0.549	0.601	0.570
C22:0	0.86	0.94	0.83	0.89	0.039	0.079	0.289	0.725
C22:5n-3	0.64	0.67	0.56	0.68	0.038	0.040	0.274	0.253
C24:0	0.35	0.38	0.33	0.35	0.022	0.193	0.162	0.672
Summations[5]								
De novo	316	344	305	332	8.5	0.001	0.144	0.995
Preformed	410	432	483	485	14.0	0.379	<0.001	0.477
Mixed	395	401	455	472	12.4	0.268	<0.001	0.600
SFA	794	827	871	912	21.6	0.070	<0.001	0.835

Table 2.5 (continued). Milk fatty acid (FA) yield (g/d) of lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or from protein and fat (HP/HF)

	Treatment[1]					*P*-value[2]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM	PT	FT	PT × FT
MUFA	282	296	327	331	10.3	0.375	<0.001	0.619
PUFA	45.8	54.6	43.4	46.6	1.96	0.004	0.012	0.159

[a,b]Means within a row with no common superscripts differ ($P < 0.05$).

[1]LP/LF = basal TMR fed at 95% of control period (CNTL) ad libitum intake with no protein or fat supplement; HP/LF = TMR fed at 95% of CNTL ad libitum intake supplemented daily with 2.0 kg SoyPass + RaPass on a DM basis; LP/HF = TMR fed at 95% of CNTL ad libitum intake supplemented daily with 0.68 kg Hidropalm on a DM basis; HP/HF = TMR fed at 95% of CNTL ad libitum intake supplemented daily with 2.0 kg SoyPass + RaPass and 0.68 kg Hidropalm on a DM basis. For all treatments n = 14.

[2]PT = effect of energy from protein; FT = effect of energy from fat.

[3]C18:1 *cis*-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, as these 2 FA could not be separated in the analysis. The proportion of C18:1 *trans*-12 is considered negligible.

[4]Total CLA consists of mainly C18:2 *cis*-9,*trans*-11.

[5]De novo-synthesized FA (<16 carbons) originate from mammary de novo synthesis, preformed FA (>16 carbons) originate from mammary plasma extraction, mixed FA (16 carbons) originate from both de novo-synthesized and preformed FA. Sum of the respective SFA, MUFA, and PUFA reported in this table.

Apparent Total-Tract Digestibility

Efficacy of energy supplementation from protein or fat for milk production is related to the digestibility of the added component and its effect on availability of other nutrients. Therefore, a second objective was to assess the effects of RP protein and rumen-inert fat on digestibility. Improved energy utilization is one of the primary purposes for supplementing saturated fat to dairy cow diets. Fat- and protein-corrected milk yield was stimulated to the same extent in response to isoenergetic supplementation of extra NE_L from RP protein and rumen-inert fat, and feed energy efficiency, calculated as milk energy as a proportion of digestible energy intake, did not differ. Therefore, efficiency of energy use for milk production was similar between aminogenic and lipogenic diets. Feed efficiency in the present study increased in response to FT as a result of increased milk production stimulated by the fat-containing TMR, which supplied more energy at a similar DMI (Table 2.3). Many others have reported increased feed efficiency when supplemental fat is fed (Lock et al., 2013; Rico et al., 2014; Boerman et al., 2015) or infused postruminally (Oldick et al., 1997).

Energy from protein and fat had little effect on nutrient ATTD (**Table 2.6**). The HP diets had lower dietary crude fat contents than the LP diets (Table 2.2). Therefore, the decrease in crude fat digestibility in response to PT may reflect the relatively higher contribution of endogenous fat losses to fecal fat excretion at low dietary fat contents (Kil et al., 2010). Fat supplementation has been associated with a reduction in nutrient digestibility (Harvatine and Allen, 2006b), but the effect depends highly on the particular nutrient, the location in the digestive tract where digestibility is measured, the amount of fat added, and the degree of saturation of the FA fed (Palmquist and Jenkins, 1980; Jenkins and Jenny, 1989; Pantoja et

al., 1995). Adding a high level of fat to a diet may increase apparent fat digestibility if endogenous fat becomes a smaller part of total fecal fat. In contrast, apparent digestibility may decrease if the true digestibility of the fat supplement is lower than that of the basal diet. Recently, Weld and Armentano (2017) showed that the inclusion of saturated fats to dairy rations in 38 studies did not affect total-tract NDF digestibility. The level of fat supplementation in the present experiment (i.e., 36 g/kg of DM) was similar to that in the meta-analysis of Weld and Armentano (2017; on average, 33 g of FA/kg of DM, ranging from 11 to 66 g of FA/kg of DM). With our fat supplement, we observed no effect of FT or of a PT × FT interaction on ATTD of DM, OM, NDF, crude fat, starch, or gross energy, which agrees with other studies where saturated fats were fed or infused postruminally (Chan et al., 1997; Oldick et al., 1997; Bremmer et al., 1998). However, in contrast to these studies, we observed a small positive effect of FT on CP digestibility. Palmquist et al. (1993b) suggested that increased N digestibility with fat supplementation might be associated with the lower starch contents generally found in fat-supplemented diets. Although starch content was similar between the HP/LF and LP/HF diets, lower DMI on LP/HF could have slightly reduced starch entering the rumen. Under these conditions, microbial protein synthesis in the rumen was expected to be lower, and N loss through NH_3-N production was expected to be higher. If intestinal absorption of NH_3-N is higher compared with that of N originating from synthesized microbial protein, apparent N digestibility will increase.

Table 2.6. Apparent total-tract digestibility (%) of nutrients in lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or from protein and fat (HP/HF)[1]

	Treatment[2]					*P*-value[3]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM	PT	FT	PT × FT
DM	71.0	72.8	72.0	72.3	1.08	0.348	0.810	0.530
OM	73.0	73.7	73.9	73.9	0.75	0.598	0.460	0.638
CP	67.1	67.5	68.5	69.2	0.65	0.389	0.017	0.747
NDF	58.7	62.0	61.5	62.0	1.41	0.178	0.332	0.332
Crude fat	66.1	61.9	67.7	64.0	1.33	0.006	0.159	0.858
Starch	98.2	98.0	98.2	98.0	0.09	0.085	0.759	0.528
Gross energy	70.3	70.8	71.4	71.0	0.80	0.915	0.414	0.606

[1]Data are least squares means calculated from feed and feces sampled during the final week of each period using data from the final week of the control period (CNTL) as covariates for each parameter.

[2]LP/LF = basal TMR fed at 95% of CNTL ad libitum intake with no protein or fat supplement; HP/LF = TMR fed at 95% of CNTL ad libitum intake supplemented daily with 2.0 kg SoyPass + RaPass on a DM basis; LP/HF = TMR fed at 95% of CNTL ad libitum intake supplemented daily with 0.68 kg Hidropalm on a DM basis; HP/HF = TMR fed at 95% of CNTL ad libitum intake supplemented daily with 2.0 kg SoyPass + RaPass and 0.68 kg Hidropalm on a DM basis. For all treatments n = 14.

[3]PT = effect of energy from protein; FT = effect of energy from fat.

CONCLUSIONS

This study demonstrated independent and additive stimulation of milk yield when protein and fat were supplemented at isoenergetic levels. Energy from RP protein stimulated the greatest response in milk protein yield. Energy from rumen-inert hydrogenated palm FA increased feed efficiency and stimulated milk fat yield by incorporation of LCFA into milk fat and reduction of mammary de novo FA synthesis. Energy from fat improved N utilization at high and low MP levels. In addition, milk urea concentration was identified as a poor indicator of actual N efficiency when fat supplementation was used to increase energy intake. Energy from fat produced the same level of lactose yield as energy from protein. During fat supplementation, repartitioning of intramammary glucose in support of lactose synthesis may have been allowed in part through decreased synthesis of de novo FA, but major glucose sequestration by the gland was likely stimulated by arterial glucose concentrations and local mammary gland kinetic mechanisms. These mechanisms should be investigated to elucidate the dynamics of mammary glucose partitioning when lipogenic substrates change milk precursor availability.

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Chapter 3

Mammary gland utilization of amino acids and energy metabolites differs when dairy cow rations are isoenergetically supplemented with protein and fat

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ABSTRACT. Mammary gland utilization of AA and other metabolites in response to supplemental energy from protein (PT) and supplemental energy from fat (FT) was tested in a 2 × 2 factorial arrangement using a randomized complete block design. Fifty-six Holstein-Friesian dairy cows were adapted during a 28-d control period to a basal total mixed ration consisting of 34% grass silage, 33% corn silage, 5% grass hay, and 28% concentrate on a dry matter (DM) basis. Experimental rations were fed for 28 d immediately following the control period and consisted of (1) low protein, low fat (LP/LF), (2) high protein, low fat (HP/LF), (3) low protein, high fat (LP/HF), and (4) high protein, high fat (HP/HF). To obtain the high protein (HP) and high fat (HF) diets, intake of the basal ration was restricted and supplemented isoenergetically [net energy (MJ/d) basis] with 2.0 kg/d rumen-protected protein (soybean + rapeseed, 50:50 mixture on a DM basis) and 0.68 kg/d hydrogenated palm fatty acids on a DM basis. Arterial and venous blood samples were collected on d 28 of both periods. Isoenergetic supplements (MJ/d) of protein and fat independently and additively increased milk yield, PT increased protein yield, and FT increased fat yield. A PT × FT interaction affected arterial concentration of all essential amino acid (EAA) groups, where they increased in response to PT by a greater magnitude at the LF level (on average 35%) compared with the HF level (on average 14%). Mammary gland plasma flow was unaffected by PT or FT. Supplementation with PT tended to decrease mammary clearance of total EAA and decreased group 1 AA clearance by 19%. In response to PT, mammary uptake of total EAA and group 2 AA increased 12 and 14%, respectively, with significantly higher uptake of Arg, Ile, and Leu. Energy from fat had no effect on mammary clearance or uptake of any AA group. The mammary gland uptake: milk protein output ratio was not affected by FT, whereas PT increased this ratio for EAA and group 2 AA. Arterial plasma insulin concentration decreased in response to FT, in particular on the HP/HF diet, as indicated by a PT × FT interaction. Arterial concentrations of nonesterified fatty acids, triacylglycerol, and long-chain fatty acids increased in response to FT, and concentrations of β-hydroxybutyrate and acetate decreased in response to FT only at the HP level. Mammary clearance and uptake of triacylglycerol and long chain fatty acids increased in response to FT. Energy from PT and FT increased lactose yield despite no change in arterial glucose concentration or mammary glucose uptake. Mammary-sequestered glucose with PT or FT was used in the same amount for lactose synthesis, and a positive net mammary glucose balance was found across all treatments. Results presented here illustrate metabolic flexibility of the mammary gland in its use of aminogenic versus lipogenic substrates for milk synthesis.

Key words: amino acid, hydrogenated palm fatty acid, protein synthesis, lactose synthesis, mammary gland

INTRODUCTION

Lactating mammary glands have an obligatory requirement for glucose as a substrate to synthesize lactose. Lactose synthesis represents the major fate of glucose metabolism in mammary epithelial cells, and glucose oxidation facilitates the synthesis of other milk components such as fatty acids (FA) and protein (Mepham, 1987; Xiao and Cant, 2005). A relationship exists between MP supply and lactose yield independent of glucose supply, where infusions of casein or AA mixtures increase both milk protein and lactose yield (Galindo et al., 2011; Nichols et al., 2016). This relationship suggests that AA supplementation has an effect on whole-body energy partitioning and mammary gland metabolism (Lemosquet et al., 2009a; Lapierre et al., 2010). Rumen-protected protein sources are fed with the objective of increasing AA availability for absorption and mammary gland extraction at first pass, but because milk synthesis is an energy-demanding process, AA may be oxidized if the concomitant supply of energy for productive purposes is lacking. Alternatively, supply of non-AA energy precursors can optimize the use of available AA for milk protein synthesis and alter the kinetics of mammary AA uptake (Raggio et al., 2006; Rius et al., 2010a). Infusions of propionate, starch, and glucose into the digestive tract, with and without casein or AA infusion, have been studied extensively (Lemosquet et al., 2009a; Rius et al., 2010b; Nichols et al., 2016), but few studies exist examining mammary gland kinetics in response to postruminal fat supplementation.

Lactose yield from dairy cows on lipogenic diets is equal to or higher than that with glucogenic diets in some studies (Hammon et al., 2008; Lohrenz et al., 2010) but not in all (Boerman et al., 2015; van Hoeij et al., 2017). Long-chain fatty acids (LCFA) do not directly contribute to glucose precursors necessary for lactose synthesis, but increased mammary extraction of LCFA can decrease de novo FA synthesis by the gland (Grummer and Carroll, 1991; Chilliard, 1993; Hammon et al., 2008) which may reduce glucose requirements for oxidative catabolism and increase supply of energy for lactose and protein synthesis. Cant et al. (1993a) fed a mix of saturated and unsaturated fats and observed increased mammary uptake of triacylglycerides (TAG) and output of LCFA into milk, higher lactose yield, and increased mammary glucose utilization for lactose. Previous work has quantified mammary AA use in response to largely unsaturated fat sources (Casper and Schingoethe, 1989; Casper et al., 1990; Cant et al., 1993b), but there is a paucity of recent data characterizing AA responses to rumen-inert fat supplements.

Intramammary metabolism must be flexible to derive substrates for the regulation of milk volume and composition based on supply of aminogenic, lipogenic, or glucogenic precursors. In an experiment to test this concept (Nichols et al., 2018), isoenergetic levels of protein or fat supplementation increased total milk, lactose, protein (tendency with fat supplementation), and fat yield (tendency with protein supplementation). The increase in milk fat yield with fat supplementation was associated with a shift toward incorporation of

LCFA and decreased de novo FA synthesis. The aim of the present work was to investigate metabolite utilization by the mammary gland underlying these observed milk production responses. The current study used a practical and relatively large-scale approach to measure mammary gland metabolism. We expected AA from protein to support mammary protein synthesis and, if acting as a glucogenic substrate, to yield glucose in support of lactose synthesis. This study is also unique in its characterization of mammary gland kinetics with fat supplementation at low and high MP levels. We hypothesized that, compared with low-fat diets, energy from fat would stimulate mammary AA sequestration or reduce AA catabolism in support of milk protein synthesis and increase glucose sequestration in support of lactose synthesis.

MATERIALS AND METHODS

Experimental Design

Experimental procedures were approved by the Animal Care and Use Committee for Nutreco Nederland B.V. (Amersfoort, the Netherlands) and conducted under the Dutch Law on Animal Experiments. The experimental design, animal housing, diets, and feed chemical analyses have been described in detail by Nichols et al. (2018). Briefly, 56 Holstein-Friesian cows (20 primiparous, 138 ± 64 DIM; 36 multiparous, 3.7 ± 1.8 lactations, 181 ± 93 DIM) were used in a randomized complete block design where supplemental energy from protein (**PT**) or supplemental energy from fat (**FT**) was tested in a factorial arrangement over 2 successive periods (control and experimental), each consisting of 21 d of diet adaptation and 7 d of data collection. A basal diet was fed as a TMR during the control period, consisting of 34% grass silage, 33% corn silage, 5% grass hay, and 28% concentrate on a DM basis, and was designed to meet NE_L and MP requirements for cows of 650 kg of BW consuming 22 kg of DM/d producing 30 kg of milk/d containing 45 g/kg fat and 35 g/kg protein. Cows were blocked by parity, DIM, and DMI of the final 7 d of the control period and within blocks were randomly assigned to 1 of 4 diets for the experimental period: (1) low protein, low fat (**LP/LF**; 95% MP, 95% NE_L), (2) high protein, low fat (**HP/LF**; 131% MP, 107% NE_L), (3) low protein, high fat (**LP/HF**; 95% MP, 107% NE_L), and (4) high protein, high fat (**HP/HF**; 131% MP, 119% NE_L), where MP and NE_L are expressed relative to animal requirements in the control period. For all treatments, basal diet intake for individual cows was restricted to 95% of their ad libitum intake recorded during the control period. To obtain the high protein (HP) and high fat (HF) diets, a 2.0-kg 50:50 mixture (DM basis) of rumen-protected (RP) soybean meal and rapeseed meal (both rumen protected by xylose treatment; SoyPass + RaPass; Borregaard LignoTech, Sarpsborg, Norway) and 0.68 kg of rumen-inert hydrogenated LCFA (85% free FA and 15% triglycerides; 50% C16:0 and 47% C18:0; Hidropalm; Norel, Madrid, Spain) on a DM basis were supplemented into the concentrate portion of each basal TMR such that additional daily intake of MP and NE_L arose from the supplement addition. Therefore, differences in

DMI between each diet were created by design (**Table 3.1**) to allow isoenergetic intake of protein and fat with HP and HF. Ingredient and chemical compositions of the control and experimental diets are presented in Table 3.1. The TMR were mixed and distributed once daily at 1000 h via electronic intake boxes (Insentec, Marknesse, the Netherlands) that controlled and recorded intake of individual animals. Cows were milked twice daily at 530 and 1630 h, and milk production was recorded electronically.

Table 3.1. Formulated TMR component intake, calculated chemical composition, and DMI during the control period and during the experimental period for the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or protein and fat (HP/HF)[1,2]

Item	Control	LP/LF	HP/LF	LP/HF	HP/HF
Ingredients, kg of DM/d					
Grass silage	7.1	6.7	6.7	6.7	6.7
Corn silage	6.9	6.5	6.5	6.5	6.5
Grass hay	1.00	0.98	0.98	0.98	0.98
Concentrate[3]	5.8	5.5	5.5	5.5	5.5
SoyPass + RaPass[4]	-	-	2.0	-	2.0
Hidropalm[5]	-	-	-	0.68	0.68
Total predicted DMI, kg/d	20.8	19.7	21.7	20.5	22.5
Observed DMI, kg/d	20.1	19.7	21.4	19.5	21.4
NE_L,[6] MJ/kg of DM					
Concentrate	7.22	7.19	7.57	8.95	8.63
TMR	6.68	6.68	6.85	7.23	7.26
DVE[7]					
Concentrate	152	152	205	131	178
TMR	87	87	111	83	105

[1]Values for TMR were calculated based on ration composition and calculated values obtained for roughages and concentrate. Calculated values for grass silage and corn silage were obtained from near-infrared spectroscopy analysis (Eurofins Agro, Wageningen, the Netherlands), and values for grass hay were obtained from CVB (2008). Concentrate values including SoyPass, RaPass, and Hidropalm were estimated from raw material composition from CVB (2008).

[2]LP/LF = basal TMR fed at 95% of control ad libitum intake with no protein or fat supplement; HP/LF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass (Borregaard LignoTech, Sarpsborg, Norway) on a DM basis; LP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 0.68 kg of Hidropalm (Norel Animal Nutrition, Madrid, Spain) on a DM basis; HP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass and 0.68 kg of Hidropalm on a DM basis.

[3]Portion of concentrate excluding rumen-protected protein and hydrogenated palm fatty acid supplements.

[4]A 50:50 mix (DM basis) of SoyPass (xylose-treated rumen-protected soybean meal) and RaPass (xylose-treated rumen-protected rapeseed meal) manufactured by Borregaard LignoTech (Sarpsborg, Norway).

[5]Hidropalm (hydrogenated palm fatty acids; 85% free fatty acids and 15% triglycerides; 50% C16:0 and 47% C18:0) manufactured by Norel Animal Nutrition (Madrid, Spain).

[6]Calculated with the VEM system (CVB, 2008).

[7]Intestinal digestible protein (CVB, 2008).

Milk and Blood Collection and Analysis

Milk sampling for daily composition analysis is described by Nichols et al. (2018). For analyses of mammary gland metabolite uptake, milk samples were collected from individual cows during the final week of each period in the afternoon milking on d 25 and 27, pooled into a weekly afternoon sample by individual cow, stored at 4°C, and analyzed within 3 d. On d 28 of each period at 0800, 1000, 1200, and 1400 h, blood samples were collected by venipuncture into 10-mL sodium heparin and potassium EDTA Vacutainers (Becton Dickinson, Franklin Lakes, NJ) concurrently from the coccygeal vessels, where arteriovenous (AV) differences across the tail are assumed to be negligible, thus representing mammary arterial supply (Emery et al., 1965), and from the subcutaneous abdominal vein of each cow. Samples were collected from the left and right subcutaneous abdominal veins, alternating at each time point, to account for differences between sides and to avoid oversampling a particular area. Collection tubes were immediately placed in ice and centrifuged at 3,000 × *g* for 15 min at 4°C. Plasma was transferred into polypropylene tubes and frozen at −20°C until analysis. Pooled afternoon milk samples were analyzed weekly for fat, crude protein (CP), lactose, and milk urea by mid-infrared spectroscopy (ISO method 9622; ISO, 2013; Qlip NV, Zutphen, the Netherlands). Arterial and venous plasma samples were pooled over time and by sampling site for each cow by period and analyzed for glucose, β-hydroxybutyrate (BHB), nonesterified FA (NEFA), TAG, and acetate according to the methods of Weekes et al. (2006) and for urea (kit no. MAK006; Sigma Chemical Co., Oakville, ON, Canada). Long-chain FA concentrations were calculated on a molar basis as 3 × TAG + NEFA. Immunoassay was used for analysis of growth hormone (kit no. CSB-E13443B; Cusabio, Wuhan, China), IGF-1 (kit no. CSB-E08893b; Cusabio), leptin (kit no. CSBE06771b; Cusabio), and insulin (kit no. 90060; Crystal Chem Inc., Downers Grove, IL). Amino acid concentrations in plasma samples collected at 1000, 1200, and 1400 h were analyzed using ultra-performance liquid chromatography in conjunction with Empower Chromatography Data software (Waters Corp., Milford, MA) according to the protocol described by Boogers et al. (2008).

Calculations and Statistical Analysis

Plasma AA concentrations were averaged over the 3 analyzed sampling times. Milk CP was assumed to consist of 94.5% true protein (DePeters and Ferguson, 1992). All following calculations were based on this estimate of true protein yield. Mammary plasma flow (MPF) across the whole udder was estimated according to the Fick principle using Phe and Tyr as internal markers (Cant et al., 1993b), where MPF (L/h) = [milk Phe + Tyr output (µmol/h)] / [AV Phe + Tyr difference (µmol/L)], with an allowance for 3.37% contribution from blood-derived proteins (Lapierre et al., 2012). Milk output of Phe + Tyr was estimated from the afternoon milk protein yield using the mean Phe and Tyr contents reported by Mepham (1987) and Lapierre et al. (2012). Mammary clearances of metabolites were calculated from the model of Hanigan et al. (1998), where clearance (L/h) = (AV difference × MPF)/venous

concentration. Uptakes (mmol/h) of metabolites across the mammary glands were calculated as the product of their plasma AV differences and MPF. Positive AV differences and uptakes indicate metabolite removal from plasma, whereas negative values indicate net metabolite release from the mammary glands. Mammary gland nutrient balances were calculated using mean milk protein AA composition reported by Mepham (1987) and Lapierre et al. (2012) for AA uptake:output ratios (U:O) and according to estimations of Dijkstra et al. (1996) for glucose, 2C compounds (acetate and BHB), and LCFA. Of C16 FA in milk, 50% were assumed to be synthesized de novo from 2C compounds and 50% were assumed to be sequestered as preformed FA. The molecular weight of FA ≥16C in blood was calculated according to their molecular weight in milk, and the 50% contribution of C16 was applied. Variances in milk and milk component production, plasma constituent concentrations, AV differences, and mammary clearances and uptakes were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) with level of protein and fat and their interaction as fixed effects and parameters measured during the control period used as covariates according to the following model:

$$Y_{ijkl} = \mu + \beta \times \mu_l + block_i + PT_j + FT_k + (PT \times FT)_{jk} + \varepsilon_{ijkl},$$

where Y_{ijkl} = observed trait, μ = overall mean, β = control covariate parameter, μ_l = observed trait in the control period of cow l, $block_i$ = random block effect (i = 1 to 14), PT_j = fixed protein effect (j = 1 to 2), FT_k = fixed fat effect (k = 1 to 2), $(PT \times FT)_{jk}$ = interaction between fixed PT and FT effects, and ε_{ijkl} = residual random error term. Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. Multiple comparisons between treatment means were made using the Tukey-Kramer method when a PT × FT interaction was detected at $P \leq 0.10$.

RESULTS

Milk Production

Daily lactation performance was presented by Nichols et al. (2018). The present paper reports milk production on an hourly basis (**Table 3.2**). Total milk yield and lactose yield increased in response to PT ($P \leq 0.01$) and FT ($P \leq 0.03$). Milk protein yield increased in response to PT ($P < 0.01$) and tended to increase in response to FT ($P = 0.05$). Milk fat yield increased in response to FT ($P < 0.01$) and tended to increase in response to PT ($P = 0.10$). Milk protein content increased in response to PT ($P < 0.01$), fat content increased in response to FT ($P = 0.02$), and lactose content was unaffected by PT or FT. Milk urea content increased in response to PT ($P < 0.01$), but the effect tended to be greater at the LF level (PT × FT interaction, $P = 0.06$).

Table 3.2. Milk and component production of lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or protein and fat (HP/HF)[1]

	Treatment[2]					*P*-value[4]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM[3]	PT	FT	PT × FT
Milk, kg/h	1.11	1.20	1.18	1.25	0.029	0.01	0.03	0.71
Crude protein, g/h	38.1	42.2	40.6	43.4	1.00	<0.01	0.05	0.50
Crude protein, g/kg	34.8	36.0	34.7	35.1	0.29	0.01	0.11	0.19
Fat, g/h	49.9	52.2	55.2	57.3	1.37	0.10	<0.01	0.93
Fat, g/kg	45.2	45.1	47.5	46.4	0.78	0.44	0.02	0.52
Lactose, g/h	50.2	54.5	54.2	57.3	1.28	0.01	0.01	0.66
Lactose, g/kg	45.4	45.7	45.8	45.7	0.18	0.45	0.35	0.28
Urea, mg/dL	21[a]	28[b]	22[a]	26[b]	0.7	<0.01	0.43	0.06

[a,b]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Data are least squares means from the final week of the experimental period using data from the final week of the control period as covariates for each parameter.

[2]LP/LF = basal TMR fed at 95% of control ad libitum intake with no protein or fat supplement; HP/LF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass (Borregaard LignoTech, Sarpsborg, Norway) on a DM basis; LP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 0.68 kg of Hidropalm (Norel Animal Nutrition, Madrid, Spain) on a DM basis; HP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass and 0.68 kg of Hidropalm on a DM basis.

[3]n = 14.

[4]PT = effect of energy from protein; FT = effect of energy from fat.

Arterial AA Concentrations, Mammary Plasma Flow, and AA Kinetics

Arterial plasma AA concentrations of total essential AA (EAA), group 1 AA, and group 2 AA were affected by a PT × FT interaction where the increase in response to protein was greater at the LF level compared with the HF level ($P \leq 0.03$; **Table 3.3**). The increase in response to PT was greater at the LF level compared with the HF level for His, Ile, Leu, and Val ($P \leq 0.05$) and tended to be greater for Lys and Phe ($P \leq 0.07$). Concentrations of all other individual EAA were unaffected by FT but increased in response to PT ($P \leq 0.04$) with the exception of Met, which was unaffected. Concentration of total non-EAA (NEAA) was unaffected by PT or FT. Individually, Pro and Tyr increased ($P < 0.01$) and Gln decreased ($P = 0.02$) in response to PT, and Gly tended to increase in response to FT ($P = 0.09$).

Arteriovenous differences of total EAA, group 1 AA, and group 2 AA were affected by a PT × FT interaction where the increase in response to PT was greater at the LF level compared with the HF level ($P \leq 0.02$; **Table 3.4**). Individually, the increase in AV difference in response to PT was greater at the LF level compared with the HF level for His, Ile, Leu, Lys, and Thr ($P \leq 0.05$) and tended to be greater for Phe and Trp ($P \leq 0.08$). Arteriovenous differences for all individual EAA were unaffected by FT, and AV difference of Arg and Val increased in response to PT ($P \leq 0.01$). Arteriovenous difference for total NEAA was affected by a PT × FT interaction where the lowest AV difference was reached at LP/LF and HP/HF and the highest was reached at HP/LF and LP/HF ($P = 0.02$). Individually, AV difference of Asn and of Gln increased ($P <$

0.01) and that of Tyr tended to increase (P = 0.06) in response to PT to a greater extent at the LF level than at the HF level, and Gly AV difference tended to reach the lowest AV difference at LP/LF and HP/HF and the highest AV difference at HP/LF and LP/HF (P = 0.08). The AV difference of Asp tended to increase (P = 0.09) in response to PT, and the AV difference of Glu decreased in response to PT and FT (P = 0.02).

Mammary plasma flow and AA clearance rates are presented in **Table 3.5**. Mammary plasma flow tended to be affected by a PT × FT interaction (P = 0.07), where the highest plasma flow was observed at LP/LF. Mammary clearance of total EAA tended to decrease in response to PT (P = 0.06) and was unaffected by FT. Clearance of group 1 AA decreased in response to PT (P < 0.01), whereas group 2 AA clearance was unaffected by PT or FT. Individually, clearance of His, Leu, and Phe decreased (all P < 0.01) in response to PT. Clearance of Met decreased in response to FT (P = 0.03). Mammary clearance of total NEAA tended to be affected by a PT × FT interaction (P = 0.07). In response to PT, Glu clearance tended to decrease more at the HF level (P = 0.08), Gln clearance tended to increase more at the LF level (P = 0.08), and Ala clearance tended to decrease (P = 0.06). Clearance of Ser increased in response to FT (P = 0.01).

Mammary gland net uptake of total EAA increased in response to PT (P = 0.03) and was unaffected by FT (**Table 3.6**). Uptake of group 1 AA was unaffected by PT or FT, but uptake of group 2 AA increased in response to PT (P = 0.02). Individually, uptakes of Arg, Ile, and Leu increased (P ≤ 0.04) and uptake of Val tended to increase (P = 0.07) in response to PT. Uptakes of individual EAA were not affected by FT. Mammary net uptake of total NEAA tended to be affected by a PT × FT interaction (P = 0.08). Individually, a PT × FT interaction affected uptake of Asn (P = 0.03), indicating an increase in response to PT at the LF level but no response at the HF level, and tended to affect uptake of Gln and Gly (P = 0.06), where they reached the lowest uptake at LP/LF and HP/HF and the highest uptake at HP/LF and LP/HF. In response to PT, uptake of Tyr increased (P = 0.05) and uptakes of Ala and Glu decreased (P ≤ 0.04). Uptake of Ser increased in response to FT (P = 0.03).

The mammary gland U:O of total EAA and group 2 AA increased in response to PT (P = 0.04; **Table 3.7**). This ratio for the group 1 AA was unaffected by PT or FT. Individually, in response to PT, the U:O of Leu tended to be affected more at the LF level (PT × FT interaction; P = 0.06). The U:O of Ile and Val increased in response to PT (P ≤ 0.05), and the U:O of Trp tended to decrease (P = 0.10). A PT × FT interaction affected NEAA U:O (P = 0.06), which numerically decreased upon increasing PT at the HF level but not at the LF level. Individually, the U:O of Asn was affected by a PT × FT interaction where the lowest values were achieved on LP/LF and HP/HF (P < 0.01); a similar tendency (P ≤ 0.08) was observed for Gln and Gly. The U:O of Ala and Glu decreased in response to PT (P ≤ 0.01), and the U:O of Glu tended to decrease (P = 0.10) and Ser tended to increase (P = 0.07) in response to FT.

Table 3.3. Arterial plasma concentrations (μ*M*) of AA in lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or protein and fat (HP/HF)[1]

	Treatment[2]					*P*-value[4]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM[3]	PT	FT	PT × FT
Arg	83	105	91	99	5.1	0.01	0.90	0.19
His	25[a]	56[b]	28[a]	47[c]	2.2	<0.01	0.15	<0.01
Ile	139[a]	168[b]	146[ab]	150[ab]	5.9	0.01	0.35	0.05
Leu	99[a]	158[b]	104[a]	139[b]	5.1	<0.01	0.17	0.03
Lys	78[a]	93[b]	84[ab]	85[ab]	3.6	0.04	0.77	0.06
Met	18	18	18	19	1.0	0.66	0.57	0.90
Phe	40[a]	49[b]	41[a]	46[b]	1.2	<0.01	0.63	0.07
Thr	94	105	94	101	4.1	0.04	0.76	0.64
Trp	42	48	44	45	1.5	0.03	0.70	0.14
Val	217[a]	310[b]	231[a]	284[b]	8.1	<0.01	0.49	0.02
Ala	207	202	220	202	7.5	0.11	0.35	0.42
Asn	71	84	77	77	4.2	0.15	0.96	0.17
Asp	6.3	7.6	6.2	6.8	0.55	0.12	0.42	0.54
Gln	106	96	104	95	3.9	0.02	0.65	0.88
Glu	114	111	114	118	3.1	0.88	0.27	0.22
Gly	240	220	254	249	12.1	0.33	0.09	0.54
Pro	75	92	80	91	3.2	<0.01	0.55	0.42
Ser	97	92	90	89	3.1	0.37	0.16	0.47
Tyr	48	58	49	53	2.2	<0.01	0.31	0.19
EAA[5]	817[a]	1109[b]	867[ac]	989[bc]	33.0	<0.01	0.30	0.02
Group 1[6]	169[a]	227[b]	178[a]	205[b]	6.3	<0.01	0.30	0.02
Group 2[7]	696[a]	941[b]	738[ac]	838[bc]	30.5	<0.01	0.32	0.03
NEAA[8]	965	966	992	973	28.8	0.75	0.56	0.75
TAA[9]	1777[a]	2084[b]	1860[a]	1956[ab]	56.0	<0.01	0.68	0.08

[a-c]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Data are least squares means from d 28 of the experimental period where measurements on d 28 of the control period are used as covariates.

[2]LP/LF = basal TMR fed at 95% of control ad libitum intake with no protein or fat supplement; HP/LF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass (Borregaard LignoTech, Sarpsborg, Norway) on a DM basis; LP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 0.68 kg of Hidropalm (Norel Animal Nutrition, Madrid, Spain) on a DM basis; HP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass and 0.68 kg of Hidropalm on a DM basis.

[3]n = 14.

[4]PT = effect of energy from protein; FT = effect of energy from fat.

[5]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

[6]Group 1 = His, Met, Phe + Tyr, Trp.

[7]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.

[8]NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, Tyr.

[9]Total AA = EAA + NEAA.

Table 3.4. Mammary gland arteriovenous differences (μ*M*) of AA in lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or protein and fat (HP/HF)[1]

	Treatment[2]					*P*-value[4]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM[3]	PT	FT	PT × FT
Arg	28	39	32	36	2.6	0.01	0.79	0.15
His	8.2[a]	12.3[b]	10.3[ab]	10.4[ab]	0.89	0.02	0.92	0.03
Ile	30[a]	42[b]	32[a]	37[ab]	1.8	<0.01	0.43	0.05
Leu	44[a]	60[b]	49[ac]	53[bc]	2.0	<0.01	0.66	0.01
Lys	43[a]	53[b]	48[ab]	47[ab]	2.0	0.06	0.77	0.02
Met	9.2	10.2	9.0	9.2	0.90	0.50	0.52	0.65
Phe	17[a]	21[b]	18[ab]	19[ab]	0.8	<0.01	0.71	0.08
Thr	23[a]	28[b]	24[ab]	25[ab]	1.1	0.03	0.43	0.05
Trp	3.7	5.0	4.2	3.9	0.46	0.21	0.45	0.06
Val	40	55	45	51	3.2	<0.01	0.94	0.15
Ala	25	20	24	18	4.0	0.21	0.67	0.97
Asn	18[a]	27[b]	23[ab]	21[ab]	2.0	0.11	0.84	<0.01
Asp	1.9	3.4	1.9	2.1	0.50	0.09	0.21	0.18
Gln	19[a]	27[b]	23[ab]	21[ab]	1.8	0.06	0.53	<0.01
Glu	35	32	32	27	1.8	0.02	0.02	0.32
Gly	-8.2	12.5	10.5	0.89	8.41	0.51	0.68	0.08
Pro	8.4	10.4	11.0	11.8	1.76	0.45	0.25	0.74
Ser	16	19	20	18	2.2	0.96	0.42	0.25
Tyr	16[a]	20[b]	17[ab]	18[ab]	0.8	<0.01	0.46	0.06
EAA[5]	235[a]	323[b]	266[ac]	284[bc]	11.3	<0.01	0.73	<0.01
Group 1[6]	54[a]	67[b]	58[ab]	58[ab]	3.0	0.02	0.34	0.02
Group 2[7]	198[a]	275[b]	226[ac]	243[bc]	9.9	<0.01	0.83	<0.01
NEAA[8]	125	172	163	141	14.4	0.41	0.80	0.02
TAA[9]	359[a]	495[b]	429[ab]	425[ab]	22.1	<0.01	0.99	<0.01

[a-c]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Data are least squares means from d 28 of the experimental period where measurements on d 28 of the control period are used as covariates.

[2]LP/LF = basal TMR fed at 95% of control ad libitum intake with no protein or fat supplement; HP/LF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass (Borregaard LignoTech, Sarpsborg, Norway) on a DM basis; LP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 0.68 kg of Hidropalm (Norel Animal Nutrition, Madrid, Spain) on a DM basis; HP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass and 0.68 kg of Hidropalm on a DM basis.

[3]n = 14.

[4]PT = effect of energy from protein; FT = effect of energy from fat.

[5]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

[6]Group 1 = His, Met, Phe + Tyr, Trp.

[7]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.

[8]NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, Tyr.

[9]Total AA = EAA + NEAA.

Table 3.5. Whole-mammary gland plasma flow and clearances of AA in lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or protein and fat (HP/HF)[1]

	Treatment[2]					*P*-value[4]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM[3]	PT	FT	PT × FT
Plasma flow, L/h	706	604	654	666	38.7	0.16	0.87	0.07
Clearance, L/h								
Arg	353	404	367	420	42.5	0.12	0.64	0.98
His	778	118	620	330	129.4	<0.01	0.82	0.17
Ile	194	203	191	231	15.9	0.13	0.43	0.34
Leu	614	373	631	425	43.9	<0.01	0.40	0.66
Lys	855	835	911	826	76.3	0.49	0.76	0.67
Met	818	1113	708	691	123.2	0.23	0.03	0.18
Phe	528	448	567	478	33.0	<0.01	0.23	0.87
Thr	230	204	243	233	18.9	0.35	0.27	0.68
Trp	71	70	62	67	7.9	0.81	0.42	0.75
Val	150	128	165	153	15.2	0.28	0.20	0.74
Ala	104	67	101	71	16.9	0.06	0.97	0.86
Asn	233	274	283	255	24.0	0.77	0.46	0.12
Asp	364	861	443	247	268.6	0.58	0.33	0.21
Gln	141[a]	245[b]	193[ab]	204[ab]	25.2	0.03	0.82	0.08
Glu	270[ab]	265[ab]	275[a]	205[b]	20.2	0.04	0.13	0.08
Gly	-3.0	33.4	51.1	3.1	25.67	0.82	0.65	0.11
Pro	94	80	114	97	15.1	0.31	0.23	0.95
Ser	138	141	195	179	18.4	0.73	0.01	0.62
Tyr	350	324	377	344	24.9	0.23	0.34	0.88
EAA[5]	293	243	299	269	21.3	0.06	0.44	0.63
Group 1[6]	309	249	326	263	20.7	<0.01	0.47	0.94
Group 2[7]	296	247	298	276	23.0	0.12	0.48	0.55
NEAA[8]	101	124	139	113	13.0	0.94	0.30	0.07
TAA[9]	173	181	204	185	14.0	0.69	0.20	0.32

[a,b]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Data are least squares means calculated from measurements on d 28 of the experimental period where calculations from d 28 of the control period are used as covariates.

[2]LP/LF = basal TMR fed at 95% of control ad libitum intake with no protein or fat supplement; HP/LF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass (Borregaard LignoTech, Sarpsborg, Norway) on a DM basis; LP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 0.68 kg of Hidropalm (Norel Animal Nutrition, Madrid, Spain) on a DM basis; HP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass and 0.68 kg of Hidropalm on a DM basis.

[3]n = 14.

[4]PT = effect of energy from protein; FT = effect of energy from fat.

[5]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

[6]Group 1 = His, Met, Phe + Tyr, Trp.

[7]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.

[8]NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, Tyr.

[9]Total AA = EAA + NEAA.

Table 3.6. Mammary gland uptakes (mmol/h) of AA in lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or protein and fat (HP/HF)[1]

	Treatment[2]					*P*-value[4]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM[3]	PT	FT	PT × FT
Arg	18.7	23.5	21.6	24.2	2.05	0.04	0.31	0.54
His	5.4	7.1	6.8	6.8	0.58	0.13	0.33	0.16
Ile	19.5	23.7	21.4	24.8	1.36	<0.01	0.26	0.75
Leu	29.7	34.5	31.9	35.2	1.86	0.01	0.32	0.63
Lys	28.8	30.9	30.7	31.7	1.84	0.29	0.35	0.69
Met	6.4	6.0	5.8	5.8	0.55	0.64	0.47	0.70
Phe	11.4	12.1	12.1	12.8	0.60	0.20	0.19	0.93
Thr	14.8	16.1	16.4	16.3	0.93	0.41	0.22	0.38
Trp	2.4	3.0	2.5	2.6	0.25	0.14	0.50	0.42
Val	25.9	31.5	30.0	34.2	2.61	0.07	0.20	0.78
Ala	17.4	11.8	18.5	12.1	2.82	0.04	0.80	0.89
Asn	11.6[a]	15.7[b]	15.0[ab]	14.2[ab]	1.19	0.14	0.40	0.03
Asp	1.4	2.0	1.3	1.5	0.30	0.18	0.25	0.48
Gln	12.2	16.3	15.1	13.9	1.47	0.31	0.86	0.06
Glu	22.6	19.6	22.0	18.2	1.48	0.01	0.42	0.75
Gly	-4.3	6.4	9.0	0.5	5.00	0.82	0.47	0.06
Pro	5.6	5.8	7.6	7.7	1.18	0.89	0.11	0.96
Ser	9.7	10.2	13.0	12.4	1.19	0.99	0.03	0.65
Tyr	10.5	11.8	11.2	11.9	0.54	0.05	0.42	0.54
EAA[5]	161.4	187.6	175.1	189.3	10.54	0.03	0.39	0.50
Group 1[6]	35.6	38.9	37.9	38.3	1.92	0.32	0.63	0.41
Group 2[7]	136.5	160.2	148.3	163.0	9.42	0.02	0.37	0.57
NEAA[8]	81.9	98.6	113.1	93.0	10.13	0.86	0.21	0.08
TAA[9]	241.9	287.3	288.2	281.4	18.66	0.26	0.23	0.12

[a,b]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Data are least squares means calculated from measurements on d 28 of the experimental period where calculations from d 28 of the control period are used as covariates.

[2]LP/LF = basal TMR fed at 95% of control ad libitum intake with no protein or fat supplement; HP/LF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass (Borregaard LignoTech, Sarpsborg, Norway) on a DM basis; LP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 0.68 kg of Hidropalm (Norel Animal Nutrition, Madrid, Spain) on a DM basis; HP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass and 0.68 kg of Hidropalm on a DM basis.

[3]n = 14.

[4]PT = effect of energy from protein; FT = effect of energy from fat.

[5]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

[6]Group 1 = His, Met, Phe + Tyr, Trp.

[7]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.

[8]NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, Tyr.

[9]Total AA = EAA + NEAA.

Table 3.7. Mammary gland AA uptake:milk protein output ratios in lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or protein and fat (HP/HF)[1]

	Treatment[2]					*P*-value[4]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM[3]	PT	FT	PT × FT
Arg	2.96	3.06	2.70	2.86	0.159	0.42	0.18	0.85
His	0.98	1.01	1.02	0.94	0.033	0.40	0.75	0.11
Ile	1.20	1.36	1.20	1.36	0.045	<0.01	0.95	0.99
Leu	1.05[a]	1.18[b]	1.09[ac]	1.15[bc]	0.022	<0.01	0.66	0.06
Lys	1.36	1.34	1.38	1.31	0.036	0.20	0.97	0.42
Met	0.91	0.92	0.91	0.88	0.035	0.79	0.60	0.57
Phe	1.05	1.03	1.05	1.04	0.014	0.43	0.59	0.47
Thr	0.99	1.01	1.02	0.99	0.030	0.81	0.96	0.36
Trp	1.09	0.92	0.96	0.85	0.095	0.10	0.25	0.74
Val	1.24	1.42	1.31	1.44	0.077	0.05	0.56	0.78
Ala	1.23	0.78	1.18	0.78	0.163	0.01	0.87	0.90
Asn	1.01	1.25	1.24	1.07	0.079	0.58	0.70	<0.01
Asp	0.09	0.12	0.08	0.08	0.020	0.50	0.21	0.54
Gln	0.52	0.63	0.60	0.53	0.052	0.69	0.85	0.08
Glu	0.56	0.42	0.50	0.39	0.031	<0.01	0.10	0.56
Gly	-0.55	0.67	0.98	0.05	0.568	0.81	0.43	0.07
Pro	0.17	0.17	0.21	0.21	0.030	0.99	0.20	0.90
Ser	0.45	0.45	0.58	0.51	0.049	0.52	0.07	0.51
Tyr	0.95	0.97	0.96	0.96	0.013	0.43	0.59	0.47
EAA[5]	1.17	1.29	1.22	1.25	0.034	0.04	0.91	0.20
Group 1[6]	0.92	0.94	0.93	0.90	0.022	0.73	0.38	0.19
Group 2[7]	1.24	1.38	1.29	1.34	0.042	0.04	0.82	0.27
NEAA[8]	0.45	0.50	0.59	0.46	0.049	0.44	0.38	0.06
TAA[9]	0.76	0.84	0.86	0.79	0.035	0.77	0.45	0.05

[a-c]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Data are least squares means calculated from measurements on d 28 of the experimental period where calculations from d 28 of the control period are used as covariates. The AA composition in milk protein was estimated using Mepham (1987) and Lapierre et al. (2012).

[2]LP/LF = basal TMR fed at 95% of control ad libitum intake with no protein or fat supplement; HP/LF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass (Borregaard LignoTech, Sarpsborg, Norway) on a DM basis; LP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 0.68 kg of Hidropalm (Norel Animal Nutrition, Madrid, Spain) on a DM basis; HP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass and 0.68 kg of Hidropalm on a DM basis.

[3]n = 14.

[4]PT = effect of energy from protein; FT = effect of energy from fat.

[5]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

[6]Group 1 = His, Met, Phe + Tyr, Trp.

[7]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.

[8]NEAA = Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, Tyr.

[9]Total AA = EAA + NEAA.

Other Metabolites and Hormones: Arterial Concentrations and Kinetics

Arterial plasma glucose concentration was unaffected by PT or FT (**Table 3.8**). A PT × FT interaction affected BHB and acetate concentrations ($P \leq 0.04$), where they increased in response to PT, but only at the LF level. Concentrations of NEFA, TAG, and LCFA increased in response to FT ($P < 0.01$) and were unaffected by PT. Urea concentration increased in response to PT ($P < 0.01$) and was unaffected by FT. All hormone concentrations were unaffected by PT or FT except insulin, which decreased in response to FT but only at the HP level (PT × FT interaction, $P < 0.01$).

Mammary AV difference, clearance, and uptake of glucose were unaffected by PT or FT (**Table 3.9**). A PT × FT interaction affected AV difference of BHB ($P = 0.03$) and tended to affect the AV difference of acetate ($P = 0.08$), where the lowest AV difference was reached at LP/LF and HP/HF and the highest AV difference was reached at HP/LF and LP/HF. Mammary AV difference, clearance, and uptake of NEFA were unaffected by PT or FT. Mammary AV difference ($P \leq 0.01$), clearance ($P \leq 0.05$), and uptake ($P < 0.01$) of TAG and LCFA increased in response to FT. Arteriovenous difference, clearance, and uptake of LCFA tended to decrease ($P \leq 0.09$) in response to PT.

In response to FT, calculated glucose output as milk lactose tended to increase ($P = 0.09$; **Table 3.10**) and glucose required for fat synthesis increased ($P = 0.04$), whereas PT did not affect these variables. Glucose uptake not required for lactose and fat synthesis was not affected by PT or FT. Milk output of 2C compounds increased in response to PT ($P = 0.01$), and output of LCFA increased in response to FT ($P < 0.01$). Calculated 2C balance was not affected by PT or FT. Long-chain FA uptake did not cover requirements for LCFA output on any treatment and became more negative in response to PT ($P = 0.01$).

Table 3.8. Arterial plasma concentrations of metabolites and hormones in lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or protein and fat (HP/HF)[1]

	Treatment[3]					*P*-value[5]		
Item[2]	LP/LF	HP/LF	LP/HF	HP/HF	SEM[4]	PT	FT	PT × FT
Glucose, m*M*	2.62	2.66	2.61	2.68	0.060	0.39	0.96	0.76
BHB, m*M*	0.75[a]	0.92[b]	0.76[ab]	0.64[a]	0.045	0.56	<0.01	<0.01
Acetate, m*M*	1.63[ab]	1.85[a]	1.65[ab]	1.47[b]	0.094	0.84	0.06	0.04
NEFA, µ*M*	96	90	117	115	5.0	0.36	<0.01	0.61
TAG, µ*M*	52	52	64	60	2.5	0.30	<0.01	0.37
LCFA, µ*M*	254	246	309	295	9.4	0.20	<0.01	0.77
Urea, m*M*	4.56	5.61	4.63	5.51	0.173	<0.01	0.93	0.61
GH, µg/L	3.37	3.48	3.47	3.71	0.302	0.56	0.60	0.83
IGF-1, µg/L	3.15	3.20	3.21	3.66	0.277	0.38	0.35	0.47
Leptin, µg/L	6.51	6.47	6.57	6.48	0.071	0.37	0.59	0.74
Insulin, µg/L	1.31[ab]	1.49[a]	1.22[bc]	1.11[c]	0.052	0.53	<0.01	<0.01

[a-c]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Data are least squares means from d 28 of the experimental period where measurements on d 28 of the control period are used as covariates.

[2]NEFA = nonesterified fatty acids; TAG = triacylglycerol; LCFA = long-chain fatty acids; GH = growth hormone.

[3]LP/LF = basal TMR fed at 95% of control ad libitum intake with no protein or fat supplement; HP/LF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass (Borregaard LignoTech, Sarpsborg, Norway) on a DM basis; LP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 0.68 kg of Hidropalm (Norel Animal Nutrition, Madrid, Spain) on a DM basis; HP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass and 0.68 kg of Hidropalm on a DM basis.

[4]n = 14.

[5]PT = effect of energy from protein; FT = effect of energy from fat.

Table 3.9. Mammary gland arteriovenous (AV) differences, clearances, and uptakes of metabolites in lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or protein and fat (HP/HF)[1]

	Treatment[3]					*P*-value[5]		
Item[2]	LP/LF	HP/LF	LP/HF	HP/HF	SEM[4]	PT	FT	PT × FT
Glucose								
AV difference, m*M*	0.75	0.86	0.83	0.82	0.047	0.30	0.60	0.21
Clearance, L/h	268	298	314	314	28.5	0.58	0.26	0.59
Uptake, mmol/h	508	511	539	549	43.4	0.87	0.41	0.93
BHB								
AV difference, m*M*	0.21	0.29	0.28	0.17	0.041	0.81	0.56	0.03
Clearance, L/h	337	389	473	255	134.6	0.55	0.99	0.32
Uptake, mmol/h	137	168	173	116	30.2	0.67	0.79	0.15
Acetate								
AV difference, m*M*	0.96	1.19	1.06	0.95	0.095	0.55	0.45	0.08
Clearance, L/h	1066	1171	1207	1301	170.2	0.54	0.39	0.97
Uptake, mmol/h	669	712	678	659	77.9	0.87	0.76	0.66
NEFA								
AV difference, µ*M*	-19	-25	-28	-30	4.3	0.36	0.14	0.65
Clearance, L/h	-136	-123	-121	-141	19.6	0.85	0.93	0.39
Uptake, mmol/h	-15.6	-16.4	-17.8	-21.4	3.5	0.51	0.29	0.69
TAG								
AV difference, µ*M*	24	23	36	29	2.0	0.10	<0.01	0.11
Clearance, L/h	544	507	810	703	68.0	0.28	<0.01	0.60
Uptake, mmol/h	15.4	13.6	22.0	19.7	1.4	0.13	<0.01	0.84
LCFA								
AV difference, µ*M*	51	46	80	57	7.7	0.06	0.01	0.24
Clearance, L/h	165	131	223	172	24.7	0.09	0.05	0.74
Uptake, mmol/h	31.0	25.7	48.3	36.1	4.76	0.07	<0.01	0.47

[1]Data are least squares means calculated from measurements on d 28 of the experimental period where calculations from d 28 of the control period are used as covariates.

[2]NEFA = nonesterified fatty acids; TAG = triacylglycerol; LCFA = long-chain fatty acids.

[3]LP/LF = basal TMR fed at 95% of control ad libitum intake with no protein or fat supplement; HP/LF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass (Borregaard LignoTech, Sarpsborg, Norway) on a DM basis; LP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 0.68 kg of Hidropalm (Norel Animal Nutrition, Madrid, Spain) on a DM basis; HP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass and 0.68 kg of Hidropalm on a DM basis.

[4]n = 14.

[5]PT = effect of energy from protein; FT = effect of energy from fat.

Table 3.10. Calculated mammary gland nutrient balance in lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or protein and fat (HP/HF)[1]

	Treatment[2]					*P*-value[4]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM[3]	PT	FT	PT × FT
Glucose uptake, g/h	91	92	97	99	7.9	0.87	0.41	0.93
Required for lactose, g/h	54	56	59	60	2.5	0.55	0.09	0.92
Required for fat, g/h	17	18	19	19	0.9	0.45	0.04	0.39
Excess,[5] g/h	22	17	22	21	6.2	0.62	0.78	0.73
2C uptake,[6] g/h	53	60	58	51	6.7	0.95	0.72	0.33
2C output, g/h	21	23	22	24	0.59	0.01	0.12	0.82
2C balance, g/h	32	37	36	27	6.3	0.81	0.59	0.26
LCFA[7] uptake, g/h	8.8	7.3	13.8	10.3	1.34	0.07	<0.01	0.47
LCFA output, g/h	25	26	30	30	0.8	0.36	<0.01	0.75
LCFA balance, g/h	-16	-20	-16	-20	1.7	0.01	0.92	0.99

[1]Estimated based on calculations of Dijkstra et al. (1996).

[2]LP/LF = basal TMR fed at 95% of control ad libitum intake with no protein or fat supplement; HP/LF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass (Borregaard LignoTech, Sarpsborg, Norway) on a DM basis; LP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 0.68 kg of Hidropalm (Norel Animal Nutrition, Madrid, Spain) on a DM basis; HP/HF = TMR fed at 95% of control ad libitum intake supplemented daily with 2.0 kg of SoyPass + RaPass and 0.68 kg of Hidropalm on a DM basis.

[3]n = 14.

[4]PT = effect of energy from protein; FT = effect of energy from fat.

[5]Uptake − required for lactose − required for fat.

[6]2C = acetate + BHB.

[7]Long-chain fatty acid.

DISCUSSION

Results presented here complement previously reported daily lactation performance, digestibility, and milk FA composition (Nichols et al., 2018). In the present paper, we report milk yield and composition on an hourly basis where PT and FT independently and additively stimulated total milk and lactose yield, PT increased protein yield and tended to increase fat yield, and FT increased fat yield and tended to increase protein yield. Dry matter intake did not differ from the designed differences, as described by Nichols et al. (2018). Therefore, delivery of aminogenic and lipogenic nutrients as precursor substrates in circulation for mammary gland use arose from dietary supplementation of RP protein or rumen-inert hydrogenated palm FA. Here we investigated how local mammary mechanisms were affected to produce similar levels of milk lactose, protein, and fat when aminogenic versus lipogenic diets were fed.

Mammary Glucose Balance

Lactose yield increased independently and additively in response to PT and FT. A relationship exists between protein supplementation and lactose yield where AA increase whole-body appearance of glucose through increased true appearance across the splanchnic

tissues, mainly through hepatic gluconeogenesis (Galindo et al., 2011). Many research groups have reported increases in whole-body glucose appearance, total milk yield, and lactose yield in response to increased postruminal AA supply (Clark et al., 1977; Lemosquet et al., 2009a; Galindo et al., 2011). Thus, we hypothesized that AA from PT would increase hepatic glucose production to support milk lactose yield, which would be reflected in higher arterial glucose and mammary glucose uptake. However, the approximately 90 g/d increase in lactose yield observed on HP compared with LP diets did not coincide with an increase in mammary glucose uptake. Given that fat yield, and therefore presumably glucose requirements for glycerol and NADPH related to de novo FA synthesis, did not decrease, glucose use for other purposes must have decreased. The calculated excess glucose uptake relative to that required for lactose and fat synthesis decreased numerically (~70 g/d) on HP compared with LP diets, which may indicate reduced contribution of glucose to glycolysis and the tricarboxylic acid cycle. In contrast to AA, LCFA do not yield direct glucose precursors. With regards to the effect of FT on lactose yield, we hypothesized, in line with others (Grummer and Carroll, 1991; Chilliard, 1993; Hammon et al., 2008), that reduction in mammary de novo FA synthesis and the associated glucose utilization to provide NADPH for this synthesis could have contributed glucose for lactose production on HF diets. However, even with the assumptions that 100% NADPH required for de novo FA synthesis comes from glucose and that some mixed-source C16 FA shifted from de novo to preformed FA, this contribution remains small (~12 g/d; Nichols et al., 2018) and cannot have covered the approximately 80 g/d increase in lactose yield observed on HF compared with LF diets. Therefore, it could again be suspected that glucose was made available for lactose synthesis through increased arterial concentration or mammary glucose uptake in response to FT. However, in contrast to these hypotheses, we found no effect of FT on plasma glucose concentration or mammary glucose uptake.

Overall, the mammary gland sequestered glucose in excess of estimated requirements for lactose and fat synthesis, assuming that 1.05 and 0.31 g of glucose is required to synthesize 1 g of lactose and fat, respectively (Dijkstra et al., 1996). On average across all diets, 61% of glucose uptake contributed to lactose synthesis. This level of transfer efficiency is similar to that of Cant et al. (1993a), who observed 57 and 77% transfer of mammary extracted glucose into lactose when casein was infused abomasally with and without dietary fat supplementation, respectively. However, it appears that when mammary glucose balance is positive, priority for glucose use is not directed infinitely toward further stimulation of lactose synthesis. Indeed, when Cant et al. (2002) infused glucose into the external iliac artery, lactose yield increased 18%, whereas glucose uptake increased 39%. Similarly, Rigout et al. (2002) duodenally infused glucose in graded amounts and observed excess mammary glucose uptake relative to lactose output at all doses. Of measured glucose uptake in this study, FT increased glucose required for fat synthesis, but this is likely an overestimation

reflected in the calculation because FT also decreased de novo FA synthesis in the present experiment (Nichols et al., 2018). However, although mammary net LCFA uptake increased in response to fat, it did not meet the requirements for LCFA (≥16C) production. Net LCFA uptake did not equilibrate with LCFA output in milk on any treatment in this experiment, but 2C balance was positive. The positive 2C balance may indicate synthesis of C16 from acetate and BHB, which may have been released from the gland in venous drainage, giving rise to the observed net release of NEFA. This synthesis and release would contribute to the observed shortfall in net mammary supply of LCFA from blood relative to milk LCFA. A possible use for excess glucose uptake not sequestered in lactose may have been oxidation to support glycerol synthesis and to yield NADPH for C16 synthesis from excess acetate to compensate for the apparently deficient preformed LCFA uptake (Dils, 1983). Excess mammary glucose also is oxidized to provide carbon for NEAA synthesis and to yield ATP for fat, protein, and lactose synthesis (Smith et al., 1983; Qiao et al., 2005). In fed goats and cows, respectively, 25 and 11% of intramammary glucose was oxidized, which contributed 39 and 24% of CO_2 produced by the gland (Bickerstaffe et al., 1974; Linzell, 1974).

Although mammary priority for glucose is high relative to other body tissues, rates of glucose sequestration and secretion of lactose in milk appear not to be correlated with circulating glucose concentrations in lactating dairy cows (Cant et al., 1993a; Nielsen et al., 2001; Qiao et al., 2005). In agreement, glucose clearance in this study was not affected by PT or FT, suggesting that capacity of glucose transport did not limit glucose uptake. Translocation of glucose into mammary cells is faster than its rate of metabolism (Xiao and Cant, 2005), and extraction can be influenced by factors such as mammary biosynthetic capacity rather than physiological substrate supply (Miller et al., 1991; Cant et al., 1993a). Glucose uptake covered requirements for lactose yield without supplementation of PT or FT, as evidenced by the excess glucose uptake on LP/LF. As such, stimulation of lactose yield with protein and fat must have been driven by factors other than mammary glucose supply. In line with this observation, Lemosquet et al. (2009a) infused propionate and casein and observed no increase in glucose uptake, but lactose yield increased in response to casein. Lemosquet et al. (2009b) and Lapierre et al. (2010) suggested that glucose availability is not always the driving mechanism to increase lactose synthesis, as evidenced when whole-body glucose appearance increases with no appreciable change in lactose yield. Lactose synthesis may be largely regulated by mammary mechanisms independent of glucose supply, such as glucose phosphorylation and transport capacity, and concentrations of metabolites glucose-6-P and glucose-1-P (Xiao and Cant, 2005). The synthetic processes for milk protein and fat may also play a role in determining total milk yield through stimulatory effects on cell signaling pathways dictating overall mammary synthetic capacity (Nichols et al., 2017) or through effects on hormones and enzymes controlling flux through the lactose synthesis pathway (Anderson et al., 2007). Taken together, results of previous studies along with data

presented here suggest that although glucose is necessary to support lactose yield, glucose availability per se is not the driving force stimulating lactose synthesis in mid-lactation cows, and that mammary regulation of milk production is dependent on factors other than mammary glucose supply.

AA Utilization with Energy from Protein

Total plasma EAA concentration increased in response to PT, in particular at the LF level. Arterial concentrations of all individual EAA increased in response to PT, with the exception of Met. This indicates intestinal absorption of the RP protein supplement in response to PT, agrees with our hypothesis that AA from protein stimulated the increase in milk protein yield, and agrees with previously reported positive effects of postruminal protein supply on plasma AA concentrations (Doepel and Lapierre, 2010; Rius et al., 2010b; Nichols et al., 2016). Considering the classification of AA as group 1 (His, Met, Phe + Tyr, Trp) or group 2 (Arg, Ile, Leu, Lys, Thr, Val) based on their respective U:O ratios close to or greater than unity (Mepham, 1987), nuances in mammary AA metabolism can be examined. In the present study, U:O of total group 1 AA was less than unity. Previous studies have shown variability in this canonical 1:1 relationship during abomasal infusions of casein or of EAA mixtures in the profile of casein (Raggio et al., 2006; Doepel and Lapierre, 2010, 2011). A novel element of the current study is that it used a relatively large number of cows managed under a more practical setting relative to several mammary net balance studies that have been conducted previously. As such, we expected a certain level of variation in this pattern. Factors such as lack of steady-state feeding, fewer blood samples taken over the milking interval, and larger cow variation are limitations of the current study and place a limit of accuracy on the U:O measurements that should be accepted. Although the absolute values for U:O for the group 1 AA and for total AA indicate some discrepancy on the whole, the relative differences in response to PT (no effect on the total group 1 AA, increased group 2 AA) align with other studies performing mammary AA net balance under conditions of supplemental MP (Raggio et al., 2006; Haque et al., 2015). Previous mammary balance studies have produced total AA balances greater than or equal to unity (Raggio et al., 2006; Doepel and Lapierre, 2010), but some studies present values near 0.90 (Larsen et al., 2014, 2015), with values ranging from 0.83 to 0.96 within a study (Larsen et al., 2014). Furthermore, in the current study, when U:O for total AA was calculated on an N basis, the ratio increased to 0.85, 0.91, 0.93, and 0.88 for LP/LF, HP/LF, LP/HF, and HP/HF, respectively. On average, 11% of milk AA-N was not accounted for, which either was derived from unquantified N sources in the mammary gland such as non-protein N or AA peptides, or represents a discrepancy in our practical mammary net balance (Bequette et al., 1998).

Increased MP supply yields increased blood AA concentrations and transfer of AA into milk, but also increased AA catabolism (Lapierre et al., 2005). In response to PT, arterial concentration of group 1 AA increased, but mammary uptake was not affected and clearance

tended to decrease. Decreased mammary affinity for group 1 AA suggests that their potential for transport into mammary cells was maximized below the HP level. Although protein did contribute to absorbed group 1 AA, a lack of concomitant mammary uptake might allow hepatic catabolism of these AA at the HP level. Group 1 AA are subject to hepatic removal, which is also linked with mammary requirements (Lapierre et al., 2005), and hepatic flux of His, Met, and Phe in particular responds to MP supply by increasing linearly with increased MP level (Raggio et al., 2004). This agrees with our observation of decreased mammary affinity for total group 1 AA and increased plasma urea concentration in response to PT, and may suggest that in this case hepatic catabolism is responding to mammary gland utilization (Lobley and Lapierre, 2003). The increase in arterial concentration of group 2 AA in response to PT was accompanied by increased mammary uptake. Increased milk protein yield stimulated by PT was likely supported by mammary extraction of group 2 AA, of which the U:O ratio increased in response to PT. Excess uptake of group 2 AA is used for intramammary NEAA synthesis (Mepham, 1987; Lapierre et al., 2012) in support of milk protein. Our observations agree with those of others when EAA supply is abundant and group 2 AA uptake contributes to NEAA synthesis (Raggio et al., 2006; Doepel and Lapierre, 2010, 2011) and are supported here by no appreciable change in NEAA uptake on HP diets and increased milk protein yield in response to PT.

AA Utilization with Energy from Fat

A second novel element of this study is the investigation of mammary gland metabolite utilization during fat supplementation at 2 levels of MP supply. In response to FT, milk protein yield tended to increase, but no significant differences in AA uptake, with the exception of Ser, or U:O of any AA group, were found. At the LP level, the addition of HF numerically increased milk protein yield and uptake of all AA groups compared with LP/LF. Although statistical differences were not detected, this magnitude of extra uptake would support the tendency for milk protein yield to increase by 1.8 g/h in response to FT. When HP and HF were supplied together, the dynamics of mammary AA uptake and capacity for milk protein synthesis achieved by PT was not affected by FT. A PT × FT interaction affected arterial concentrations of all EAA groups where the increase in response to protein was greater at the LF level compared with the HF level, but the same level of EAA uptake was achieved by HP regardless of dietary fat level. For arterial concentrations to be lower on HP/HF with the same level of protein intake as HP/LF with no detectable increase in mammary gland uptake, the addition of HF to HP must have affected intestinal EAA absorption, metabolism at the level of the portal-drained viscera or liver, or their partitioning toward extramammary peripheral tissues. Plasma insulin concentration was decreased by FT and lowest with HF at the HP level, which suggests that AA partitioning toward extramammary peripheral tissues was not stimulated. Hammon et al. (2008) did not report significant changes in flux of any AA through the portal-drained viscera when a fat-supplemented diet was compared with a

starch-based diet. Taken together, these findings suggest that changes at the level of intestinal EAA absorption may be the mechanism resulting in lower plasma EAA concentrations when protein is supplemented at the HF level. To our knowledge, no study exists comparing portal-drained viscera or liver flux in mid-lactation cows under conditions of supplementation of extra protein and saturated fat. Regardless of the utilization of EAA when HF is added to HP, the addition of HF to HP did not affect plasma urea concentration, indicating that the same level of AA catabolism occurred at the LF or HF level. Overall, our hypotheses that FT would stimulate mammary AA sequestration or reduce AA catabolism in support of milk protein synthesis were not confirmed, as neither mammary AA sequestration nor milk protein yield were significantly increased and AA catabolism did not decrease in response to FT.

Energetic Substrate Shift with Energy from Protein or Fat

Arterial glucose concentration was not affected by PT or FT despite the change in energy supply. If PT stimulated glucose appearance from AA, the higher insulin concentration with HP at the LF level agrees with signaling to extramammary insulin-sensitive tissues such as skeletal muscle and adipose to clear extra glucose from circulation. In contrast, insulin resistance from fat supplementation lessens glucose utilization by peripheral tissues and possibly increases gluconeogenesis from lactate, pyruvate, or AA through glucagon signaling (Benson et al., 2002; Hammon et al., 2008). Availability of LCFA with fat also could stimulate peripheral tissue oxidation of acetate and in turn reduce glucose oxidation. The PT × FT interaction affecting insulin concentration, resulting in the lowest concentration when HP and HF are supplied together, aligns with the interaction on acetate concentration and suggests reduced glucose storage, a shift in energy precursor for extramammary tissues, and stimulation of lipolysis, which agrees with numerically highest glucose concentration and mammary glucose uptake, lactose yield, and fat yield on HP/HF.

Arterial concentrations of NEFA and TAG increased in response to fat, in agreement with observations of others (Hammon et al., 2008; Boerman et al., 2015; Nichols et al., 2019), and with the absorption of LCFA from the digestive tract. Plasma NEFA concentrations reflect the increase in endogenous lipid flux on HF diets, possibly caused by enhanced lipolytic activity in the liver and in adipose tissue during fat feeding (Chilliard, 1993), and may have resulted from their incomplete uptake by peripheral tissues after TAG hydrolysis (Grum et al., 1996). Increased mammary AV difference of TAG suggests that mammary TAG hydrolysis increased in response to fat (Cant et al., 1993a). Arterial BHB concentration increased in response to PT, but only at the LF level. Elevated BHB concentrations might be associated with increased arterial concentrations of ketogenic AA Leu and Lys on HP/LF or decreased BHB utilization by nonhepatic tissues (Ørskov et al., 1999), or could be induced by increased butyrate production in the rumen, although the latter is unlikely as DM and neutral detergent fiber digestibility were not affected by PT or FT (Nichols et al., 2018). The interaction on BHB

concentration that mirrors that of insulin and acetate suggests that BHB is also responsive to the mechanism of energy precursor repartitioning that seems to occur when energy is supplemented from protein and fat together.

CONCLUSIONS

Isoenergetic supplementation of aminogenic or lipogenic substrates modified the profile of AA and other metabolites available for mammary metabolism. Energy from protein increased arterial EAA concentration but to a greater extent at the LF level compared with the HF level. This suggests that supplementation with energy from fat alters EAA absorption across the gut or utilization of EAA by extramammary tissues, because the same level of mammary EAA uptake was stimulated by HP regardless of dietary fat level. Milk protein yield was supported by increased uptake of group 2 AA in response to energy from protein. Supplementing RP protein and hydrogenated palm FA does not further enhance mammary glucose balance in mid-lactation dairy cows but does increase milk lactose yield. Thus, it appears that factors other than mammary glucose supply stimulate lactose yield when extra energy is supplemented from protein and fat. Further investigation of intramammary synthetic pathways is warranted to obtain a more clear view of how lactose synthesis is affected by AA and other metabolites when mammary glucose supply is not limiting.

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Chapter 4

Energy metabolism and secretory cell differentiation in mammary cells of lactating dairy cows respond differently to dietary supplementation with energy from protein and fat

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ABSTRACT. Secretory capacity of bovine mammary glands is made possible by a high number of secretory cells and their flexibility in use of metabolites for milk component production. This study used RNA isolated from milk fat to measure expression of genes involved in energy-yielding pathways, the unfolded-protein response, and secretory cell differentiation in mammary glands of lactating cows in response to supplemental energy from protein (PT) and fat (FT) tested in a 2 × 2 factorial arrangement. The hypothesis was that expression of genes in the branched-chain AA catabolic pathway and tricarboxylic acid (TCA) cycle would be affected by PT and FT based on mammary gland use of different energy types (aminogenic versus lipogenic) to synthesize milk components. It was also hypothesized that the response of genes related to endoplasmic reticulum (ER) homeostasis and secretory cell differentiation would reflect the increase in milk production achieved by PT and FT. To evaluate these hypotheses, 56 multiparous Holstein-Friesian dairy cows were adapted during a 28-d control period to a basal total mixed ration consisting of 34% grass silage, 33% corn silage, 5% grass hay, and 28% concentrate on a dry matter basis. Experimental rations were fed for 28 d immediately following the control period and consisted of (1) low protein, low fat (LP/LF), (2) high protein, low fat (HP/LF), (3) low protein, high fat (LP/HF), or (4) high protein and high fat (HP/HF). To obtain the high protein (HP) and high fat (HF) diets, intake of the basal ration was restricted and supplemented isoenergetically (net energy basis) with 2.0 kg/d rumen-protected protein (soybean + rapeseed, 50:50 mixture on dry matter basis) and 0.68 kg/d hydrogenated palm fatty acids on a dry matter basis. RNA was isolated from milk fat samples collected on d 27 of each period and subject to real-time quantitative PCR. Energy from protein increased expression of *BCAT1* (branched-chain amino acid transferase 1) mRNA, but only at the LF level, and tended to decrease expression of mRNA encoding the main subunit of the branched-chain keto-acid dehydrogenase complex. mRNA expression of malic enzyme, a proposed channeling route for AA though the TCA cycle, was decreased by PT, but only at the LF level. Expression of genes associated with de novo fatty acid synthesis were not affected by PT or FT. Energy from fat had no independent effect on genes related to ER homeostasis or secretory cell differentiation. At the LF level, PT activated *XBP1* (X-box binding protein 1) mRNA. At the HF level, PT increased mRNA expression of the gene encoding GADD34 (growth arrest and DNA damage-inducible 34). These findings support our hypothesis that mammary cells use aminogenic and lipogenic precursors differently in support of milk component production when AA and fatty acid supply is altered by dietary intervention. They also suggest that mammary cells respond to increased AA supply through mechanisms of adaptive ER homeostasis and secretory cell differentiation, dependent on the presence of extra energy from fat supplementation.

Key words: mammary cell, unfolded protein response, tricarboxylic acid cycle, rumen-protected protein, hydrogenated palm fatty acid

INTRODUCTION

Milk synthesis exemplifies the intersection between whole-body and mammary metabolism involving glycolysis, hexoneogenesis, lipogenesis, and AA synthesis, but regulation of these pathways has not been characterized to the same extent in lactating mammary glands as in other metabolic tissues, such as the liver. The bovine mammary gland is unique compared with non-ruminant animals in that it uses acetate instead of glucose to synthesize fatty acids (FA; Bauman et al., 1970). The virtual absence of glucose-6-phosphatase in lactating bovine mammary tissue is consistent with its inability to convert gluconeogenic substrates (including lactate, pyruvate, glycerol and glutamate) to glucose (Scott et al., 1976). Whilst the glucose moiety of lactose appears to primarily arise from free glucose, the galactose moiety of lactose may originate partly from hexose phosphate intermediates (Wood et al., 1965). Studies injecting ^{14}C-labelled glycerol unilaterally into one pudic artery of a lactating cow indicated synthesis of galactose via the pentose phosphate pathway (Wood et al., 1958). Energy-yielding pathways in bovine mammary cells must be flexible to allow balance of tricarboxylic acid (TCA) cycle intermediates in response to changing dietary supply of aminogenic and lipogenic substrate for milk production. Enzymes regulating intermediate flux through the TCA cycle and expression of their genes respond to available substrate (Owen et al., 2002), but have gone largely unexplored in bovine mammary secretory cells.

Bovine mammary glands have a massive secretory capacity made possible by a high number of secretory cells, but the intracellular mechanisms by which metabolites and hormones stimulate milk component synthesis are incompletely understood. The mammalian target of rapamycin complex 1 (mTORC1) and integrated stress response (ISR) network have been implicated in acute regulation of protein synthesis in mammary epithelial cells in vitro (Burgos et al., 2010; Appuhamy et al., 2011; Burgos et al., 2013) and in vivo within 36 h (Rius et al., 2010; Toerien et al., 2010), but activation of mTORC1 and ISR signaling networks in mammary tissue does not explain the milk protein yield response to chronic nutritional intervention over several days (Curtis et al., 2014; Doelman et al., 2015). Instead, endoplasmic reticulum (ER) biogenesis and secretory cell differentiation may be activated in response to long-term nutritional intervention (Nichols et al., 2017; Cant et al., 2018). Expression of mRNA coding for constituent proteins of the unfolded-protein response (UPR) suggested that 5-d abomasal EAA infusions initiated a non-stress-related cellular adaptation to support milk protein secretion from mammary cells (Nichols et al., 2017).

Mammary biopsy is a common technique to obtain tissue for in vivo cell signaling analysis (Curtis et al., 2014; Doelman et al., 2015; Nichols et al., 2017). Disadvantages to mammary biopsy are the level of invasiveness and the post-operative recovery and care required, which pose limits on the frequency of sampling, the physiological state of the animal at the time of the procedure, and the number of animals upon which this procedure

can be performed. An alternative source of mRNA is secretory cell cytoplasm trapped within milk fat globules during apocrine secretion (Hutson and Patton, 1990). Compared with that captured from a core biopsy, the transcriptome of milk fat RNA may be more representative of all secretory cells of the udder (Maningat et al., 2007; Lemay et al., 2013; Cánovas et al., 2014). Cytoplasmic material is a small fraction of the bovine milk fat globule (Hutson and Patton, 1990), but the high fat content of ruminant milk allows sufficient RNA to be obtained for gene expression analysis (Brenaut et al., 2012; Abdelatty et al., 2017). Compared with biopsy, milk fat RNA collection is non-invasive and can be facilitated in a large sample size, thereby allowing further characterization of mammary function to link genomic and phenotypic data across a variety of experiments.

We recently reported results on milk production and composition, and mammary gland metabolite utilization (Nichols et al., 2018, 2019) from an experiment comparing the addition of 17 MJ/d NE_L from protein (PT) or fat (FT) to a base diet. Milk protein yield and mammary gland uptake of Arg, Ile, Leu, and Val increased in response to PT independent of FT level, and net intramammary catabolism of Ile, Leu, and Val increased, as indicated by the difference between their uptake and output in milk. Supplementation with PT also increased milk output of intramammary-synthesized de novo FA. In contrast, FT tended to increase milk protein yield, did not affect mammary net uptake or uptake:output ratios of EAA, had no effect on milk output of de novo-synthesized FA, but increased output of preformed FA in milk. Considering the responses in milk production and mammary gland AA utilization observed by Nichols et al. (2018, 2019), in the current study we investigated mammary gland expression of genes related to catabolism of AA, particularly branched-chain AA (BCAA), lipogenesis, and secretory cell differentiation in the same lactating dairy cows after 27 d of energy supplementation from protein or fat. We hypothesized that expression of genes in the BCAA catabolic pathway and TCA cycle would be affected differently by PT and FT based on the energy type (aminogenic versus lipogenic) available to the mammary gland, and that genes related to the maintenance of ER homeostasis and secretory cell differentiation would respond to nutritional intervention and reflect the increase in milk production achieved by supplementation with PT and FT.

MATERIALS AND METHODS

Experimental Design

Experimental procedures were approved by the Animal Care and Use Committee for Nutreco Nederland B.V. (Amersfoort, the Netherlands) and conducted under the Dutch Law on Animal Experiments. The experimental design and treatment diets have been thoroughly described elsewhere (Nichols et al., 2018). Briefly, 56 Holstein-Friesian cows (20 primiparous, 138 ± 64 DIM; 36 multiparous, 3.7 ± 1.8 lactations, 181 ± 93 DIM) were used in a randomized complete block design where supplemental energy from protein (PT) or fat (FT) was tested

in a factorial arrangement over two successive periods (control and experimental) each consisting of 21 d of diet adaptation and 7 d of data collection. A basal diet (34% grass silage, 33% corn silage, 5% grass hay, and 28% concentrate on a DM basis) designed to meet NEL and MP requirements was fed as a TMR during the control period. Cows were blocked by parity, DIM, and DMI of the final 7 d of the control period, and within blocks were randomly assigned to 1 of 4 diets for the experimental period: 1) low protein, low fat (**LP/LF**; 95% MP, 95% NE_L), 2) high protein, low fat (**HP/LF**; 131% MP, 107% NE_L), 3) low protein, high fat (**LP/HF**; 95% MP, 107% NE_L), or 4) high protein and high fat (**HP/HF**; 131% MP, 119% NE_L), where MP and NE_L are expressed relative to animal requirements in the control period. For all treatments, basal diet intake for individual cows was restricted to 95% of their ad libitum intake recorded during the control period. To obtain the high protein (HP) and high fat (HF) diets, a 2.0 kg 50:50 mixture (DM basis) of rumen-protected (RP) soybean meal and rapeseed meal (SoyPass + RaPass; both rumen-protected by xylose-treatment; Borregaard LignoTech, Sarpsborg, Norway) and 0.68 kg rumen-inert hydrogenated LCFA (Hidropalm; Norel, Madrid, Spain) on a DM basis were supplemented into the concentrate portion of each basal TMR such that additional daily intake of MP and NEL arose from the supplement addition. The TMR were mixed and distributed once daily at 1000 h and cows were milked twice daily at 530 and 1630 h.

Milk Fat Collection, RNA Extraction, and Real-Time Quantitative PCR

During morning milking on d 27 of each period, 10 mL of milk was collected by hand from individual cows directly after the milking machine was removed. Samples were immediately stored at 4°C. Within 1 h of collection, milk samples were centrifuged at 2,000 × g for 10 min at 4°C to facilitate separation of the fat fraction. Approximately 1 g of the supernatant cream layer was transferred into 6 mL TRI Reagent (Sigma Aldrich, St. Louis, MO), mixed vigorously, snap frozen in liquid N_2, and stored at -80°C until RNA extraction. Total RNA was isolated from milk fat according to TRI Reagent manufacturer's instructions for handling samples with a high fat content. Total RNA concentrations and purity were determined by optical density measurement using a Nano-Drop ND-1000 (ThermoFisher Scientific). The average 260/280 absorbance ratio of total RNA samples was 1.57 ± 0.17 (mean ± SD). All samples showed an RNA integrity number greater than 6.0. An aliquot of 100 ng total RNA was reverse transcribed with Superscript III (ThermoFisher Scientific) in the presence of random hexamers (250 ng/µL; Roche, Almere, the Netherlands) and dNTPs (10 mM; Roche) at 25°C for 5 min, 50°C for 1 h, and 70°C for 15 min and cDNA was stored at -80°C until further analysis.

Primers (Integrated DNA Technologies, Coralville, IA) were designed to yield PCR amplification products of 100 to 200 bp (**Table 4.1**) with efficiency greater than 90%. Real-time quantitative PCR was carried out using 5 µl of diluted cDNA combined with a 15 µl mixture composed of 10 µl PerfeCTa SYBR Green FastMix (Quanta BioSciences, Gaithersburg,

MD), 0.4 µM forward and reverse primers, and DNase/RNase-free water with an Applied Biosystems 7300 Real Time PCR instrument. As internal standards, expression of housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), H3 histone class 3 (*H3F3A*), and ribosomal protein S9 (*RPS9*) were analyzed and NormFinder (Andersen et al., 2004) identified H3F3A as the most stable across block and treatment. Fold changes in gene expression relative to the mean of each treatment in the control period were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) where *H3F3A* expression was used as the reference gene.

Table 4.1. Primer sequences for real-time quantitative PCR[1]

Gene	Protein	Primer Sequence	NCBI Reference
ACC1	ACC1	5'-GTGAAGTTCCCTCAGGCTCTTAATC 3'- TGTCTGAGCAGATATCCACTTCC	NM_174224.2
BCAT1	BCAT1	5'- GGCCCACGATGAAGGATT 3'- AACGGTGGCTCGTGTGATTA	NM_001083644
BCKDHA	BCKDH E1-α	5'- TTTGGAGACCAAGTCGAGGC 3'- GAAATCTAGCCAGCCCACGA	NM_174506
CASP3	caspase-3	5'- CAGCGTCGTAGCTGAACGTA 3'- GTTTGCTGCATCCACGTCTG	NM_001077840
CSN2	β-Casein	5'- CAGGCCTTTCTGCTGTACCA 3'- CAAAAGTGAGGAGGGGGCAT	KC993858
DDIT3	CHOP	5'- CAAAGCCGGAACCTGAGGAG 3'- TCCTGCAGGTCCTCATACCA	NM_001078163
GAPDH	GAPDH	5'- GGGTCATCATCTCTGCACCT 3'- GGTCATAAGTCCCTCCACGA	NM_001034034
H3F3A	H3 histone class 3	5'- CCTCTACTGGAGGGGTGAAGA 3'- CAGACGCTGGAAGGGAAGTT	BT020962.1
HSPA5	BiP/GRP78	5'- TGAACGACCCCTGACGAAAG 3'- TGCGCTCCTTGAGCTTTTTG	NM_001075148
IDH1	IDH1	5'- ACACTGAGTGACTGTGTGCTC 3'- CTTGGTGACCTGGTCGTTGG	NM_181012.3
ME2	ME-m	5'- GTTCTCCCCGGTCAGTCTCCT 3'- TTTTCTCACCCCGCTTCTTGC	NM_001076814.1
PCK2	PEPCK-m	5'- GGGTGCTAGACTGGATCTGC 3'- CCTTGGGGAGGGAGAACAAC	NM_001205594.1
PPP1R15A	GADD34	5'- CAACCAGGAGACACAGAGGA 3'- ACTCTGGGTTGAAGGGAGG	NM_001046178
RPS9	RPS9	5'- CTGAAGCTGATCGGCGAGTA 3'- GGGTCTTTCTCATCCAGCGT	NM_001101152.2
*XBP1*t	XBP1 total	5'- TTCAGCCCCTCAGAGAAAGA 3'- CTCCCAAGAATGGTCTGCAT	BC102639
*XBP1*s	XBP1 spliced	5'- TGCTGAGTCCGCAGCAGGTG 3'- AGAATGCCCAACAGGATGTC	BC102639
*XBP1*u	XBP1 unspliced	5'- AGACTACGTGCACCTCTGCAG 3'- AGAATGCCCAACAGGATGTC	BC102639

[1]ACC1 = acetyl-CoA carboxylase; BCAT1 = branched-chain aminotransferase 1; BCKDH E1-α = branched-chain keto-acid dehydrogenase e1, α subunit; CHOP = C/EBP homologous protein; DDIT3 = DNA damage-inducible transcript 3; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; HSPA5 = heat-shock protein A5; BiP/GRP78 = 78 kDa glucose-regulated protein; IDH1 = isocitrate dehydrogenase 1, cytosolic; ME2 = malic enzyme 2, mitochondrial; ME-m = mitochondrial malic enzyme; PCK = phosphoenolpyruvate carboxykinase 2, mitochondrial; PEPCK-m = mitochondrial phosphoenolpyruvate carboxykinase; PPP1R15A = protein phosphatase 1 regulatory subunit 15A; GADD34 = growth arrest and DNA damage-inducible 34; RPS9 = ribosomal protein S9; XBP1 = X-box binding protein 1.

Statistical Analysis

Variances in gene expression were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) where level of protein and fat and their interaction were included as fixed effects and block was included as a random factor. Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. Multiple comparisons between treatment means were made using the Tukey-Kramer method when a PT × FT interaction was detected at $P \leq 0.10$.

RESULTS AND DISCUSSION

Gene expression was analyzed from RNA captured in milk fat globules. It has been demonstrated that this technique yields comparable RNA profiles and gene expression to that obtained from mammary tissue in lactating primates, cows, and goats (Maningat et al., 2007; Lemay et al., 2013; Cánovas et al., 2014). Using a similar collection technique as described in the current study, Brenaut et al. (2012) showed that RNA obtained from caprine milk fat globules quantitatively represented the transcriptional information found in mammary epithelial cells. The quality of RNA obtained in the current study, measured by purity and RNA integrity, is comparable to previous studies that have reported gene expression and transcriptome measurements from milk fat RNA (Brenaut et al, 2012; Cánovas et al., 2014; Abdelatty et al., 2017). Regarding its suitability for investigation of gene expression in response to nutritional intervention, Abdelatty et al. (2017) analyzed gene expression from milk fat RNA after 60% feed restriction in lactating dairy cows and detected changes in mRNA expression of several lipogenic genes. In the current study, genes presented in **Table 4.3** were selected in order to relate the observed nutrient effects on milk production (**Table 4.2**) to mammary cell energy metabolism. Genes presented in **Table 4.4** are related to the control of ER homeostasis and differentiation of cells towards the secretory phenotype, and were selected based on their previous identification by Nichols et al. (2017) to be responsive to postruminal EAA infusions.

Table 4.2. Milk and component yield (kg/d) from lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or from protein and fat (HP/HF)[1]

	Treatment[2]					*P*-value[3]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM	PT	FT	PT × FT
Total milk	26.6	28.7	28.4	30.0	0.70	0.01	0.03	0.71
Lactose	1.21	1.31	1.30	1.38	0.031	<0.01	0.01	0.66
Protein	0.92	1.01	0.97	1.04	0.024	<0.01	0.05	0.50
Fat	1.20	1.25	1.33	1.38	0.033	0.10	<0.01	0.93

[1]Data are least-squares means from the final week of the experimental period using data from the final week of the control period as covariates for each parameter (see Nichols et al. (2018) for further details).

[2]LP/LF, basal TMR with no protein or fat supplement fed at 95% of ad libitum intake of control period; HP/LF, TMR fed at 95% of ad libitum intake of control period supplemented daily with 2.0 kg SoyPass + RaPass on a DM basis; LP/HF, TMR fed at 95% of ad libitum intake of control period supplemented daily with 0.68 kg Hidropalm on a DM basis; HP/HF, TMR fed at 95% of ad libitum intake of control period supplemented daily with 2.0 kg SoyPass + RaPass and 0.68 kg Hidropalm on a DM basis. For all treatments n = 14.

[3]PT = effect of energy from protein; FT = effect of energy from fat.

Table 4.3. Mammary gland expression (arbitrary units) of genes for milk protein, AA catabolism, and energy-yielding pathways in lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or from protein and fat (HP/HF)[1]

	Treatment[2]					*P*-value[3]		
Item	LP/LF	HP/LF	LP/HF	HP/HF	SEM	PT	FT	PT × FT
ACC1	3.96	3.62	5.31	4.18	1.120	0.49	0.37	0.72
BCAT1	0.61[a]	2.52[b]	0.86[ab]	0.55[a]	0.451	0.08	0.06	0.02
BCKDHA	0.85	0.72	1.36	0.47	0.341	0.08	0.65	0.20
CSN2	2.24	1.38	3.46	1.53	0.861	0.13	0.45	0.55
IDH1	2.66	2.61	2.81	2.12	0.700	0.49	0.74	0.54
ME2	2.99[a]	1.27[b]	1.72[ab]	2.66[ab]	0.746	0.56	0.93	0.05
PCK2	2.25	2.28	1.88	2.67	0.741	0.42	0.98	0.44

[a,b]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Values are least-squares means of fold changes in gene expression relative to the mean of each treatment in the control period calculated by the $2^{-\Delta\Delta Ct}$ method after normalizing to *H3F3A*.

[2]LP/LF, basal TMR with no protein or fat supplement fed at 95% of ad libitum intake of control period; HP/LF, TMR fed at 95% of ad libitum intake of control period supplemented daily with 2.0 kg SoyPass + RaPass on a DM basis; LP/HF, TMR fed at 95% of ad libitum intake of control period supplemented daily with 0.68 kg Hidropalm on a DM basis; HP/HF, TMR fed at 95% of ad libitum intake of control period supplemented daily with 2.0 kg SoyPass + RaPass and 0.68 kg Hidropalm on a DM basis. For all treatments n = 14.

[3]PT = effect of energy from protein; FT = effect of energy from fat.

Energy-Yielding Pathways

Intermediates of BCAA catabolism contribute anaplerotically to the TCA cycle (Owen et al., 2002). The cytosolic form of BCAA aminotransferase (BCAT), encoded by *BCAT1*, catalyzes the first step of BCAA degradation through reversible transamination of Ile, Leu, and Val, yielding their respective branched-chain α-keto acids (BCKA) and glutamate. Glutamate may be used for de novo synthesis of other NEAA, and BCKA are reaminated to BCAA, released into blood, or decarboxylated by branched-chain keto-acid dehydrogenase complex (BCKDH), of which the main E1 α-subunit is encoded by *BCKDHA*. Decarboxylation of BCKA is an irreversible and controlling step in BCAA catabolism and yields CoA derivatives which enter the TCA cycle for oxidation or further incorporation into NEAA or FA (Brosnan and Brosnan, 2006). The BCAA are taken up by bovine mammary glands in excess of their output in milk protein and are catabolized via BCAT and BCKDH (Davis and Mepham, 1976; Wohlt et al., 1977). Increasing MP or BCAA supply causes faster intramammary BCAA catabolism (Bequette et al., 1996a; Bequette et al., 1996b; Raggio et al., 2006). We hypothesized that BCAA catabolism would increase at the HP level, but to our knowledge, effects of diet (energy type and supply) on expression of genes for BCAA catabolism in bovine mammary glands have not been reported.

Expression of BCAT1 mRNA increased in response to PT, but only at the LF level (PT × FT interaction; P = 0.02; Table 4.3). This suggests upregulation of intramammary BCAA catabolism and their use for NEAA synthesis, and agrees with our findings that mammary uptake of BCAA in excess of milk output increased in response to PT, and that the deficit between output and uptake of glutamate was larger at the HF level (Nichols et al., 2019). The tendency for decreased *BCKDHA* expression in response to PT (P = 0.08) agrees with DeSantiago et al. (1998) who reported a high ratio of BCAT to BCKDH activity in rat mammary tissue, which suggests oxidation is not the fate of all BCKA after BCAT-mediated transamination. Indeed, upwards of 80% of BCAA incorporated into milk protein may be reversibly transaminated prior to incorporation (Roets et al., 1979; Roets et al., 1983). Infusing a complete mixture of AA into goats increased the rate of mammary Leu transamination without a concomitant increase in flux through BCKDH (Bequette et al., 2002). In other experiments, increasing AA supply has resulted in faster rates of both transamination and oxidation of Leu in the mammary glands (Bequette et al., 1996a; Raggio et al., 2006). Inconsistencies in the response between *BCAT1* and *BCKDHA* expression may be related to the suggestion of DeSantiago et al. (1998) that, based on differences between lactating and virgin rats, BCKA flux through mammary BCKDH is regulated primarily by its phosphorylation state and not mRNA or protein expression.

Addition of PT to the HF diet did not increase *BCAT1* expression as it did on the LF diet, suggesting that FT inhibited up-regulation of BCAT1 expression. However, excess BCAA uptake over milk output, representing net catabolism, was not affected by FT (Nichols et al.,

2019). Changes in catabolic flux are due to a combination of effects on enzyme expression and substrate concentrations. Similar to the effect on *BCAT1* expression, there were PT × FT interactions detected for arterial concentrations of the individual BCAA, acetate, BHB, and insulin, and a tendency for a PT × FT interaction on plasma flow to the gland, where PT effects were smaller or absent on HF versus LF diets (Nichols et al., 2019). Regulation on the supply of major fuels for the TCA cycle, like acetate and BHB, through physiological adaptations like mammary blood flow, may influence catabolism of other fuels independent of enzyme expression. Energy from fat increased uptake of LCFA by the mammary gland (Nichols et al., 2019). Reduced requirements for TCA cycle intermediates to generate NADPH for de novo FA synthesis in response to FT may also figure into the lack of induction of *BCAT1* expression by PT on HF diets. The net result of higher AA and LCFA supply on HP/HF was faster intramammary BCAA catabolism, but no effect on BCAT1 at the level of mRNA expression.

Interest in the mitochondrial isoforms of malic enzyme (ME), encoded by *ME2*, and phosphoenolpyruvate carboxykinase (PEPCK), encoded by *PCK2*, in mammary secretory cells arose because they allow channeling of TCA cycle intermediates derived from NEAA and group 2 AA, including Ile and Val, to pyruvate (Pongratz et al., 2007; Méndez-Lucas et al., 2014). PEPCK catalyzes the transformation of oxaloacetate to the pyruvate precursor phosphoenolpyruvate (Agca et al., 2002; Méndez-Lucas et al., 2014). The mitochondrial PEPCK isoform facilitates conversion of AA and other substrates entering at or beyond succinyl-CoA into C skeletons of NEAA, and supports oxidation in the TCA cycle via pyruvate (Scott et al., 1976; Agca et al., 2002). As such, when intramammary BCAA are used for de novo NEAA synthesis in support of milk protein yield, this pathway may be up-regulated. Mitochondrial ME converts AA-C to pyruvate, independent of glycolytic flux, to increase oxidative flux through pyruvate dehydrogenase in response to increased AA concentrations (Mandella and Sauer, 1975; Pongratz et al., 2007). This pathway appears important when glucose is used for biosynthesis and is not the main fuel for the TCA cycle (Chang and Tong, 2003), such as in mammary secretory cells. Data on gene expression or protein activity of the mitochondrial isoforms of PEPCK and ME in response to energy or protein supply are scarce in lactating bovine mammary glands. Because intramammary BCAA catabolism increased with PT, we hypothesized that *PCK2* and *ME2* expression would increase to support AA-C flux through the TCA cycle, allowing glucose to support the increase in lactose yield with PT. In response to FT, we expected no effect due to the lower AA supply to mammary cells.

There were no effects of PT or FT on *PCK2* expression ($P \geq 0.42$; Table 4.3). Expression of *ME2* was affected by a PT × FT interaction ($P = 0.05$; Table 4.3), where PT decreased *ME2* expression, in contrast to our hypothesis, but only on the LF diet. On HP/LF, intramammary BCAA transamination increased indicated by the increase in *BCAT1* expression, but oxidation of BCKA may have contributed relatively less to the AA-C pool, which is suggested by the tendency for decreased *BCKDHA* expression in response to PT. Reduced *ME2* expression

could be expected if fewer CoA derivatives from AA-C skeletons require shuttling towards pyruvate via ME. This pattern in mRNA expression of *ME2*, *BCAT1*, and *BCKDHA* may suggest a link between these enzymes in the contribution of AA catabolism to anaplerotic flux through the TCA cycle, while a potential role of PECPK remains undetermined in lactating bovine mammary glands.

Cytosolic NADP-linked isocitrate dehydrogenase (IDH) 1, encoded by *IDH1*, catalyzes the conversion of isocitrate to α-ketoglutarate (Koh et al., 2004) and generates the primary source of NADPH for de novo FA synthesis (Bauman et al., 1970; Farrell et al., 1987). Acetyl-CoA carboxylase (ACC) 1, encoded by *ACC1*, catalyzes the first step in de novo FA synthesis. Incubation with palmitic acid decreased *IDH1* expression in bovine mammary epithelial cells (Liu et al., 2006). Mammary *ACC1* expression decreases in some studies when milk fat-depressing diets are fed to dairy cows (Piperova et al., 2000; Ahnadi et al., 2002; Peterson et al., 2003), but not in all (Bernard et al., 2005; Bernard et al., 2009). In contrast to studies where diets are designed to induce milk fat depression and subsequent decreases in lipogenic gene expression are detected (Piperova et al., 2000; Baumgard et al., 2002; Peterson et al., 2003), supplementation with rumen-inert, hydrogenated palm FA in the current experiment increased milk fat yield and we observed no change in *IDH1* or *ACC1* expression in response to PT or FT ($P \geq 0.37$; Table 4.3). Although de novo FA concentration in milk fat decreased with FT, the increase in total fat yield resulted in no change in de novo FA yield (Nichols et al., 2018), in agreement with no effects on *IDH1* or *ACC1*. In contrast, PT increased de novo FA concentration in milk but had no effect on total fat yield, producing increased de novo FA yield but no increase in *IDH1* or *ACC1* expression. The effects on de novo FA synthesis in response to PT may not have been severe enough to affect *IDH1* or *ACC1* at the mRNA expression level. Furthermore, Wright et al. (2006) concluded that ACC exerts strong control on flux but not rate-limiting control over FA synthesis, which could be more heavily influenced by the enzyme FA synthase.

Mammary Cell Biogenesis

Milk protein synthesis in ruminant mammary cells is a process of translation of mRNA for the milk protein genes *CSN1S1*, *CSN1S2*, *CSN2*, *CSN3*, *LALBA*, and *BLG*. Expression of *CSN2* was measured in the current study and was not affected by PT or FT (Table 4.3). There is abundant evidence to suggest that energy source and supply do not directly affect expression of milk protein genes, but instead affect milk protein synthesis through mechanisms such as altered mRNA translation efficiency and number of milk secretory cells (Cant et al., 2018). Secretory cell number can be regulated by proliferation and apoptosis of existing udder cells, but also by the rate of epithelial cell differentiation into cells with a secretory phenotype. If this rate is affected by nutritional intervention, it may influence milk synthetic responses in vivo. Signaling cascades of the constituent UPR play a crucial role in defining the phenotype of professional secretory cells, and in their adaptation to stimuli to mitigate cell death

(Reimold et al., 2001; Huh et al., 2010; Davis et al., 2016). Previous identification of a non-stress-related UPR response in bovine mammary glands (Nichols et al., 2017) motivated investigation of genes encoding proteins involved in this pathway in the current experiment. Initiation of the UPR during protein misfolding occurs by dissociation of the chaperone protein BiP, encoded by *HSPA5* (Hetz et al., 2015), onto 3 ER transmembrane proteins initiating the 3 UPR arms, namely protein kinase R-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1). In the first UPR arm, phosphorylation of PERK activates the ISR network (Proud, 2005) suppressing global protein synthesis, decreasing the load of new proteins on the ER, and stimulating translation of activating transcription factor 4 (ATF4) and its targets, the pro-apoptotic protein CHOP, encoded by *DDIT3*, and GADD34, encoded by *PPP1R15A* (Walter and Ron, 2011). GADD34 dephosphorylates ISR constituents, counteracting the effects of PERK, to resume protein synthesis once the ER has returned to homeostatic function (Ma and Hendershot, 2003). The second arm of the UPR, through ATF6, encourages ER biogenesis and activates transcription of *XBP1* and *HSPA5* (Hetz et al., 2015). The third UPR arm, mediated by phosphorylated IRE1 after BiP release, excises *XBP1* mRNA to generate the active spliced form *XBP1*s. The product of *XBP1*s translation is a transcription factor that stimulates expression of proteins involved in differentiation of the secretory phenotype, including rough ER formation and secretory vesicle maturation (Huh et al., 2010). If UPR signaling cascades fail to promote ER functionality, pro-apoptotic signals emerge through CHOP and effector caspases (Hetz et al., 2015).

We hypothesized that arms of the UPR would be regulated separately to effect a change in mammary cell secretory capacity. A lack of effect on expression of *DDIT3*, *HSPA5*, and *XBP1*t ($P \geq 0.24$; Table 4.4) suggests that neither the second arm nor the pro-apoptotic element of the first arm of the UPR were affected by PT or FT. Expression of *PPP1R15A* increased in response to PT on the HF diet but not on the LF diet (tendency for PT × FT interaction; $P = 0.06$). As part of the UPR, *PPP1R15A* and *DDIT3* expression levels may change in parallel under the control of ATF4. For example, Nichols et al. (2017) found that expression of *PPP1R15A* and *DDIT3* decreased after 5 d of abomasal EAA infusion, indicating suppression of the first arm of the UPR in favor of greater secretory cell number. In the present experiment, the increase in expression of *PPP1R15A*, independent of *DDIT3*, does not support an adaptive UPR, but the protein product of *PPP1R15A* translation is activatory towards protein synthesis. This is partially consistent with the greater milk protein yield observed in response to PT, regardless of dietary fat level. Increased *XBP1* splicing in response to PT (increased *XBP1*s/*XBP1*u; $P < 0.01$) suggests induction of the third, IRE1-mediated arm of the UPR to enhance protein secretory capacity, although the tendency for PT × FT interaction ($P = 0.06$) indicates a response to PT on LF diets only. The proportion of *XBP1*s to *XBP1*u represents the proportion of active *XBP1* available for translation relative to

inactive *XBP1*. The proportion of *XBP1*s to *XBP1*t tended to increase with PT, but only at the LF level (PT × FT interaction; $P = 0.01$). Similarly, Nichols et al. (2017) observed a parallel increase in *XBP1*s/*XBP1*u and *XBP1*s/*XBP1*t in response to abomasal EAA infusion. Spliced XBP1 is required for terminal differentiation of progenitor cells into secretory cells (Reimold et al., 2001; Huh et al., 2010; Tsuchiya et al., 2017), and is increased at the onset of lactation in bovine mammary glands (Yonekura et al., 2018). Conditional knockout of XBP1 affected ER expansion and β-casein expression in murine mammary cells (Davis et al., 2016; Tsuchiya et al., 2017). In summary, PT increased *PPP1R15A* expression at the HF level independent of an adaptive UPR response but in line with increased protein synthesis stimulated by PT, and IRE1-mediated XBP1 splicing was induced by PT more strongly at the LF level, in line with cellular differentiation to the secretory phenotype and increased milk protein, fat, and lactose yield in response to PT.

Table 4.4. Mammary gland expression (arbitrary units) of genes for ER homeostasis and secretory cell differentiation in lactating dairy cows fed the basal diet (LP/LF) or diets supplemented with energy from protein (HP/LF), fat (LP/HF), or from protein and fat (HP/HF)[1]

	Treatment[2]					*P*-value[3]		
	LP/LF	HP/LF	LP/HF	HP/HF	SEM	PT	FT	PT × FT
CASP3	1.00	1.46	1.72	0.75	0.514	0.63	0.99	0.18
DDIT3	2.18	2.02	3.09	2.67	0.689	0.66	0.24	0.84
HSPA5	2.14	2.21	2.50	1.12	0.694	0.27	0.54	0.24
PPP1R15A	1.06^{ab}	1.24^{ab}	0.79^{b}	2.46^{a}	0.464	0.02	0.23	0.06
XBP1								
total	4.36	4.01	4.23	4.49	0.464	0.93	0.70	0.51
spliced	1.71	2.33	2.79	2.27	0.324	0.87	0.11	0.18
unspliced	2.09	2.00	2.18	1.81	0.335	0.33	0.82	0.57
*XBP1*s/*XBP1*u	0.88^{a}	1.55^{b}	1.36^{ab}	1.49^{b}	0.172	<0.01	0.10	0.06
*XBP1*s/*XBP1*t	0.43^{a}	0.83^{b}	0.62^{ab}	0.53^{ab}	0.085	0.06	0.50	0.01
*XBP1*u/*XBP1*t	0.66	0.74	0.53	0.40	0.140	0.81	0.04	0.36

^{a,b}Means within a row with no common superscripts differ ($P < 0.05$).

[1]Values are least-squares means of fold changes in gene expression relative to the mean of each treatment in the control period calculated by the $2^{-\Delta\Delta Ct}$ method after normalizing to *H3F3A*.

[2]LP/LF, basal TMR with no protein or fat supplement fed at 95% of ad libitum intake of control period; HP/LF, TMR fed at 95% of ad libitum intake of control period supplemented daily with 2.0 kg SoyPass + RaPass on a DM basis; LP/HF, TMR fed at 95% of ad libitum intake of control period supplemented daily with 0.68 kg Hidropalm on a DM basis; HP/HF, TMR fed at 95% of ad libitum intake of control period supplemented daily with 2.0 kg SoyPass + RaPass and 0.68 kg Hidropalm on a DM basis. For all treatments n = 14.

[3]PT = effect of energy from protein; FT = effect of energy from fat.

To our knowledge, the UPR in mammary cells has not been previously investigated in response to dietary fat supplementation. The XBP1 signaling cascade is activated in response to high fat conditions in liver and skeletal muscle during ER stress (Wang et al., 2006; Deldicque et al., 2010), and in adipose during lactation (Gregor et al., 2013). Independent from canonical UPR and ER-stress signaling, hepatic *XBP1* splicing was upregulated to promote downstream activation of de novo lipogenic genes (Lee et al., 2008). In response to FT in the current study, *XBP1* splicing tended to increase (*XBP1*s/*XBP1*u; $P = 0.10$) but only at the LP level (PT × FT interaction; $P = 0.06$), and the expression of *XBP1*u relative to the total expression (*XBP1*u/*XBP1*t) decreased ($P = 0.04$). Compared with liver or adipose, *XBP1* expression in mammary glands might reflect differences in tissue function between the liver or adipose where FA are stored and mammary glands where FA are secreted.

Overall, although PT increased milk protein yield independent of FT, we found that the cellular response to PT depended on FT. At the LF level, PT stimulated splicing of XBP1, suggesting secretory cell differentiation in support of milk protein secretory capacity. In response to PT at the HF level, *XBP1*s expression was maintained at a similar level as at the LF level, but stimulation of milk protein synthesis may have been supported through *PPP1R15A* expression, which increased in response to PT specifically at the HF level. In summary, when energy is supplemented from protein, mammary secretory capacity may be increased through ER biogenesis and secretory cell differentiation through activated XBP1. When extra energy is supplemented from protein and fat together, expression of *PPP1R15A* encoding GADD34 may promote increased protein secretory activity. Moreover, the changes we observed in expression of spliced *XBP1* suggest upregulation of the third UPR arm, but with no change in expression of *HSPA5*. A separate control mechanism for the XBP1 cascade has become apparent in hepatic lipogenesis (Lee et al., 2008), in adipocytes during lactation (Gregor et al., 2013), and in innate immune cells (Martinon et al., 2010). Our findings in lactating bovine mammary glands agree with Nichols et al. (2017) and show that separate components of the UPR may be affected by mammary-sequestered nutrients, but the role this plays in secretory cell differentiation, ER functionality, and milk component synthesis in mammary epithelial cells remains to be definitively established.

CONCLUSIONS

Energy from protein increased mammary catabolism of BCAA which was associated with higher expression of *BCAT1* but only at the LF level. Energy from protein tended to decrease *BCKDHA* expression, suggesting that transamination of BCAA was occurring faster than their catabolism to CoA derivatives. Energy from protein decreased *ME2* expression, also occurring only at the LF level. Taken together, these results may suggest a link between regulation of AA catabolism, specifically the BCAA, and anaplerotic flux through the TCA cycle. We detected no effects of energy from protein or fat on *PCK2* expression. Increased

yield of de novo FA stimulated by energy from protein was not associated with increased *IDH1* or *ACC1* expression. mRNA expression of these enzymes was also not affected by energy from fat, which agrees with increased preformed FA yield and no change in de novo FA yield during fat supplementation. mRNA expression of genes related to the UPR or secretory cell differentiation were affected by energy from protein that depended on dietary fat level. At the LF level, energy from protein activated *XBP1*, possibly in support of secretory cell adaptation to protein secretory load. In response to protein at the HF level, stimulation of milk protein synthesis may have been supported through *PPP1R15A* expression, encoding the GADD34 protein. Overall, our findings show that mammary cells use aminogenic and lipogenic precursors differently in support of milk component production when AA and FA supply at the gland level is altered by dietary intervention. They also suggest that mammary cells respond to increased AA supply through mechanisms of adaptive ER homeostasis and secretory cell differentiation, dependent on the presence of extra energy from fat supplementation.

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Chapter 5

Energy and nitrogen partitioning in dairy cows at low or high metabolizable protein levels is affected differently by postrumen glucogenic and lipogenic substrates

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ABSTRACT. This study tested the effects of energy from glucogenic (glucose; GG) or lipogenic (palm olein; LG) substrates at low (LMP) and high (HMP) metabolizable protein levels on whole-body energy and N partitioning of dairy cattle. Six rumen-fistulated, second-lactation Holstein-Friesian dairy cows (97 ± 13 d in milk) were randomly assigned to a 6 × 6 Latin square design in which each experimental period consisted of 5 d of continuous abomasal infusion followed by 2 d of rest. A total mixed ration consisting of 42% corn silage, 31% grass silage, and 27% concentrate (dry matter basis) was formulated to meet 100 and 83% of net energy and metabolizable protein requirements, respectively, and was fed at 90% of ad libitum intake by individual cow. Abomasal infusion treatments were saline (LMP-C), isoenergetic infusions (digestible energy basis) of 1,319 g/d of glucose (LMP-GG), 676 g/d of palm olein (LMP-LG; major fatty acid constituents are palmitic, oleic, and linoleic acid), or 844 g/d of essential AA (HMP-C), or isoenergetic infusions of 1,319 g/d of glucose + 844 g/d of essential AA (HMP-GG) or 676 g/d of palm olein + 844 g/d of essential AA (HMP-LG). The experiment was conducted in climate respiration chambers to determine energy and N balance in conjunction with milk production and composition, nutrient digestibility, and plasma constituents. Infusion of GG and LG decreased dry matter intake, but total gross energy intake from the diet plus infusions was not affected by GG or LG. Furthermore, GG or LG did not affect total milk, protein, or lactose yields. Infusing GG or LG at the HMP level did not affect milk production differently than at the LMP level. Infusion of GG stimulated energy retention in body tissue, increased plasma glucose and insulin concentrations, decreased lipogenic metabolites in plasma, and decreased milk fat yield and milk energy output. Nitrogen intake decreased and milk N efficiency increased in response to GG, and N retention was not affected. Infusion of LG tended to increase metabolizable energy intake, increased milk fat yield and milk energy output, increased plasma triacylglycerides and long-chain fatty acid concentrations, and had no effect on energy retention. Infusion of LG decreased N intake but did not affect milk N efficiency or N retention. Compared with the LMP level, the HMP level increased dry matter intake, gross and metabolizable energy intake, and total milk, fat, protein, and lactose yields. Milk energy output increased at the HMP level, and protein level did not affect total energy retention. Heat production increased at the HMP level, but only when GG and LG were infused. The HMP level increased N intake, milk N output, and plasma urea concentration, tended to increase N retention, and decreased milk N efficiency. Regardless of protein level, GG promoted energy retention and improved milk N efficiency, but not through increased milk protein yield. Infusion of LG partitioned extra energy intake into milk and had no effect on milk N efficiency.

Key words: energy balance, nitrogen balance, glucogenic, lipogenic, milk nitrogen efficiency

INTRODUCTION

The content and the type of energy and protein in lactating cow diets are important factors influencing nutrient transfer from feed into milk components, and interactions between energy and protein at the postabsorptive level are complex. Importantly, changes in metabolic oxidation of macronutrients driven by net supply or postabsorptive interactions influence substrate supply to the lactating mammary gland. Increasing absorptive AA supply increases milk protein yield, whole-body glucose appearance, and AA catabolism, but decreases milk N efficiency and increases N excreted in urine (Lapierre et al., 2010; Dijkstra et al., 2013; Arriola Apelo et al., 2014). Nitrogen fed above requirement is associated with energy loss through heat production, mainly arising from energy required for ureagenesis. Martin and Blaxter (1965) estimated that during ammonia and urea infusion, 73% of heat produced per g of N infused (15.9 kJ/g out of a total of 21.8 kJ/g) was associated with urea production from ammonia. Reed et al. (2017) estimated a reduction in digestible energy balance of 14.6 kJ per g of N fed above requirement, and an increase in heat production between 17.2 and 31.7 kJ per g of excess N. According to the meta-analysis of Spek et al. (2013a), urinary N excretion ranges from 100 to 400 g/d. Considering the mean increase in heat production per g of excess N from Reed et al. (2017), the energy lost via heat production associated with urinary N excreted in this range is 7.3 MJ/d. Energy required to process AA and excrete excess N also varies with the form in which dietary protein is supplied (i.e., rumen-degradable vs. rumen-undegradable protein; Reed et al., 2017). Furthermore, formulating diets with EAA-balanced MP in support of maximal mammary gland AA extraction and use for milk protein synthesis can improve postabsorptive N efficiency and increase milk protein yield (Haque et al., 2012; Arriola Apelo et al., 2014).

Glucogenic nutrients are highly important to intermediary metabolism during ruminant lactation because mammary glucose use is high, but direct glucose absorption from the diet is low. Glucogenic diets fed to cows in early and mid-lactation promote energy storage in the body and decrease milk energy content (van Knegsel et al., 2007a,b; Boerman et al., 2015). These shifts in energy partitioning are also observed when glucose or glucogenic precursors are infused along the digestive tract (Reynolds et al., 2001; Rigout et al., 2002a). Infusions of ruminal propionate or postruminal glucose or starch suggest variable effects of elevated glucose availability on total milk, protein, and lactose yields (Raggio et al., 2006; Rius et al., 2010a; Nichols et al., 2016). However, increasing glucogenic energy at low and high levels of protein supply can improve postabsorptive AA transfer efficiency from the gut to milk protein by reducing AA catabolism across the gut and the splanchnic bed, potentially improving mammary gland supply (Rius et al., 2010b). In combination with high circulating AA levels, glucogenic energy may also support N retention by stimulating AA partitioning toward extra-mammary tissues (Clark et al., 1977; Nichols et al., 2016; Curtis et al., 2018).

In ruminants, lipogenic nutrients appear from degradation of OM in the rumen, from dietary fat sources reaching the lower gut, or are derived from endogenous fat reserves. Lipogenic nutrients have the potential to increase metabolizable energy intake (MEI) and milk energy output through the direct transfer of dietary fatty acids (FA) into milk (Hammon et al., 2008; Boerman et al., 2015). The effectiveness of dietary fat supplementation depends on FA chain length and degree of saturation (Pantoja et al., 1996; Bremmer et al., 1998; Harvatine and Allen, 2006a). These factors affect feed intake and digestibility, but can also influence the total yield and profile of FA in milk (Enjalbert et al., 2000). Milk and lactose yields from cows fed lipogenic diets may be equal to or higher than from those fed glucogenic or low-fat diets (Cant et al., 1991; Hammon et al., 2008; Lohrenz et al., 2010), but milk protein yield is usually lower or not affected (Cant et al., 1991; Hammon et al., 2008; Boerman et al., 2015). Lipogenic diets can improve milk N efficiency (Nichols et al., 2018), but literature characterizing whole-body N balance in response to lipogenic substrates is scarce (Andrew et al., 1991; Cant et al., 1991).

In the present experiment, glucose and palm olein were abomasally infused to study the effect of postabsorptive supply of glucogenic and lipogenic energy on energy and N metabolism of dairy cattle. Concomitant infusion of EAA allowed the effects of glucose and palm olein to be compared at a low and high MP level. We hypothesized that glucogenic and lipogenic infusions would stimulate milk production equally but through differences in energy and N partitioning. We expected infusion of glucose and palm olein to affect energy balance through energy retention (ER) and milk energy output, respectively. With regard to N balance, we expected that glucose and palm olein infusions would improve milk N efficiency, but that the effects of glucose might interact with low and high MP levels more than the effects of palm olein.

MATERIALS AND METHODS

Experimental Design and Respiration Chamber Housing

The following experimental procedures were conducted under the Dutch Law on Animal Experiments in accordance with European Union Directive 2010/63. To test the effect of energy from glucogenic (glucose; **GG**) or lipogenic (palm olein; **LG**) substrates at low (**LMP**) and high (**HMP**) MP levels, 6 rumen-fistulated, second-lactation Holstein-Friesian dairy cows with an average milk production of 25.9 ± 2.87 kg/d at 97 ± 13 DIM and 633 ± 53.9 kg of BW were randomly assigned to a 6 × 6 Latin square design. Each experimental period consisted of 5 d of continuous abomasal infusion followed by 2 d of rest (**Figure 5.1**). Cows were adapted to the experimental conditions for 19 d before the first experimental period. For the first 14 d of adaptation, cows were housed individually in tie stalls for acclimatization to the diet and the restriction in movement. From d 15 of the adaptation period, cows were

housed individually in identical climate respiration chambers (CRC) for 5 d of adaptation before the first experimental period began. Cows were housed in CRC for the entire experiment to facilitate determination of gaseous exchange, energy and N balance, and apparent total-tract nutrient digestibility (ATTD). Detailed descriptions of the CRC design and gas measurements are given by Heetkamp et al. (2015) and van Gastelen et al. (2015). Briefly, each CRC compartment measured 11.8 m^2 and had a volume of 34.5 m^3. Relative humidity was maintained at 65%, temperature at 16°C, and the ventilation rate at 43 m^3/h inside each compartment. The CRC were designed with thin walls equipped with windows to allow audio and visual contact between cows and minimize the effects of social isolation on behavior and performance. Cows were exposed to 17.5 h of light per d (0530 to 2300 h).

Gas concentrations and ventilation rates were corrected for pressure, temperature, and relative humidity to arrive at standard temperature and pressure dew point volumes of inlet and exhaust air. Production of CO_2 and CH_4 and consumption of O_2 inside each compartment were calculated from the difference between inlet and exhaust gas volumes. Gas analysis measurement in this experiment was performed as described by van Gastelen et al. (2015), where 4 CRC compartments shared a single gas analyzer, but with the addition of a second gas analyzer shared by the additional 2 CRC compartments measuring gas in 6-min intervals. Calibration gases were sampled once daily instead of the inlet air. The analyzed and actual values of these calibration gases were used to correct the analyzed gas concentrations from the inlet and exhaust air of the 6 compartments. Before the experiment started, CO_2 recovery was checked by releasing known amounts of CO_2 into each compartment and comparing the known values with data from the gas analysis system to calculate the recovery. The recovered amounts of CO_2 were between 99 and 101% (100.1 ± 0.37%). Gas measurements during time points when staff entered the CRC compartments (maximum 30 min for milking, feeding, checking abomasal infusion lines) were discarded from the data analysis. Production of CO_2 and CH_4 and consumption of O_2 was assumed to be linear between the last data point before opening and the first data point after closing the CRC.

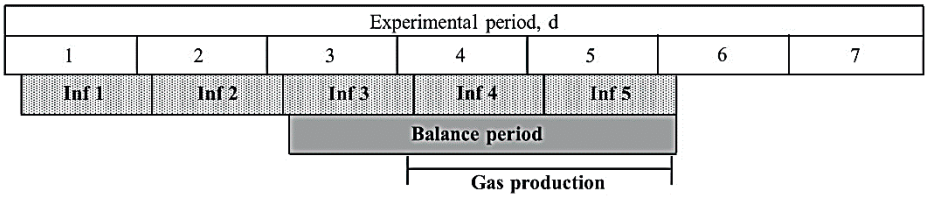

Figure 5.1. Design of a single 7-d experimental period. Inf 1 to Inf 5 = 120-h infusion period beginning at 0900 h on d 1 and ending at 0900 h on d 6 of each experimental period. The infusion period was followed by a 48-h wash-out period. Balance period = 71-h period of total manure collection with milk and feces samples from 1000 h on d 3 until 0900 h on d 6. Gas production = 48-h period of gas production and consumption measured from 0800 h on d 4 until 0800 h on d 6.

Diet, Feeding, and Treatment Infusions

Cows were fed a TMR consisting of 42% corn silage, 31% grass silage, and 27% concentrate on a DM basis (**Table 5.1**), formulated to meet 100 and 83% of NE_L and MP requirements (CVB, 2008), respectively, for cows consuming 20 kg of DM/d and producing 30 kg/d of milk containing 4.0% fat and 3.4% protein. Cows were fed ad libitum for the first 10 d of the 19-d adaptation period. Intake during the final 5 d of this 10-d ad libitum intake period was used to calculate a 10% daily intake restriction for individual cows. From d 11, cows were fed this fixed amount for the remainder of the adaptation and experimental periods described above. Fresh feed was allocated twice daily at 0530 and 1530 h by manually mixing the roughage and concentrate portions into a TMR for individual cows. The roughage portion (corn silage + grass silage) of the diet was mixed twice weekly and stored at 4°C for no longer than 4 d before feeding. The concentrate contained 0.25% titanium dioxide as an inert marker for estimation of ATTD. Feed refusals at each feeding time point were collected and weighed to determine daily feed intake. Cows had individual and free access to drinking water throughout the entire experiment. For a 34-h period over d 4 and 5 of each experimental period (0530 h on d 4 until 1530 h on d 5), cows were fed using an automated feeding system that dispensed equal portions of feed every 2 h to promote metabolic steady-state conditions in preparation for the blood sampling protocol described below.

Infusion lines were placed in the abomasum via the rumen cannula 7 d before the first experimental period and were checked daily for patency and position. The infusion device was constructed from 200 cm of braided polyvinyl chloride hose (6.4 mm i.d., 11.2 mm o.d.) attached to a rumen cannula plug at the proximal end and a flexible disc (equipped with holes to allow particle and fluid passage) at the distal end to secure its placement through the sulcus omasi. The flexible disc was 12 cm in diameter and made from plastisol (Bar Diamond Inc., Parma, ID). The infusion line (Tygon S3 E-3606, 3.2 mm i.d., 6.4 mm o.d.; Saint-Gobain Performance Plastics, Courbevoie, France) was connected between the infusate, infusion pump, and the proximal end of the abomasal infusion line with luer-to-tubing connectors (Sigma-Aldrich, St. Louis, MO). Infusion treatments were (1) 0.9% saline (**LMP-C**; 90% NE_L, 75% MP), (2) 1319 g/d of glucose (**LMP-GG**; 100% NE_L, 75% MP), (3) 676 g/d of palm olein (**LMP-LG**; 100% NE_L, 75% MP), (4) 844 g/d of EAA (**HMP-C**; 100% NE_L, 120% MP), (5) 1319 g/d of glucose + 844 g/d of EAA (**HMP-GG**; 110% NE_L, 120% MP), and (6) 676 g/d of palm olein + 844 g/d of EAA (**HMP-LG**; 110% NE_L, 120% MP), where MP and NE_L reflect the proportion of requirements met by the restricted feeding level of the diet plus the infusate. Treatment solutions were administered in 10-L batches which were replenished daily and infused via multi-channel peristaltic pumps at a rate of 6.95 mL/min to facilitate 120 h of continuous infusion (0900 h on d 1 until 0900 h on d 6 of each experimental period; Figure 5.1). The EAA infusions delivered EAA in same profile and amount as found in 1.5 kg of casein according to

Metcalf et al. (1996) and at the following rates (g/d): L-Arg (59), L-His (48), L-Ile (86), L-Val (96), L-Leu (141), L-Phe (141), DL-Met (41), L-Lys (147), L-Thr (63), and L-Trp (21). All AA were provided by Ajinomoto Eurolysine (Paris, France) and Ajinomoto Omnichem (Wetteren, Belgium) with the exception of DL-Met, which was provided by Adisseo France (Malicorne, France). As determined by GC of methyl esters, the FA composition of palm olein (weight basis) was 1.2% <C16, 43.3% C16:0, 0.1% C17:0, 1.0% C18:0, 42.5% C18:1 *cis*-9, 11.0% C18: 2n -6, 0.8% >C18:2. Daily 10-L batches of LMP-LG and HMP-LG were split into 5-L batches that were replenished twice daily to facilitate continuous infusion of palm olein, which was evenly mixed with water or with the EAA solution via continuous stirring. Daily infusion dosages (g/d) of LMP-GG, LMP-LG, and HMP-C were designed to be isoenergetic based on the gross energy (GE) content of the EAA infusion, which was calculated to be 24.3 MJ/kg based on the heat of combustion of individual EAA in the infusate. Doses (g/d) of glucose and palm olein were calculated assuming GE contents of 15.5 and 37.9 MJ/kg, respectively, and 80% digestibility of palm olein (Benson et al., 2001; NRC, 2001). Digestibility of EAA and glucose was assumed to be 100%.

Measurements and Sample Collection

Measurements of CO_2 and CH_4 production and O_2 consumption were based on data recorded from d 4 (0800 h) through d 6 (0800 h) of each experimental period, whereas energy and N balance and ATTD were based on manure and feces collection from d 3 (1000 h) through d 6 (0900 h; balance period; Figure 5.1). Each CRC compartment was cleaned at 0900 h on d 3 (taking approximately 60 min) to remove all manure collected from the end of the previous period to facilitate a fresh total collection period. At the end of each balance period (which corresponded with the end of the infusion period), cows were weighed and the manure from each compartment produced during the 71-h balance period was separately and quantitatively collected, weighed, and mixed. Manure samples were collected and stored at −20°C until analysis. In addition, to quantify contribution of N from volatilized ammonia appearing from the mixing of urine and feces in manure, samples of condensed water from the chamber heat exchanger and from 25% sulfuric acid solution (wt/wt), through which outflowing air was led to trap aerial ammonia, were collected from each CRC compartment. These samples were stored at 4°C until analysis. During the balance period, feces was collected by rectal grab sampling at 0530 and 1530 h (6 samples) and immediately pooled into a composite sample by cow, which was stored at −20°C until analysis. Feed refusals, when present, were collected during the balance period and stored at 4°C. After each balance period they were pooled by cow, sampled, and stored at −20°C until analysis.

Cows were milked twice daily at 0530 and 1530 h during the adaptation and experimental periods. Milk weight was recorded at each milking, and samples were collected at each milking into tubes containing sodium azide and stored at 4°C until analysis within 4 d. Two additional milk samples (5 g/kg of milk) were collected separately and pooled by cow

at each milking during the balance period (6 milkings). One set of samples was stored at −20°C until GE and N analyses. The second set of samples was stored at 4°C between milkings and finally split into 200-mL aliquots before storing at −20°C pending milk FA analysis. Samples of corn silage, grass silage, and concentrate were collected twice weekly during feed preparation. These samples were pooled per period and stored at −20°C until analysis. On d 5 of each experimental period, blood samples were collected from the coccygeal vessels into 10-mL sodium heparin and potassium EDTA Vacutainers (Becton Dickinson, Rutherford, NJ) at 0730, 0930, 1130, 1330, and 1530 h. After each sampling point, collection tubes were immediately placed in ice and centrifuged at 3000 × *g* for 15 min at room temperature. Plasma was pooled over sampling time points by cow and period and stored at −20°C until analysis.

Analytical Procedures

Samples of corn silage, grass silage, concentrate, manure, and feces were thawed at room temperature, oven-dried at 60°C until a constant weight was reached, and ground to pass a 1-mm screen using a Wiley mill (Peppink 100AN, Olst, the Netherlands). Wet chemical analysis for DM, ash, N, NH_3, crude fat, starch, sugars, NDF, ADF, ADL, and titanium was performed as described by Nichols et al. (2018). Crude protein content was calculated as total analyzed N × 6.25. An adiabatic bomb calorimeter (IKA-C700, Janke and Kunkel, Heitersheim, Germany) was used for determination of GE content (ISO 9831; ISO, 1998). Corn silage, grass silage, and concentrate samples were analyzed for DM, ash, N, crude fat, starch (except grass silage), sugars (except corn silage), NDF, ADF, ADL, GE, and titanium (concentrate only). Samples of refused feed were analyzed for DM. Manure samples were analyzed for DM, N, and GE. Feces samples were analyzed for DM, ash, N, crude fat, starch, NDF, GE, and titanium. In addition, samples of condensed water and the sulfuric acid solution were analyzed for N. Reported values for nutrient content of the TMR were calculated from ration composition and analyzed values obtained for the roughage and concentrate. The NE_L was calculated with the VEM (feed unit lactation) system according to Van Es (1978). For corn and grass silage, intestinal digestible protein (DVE; see Table 1), RDP balance (OEB; see Table 1), and NE_L were calculated based on the chemical composition as obtained by near-infrared spectroscopy analysis (Eurofins Agro, Wageningen, the Netherlands). For the concentrate, DVE, OEB, and NE_L were calculated based on table values for composition of the ingredients (CVB, 2008). For the TMR, these were calculated from ration composition of all roughage and concentrate ingredients.

Milk samples from the morning and afternoon milkings were analyzed separately for protein, fat, lactose, and urea by mid-infrared spectroscopy (ISO 9622; ISO, 2013; VVB, Doetinchem, the Netherlands). Pooled milk samples were analyzed for GE and N in fresh material as described above. Fatty acid composition of pooled milk samples was analyzed by GC as described by Nichols et al. (2018). Blood plasma was analyzed by the Veterinary

Diagnostic Laboratory (Utrecht University, Utrecht, the Netherlands) as described by van Knegsel et al. (2007b).

Table 5.1. Ingredient composition of TMR and analyzed and calculated chemical composition of ingredients (corn silage, grass silage, and concentrate) and complete TMR (g/kg of DM, unless otherwise noted)

	Ingredient			
Item	Corn silage	Grass silage	Concentrate[1]	TMR[2]
Inclusion	420	313	267	-
Chemical composition				
DM, g/kg	325	484	903	447
Gross energy, MJ/kg of DM	19.0	19.2	16.7	18.4
Crude ash	38	97	121	79
CP	82	162	206	140
Crude fat	33	35	26	32
NDF	354	502	309	388
ADF	197	271	206	223
ADL	11	12	6	10
Starch	349	NA[3]	257	217
Sugar	NA	72	27	29
DVE[4]	54	67	125	77
OEB[5]	-41	25	63	7
NE_L,[6] MJ/kg of DM	6.82	6.61	7.68	6.99

[1]Contained (g/kg of DM): ground corn 8% CP, 370; soy hulls, 372; soybean meal 48% CP, 116; limestone 37% Ca, 29; formaldehyde-treated soybean meal, 24; MgO, 20; urea, 20; monocalcium phosphate, 20; NaCl, 18; trace mineral and vitamin premix, 10; TiO_2 was included at 0.25% of concentrate DM.

[2]Values for TMR were calculated based on ration composition and analyzed and calculated values obtained for roughages and concentrate.

[3]NA = not analyzed.

[4]Intestinal digestible protein (CVB, 2008).

[5]Rumen degradable protein balance (CVB, 2008).

[6]NEL calculated with the VEM system (CVB, 2008).

Calculations and Statistical Analysis

Heat production (kJ/d) was calculated as 16.175 × VO_2 (L/d) + 5.021 × VCO_2 (L/d) where VO_2 and VCO_2 are volumes of O_2 consumed and CO_2 produced, respectively (Gerrits et al., 2015). Apparent total-tract digestibility was calculated considering the nutrient inflow from the diet and the treatment infusions. The infusion treatments contributed DM, ash, N, and GE. Dry matter of the infusion ingredients was assumed to be 100% plus contribution of ash in the saline (99 g/d of NaCl) and EAA infusions, and hydroxide from mixing the EAA solutions (116 g/d of NaOH and 75 g/d of HCl were used to facilitate EAA mixing). Nitrogen content of the EAA infusion was calculated based on the molar weight of N in individual EAA in the infusate. Gross energy contributions from the infusates were calculated according to the assumptions described above.

Milk yield, milk composition, and DMI were averaged over the 3-d balance period. Variances in lactation performance, milk FA composition, digestibility, energy and N balance, and plasma constituents were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The model contained main and interaction effects of infusion treatment factors (GG, LG, and AA) and period as fixed effects and cow as a random effect. Differences were considered significant at $P \leq 0.050$ and tendencies at $0.050 < P \leq 0.100$. Multiple comparisons between treatment means were made using the Tukey-Kramer method when GG × AA or LG × AA interactions were detected at $P \leq 0.100$. Treatment arrangement within the Latin square was balanced for first-order carryover effects in subsequent periods (Williams, 1949), as each treatment immediately preceded and followed every other treatment exactly once in each square. We observed no carryover effects between periods, assessed by testing for an effect of the previous treatment in the ANOVA.

RESULTS

DMI, Milk Production, and Digestibility

Infusing GG or LG at the HMP level did not affect DMI, milk production, or milk composition differently than at the LMP level (no significant GG × AA or LG × AA interactions detected; $P > 0.210$; **Table 5.2**). Dry matter intake decreased in response to GG and LG ($P \leq 0.015$) and increased at the HMP level ($P = 0.041$). Total milk yield was unaffected by GG and LG ($P > 0.610$) and increased at the HMP level ($P < 0.001$). Yields of milk protein and lactose were unaffected by GG and LG ($P > 0.280$) and increased at the HMP level ($P < 0.001$). Milk fat yield decreased in response to GG ($P < 0.001$), increased in response to LG ($P < 0.001$), and increased at the HMP level ($P = 0.023$). Infusion of GG and LG had no effect on protein or lactose content, but fat content decreased in response to GG ($P < 0.001$) and increased in response to LG ($P < 0.001$). The HMP level decreased milk fat and lactose content ($P \leq 0.001$), and increased milk protein content ($P < 0.001$). Fat- and protein-corrected milk yield decreased in response to GG ($P = 0.027$), increased in response to LG ($P = 0.021$), and increased at the HMP level ($P < 0.001$). Milk urea content was unaffected by GG or LG ($P > 0.240$) and increased at the HMP level ($P = 0.017$).

For ATTD, no significant GG × AA or LG × AA interactions were detected ($P > 0.291$; **Table 5.3**). Infusion of GG decreased crude fat digestibility ($P < 0.001$) and tended to increase GE digestibility ($P = 0.097$). Infusion of LG increased crude fat digestibility ($P < 0.001$). The HMP infusion level increased CP and GE digestibility ($P \leq 0.001$) and tended to increase DM digestibility ($P = 0.075$). Apparent total-tract digestibility of OM, NDF, and starch were unaffected by treatment.

Table 5.2. Performance of lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d[1]

	Treatment[2]							*P*-value[3]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
DMI,[4] kg/d	17.2	15.8	15.7	17.3	16.4	16.8	0.75	0.007	0.015	0.041	0.518	0.218
Yield												
Milk, kg/d	25.1	24.9	24.5	28.6	29.5	28.8	1.58	0.613	0.686	<0.001	0.369	0.541
Fat, g/d	1091	963	1229	1160	1037	1300	53.1	<0.001	<0.001	0.023	0.941	0.979
Protein, g/d	794	778	753	982	1016	973	47.4	0.702	0.283	<0.001	0.289	0.508
Lactose, g/d	1180	1182	1155	1301	1338	1309	74.7	0.543	0.792	<0.001	0.585	0.618
Composition, %												
Fat	4.42	3.93	5.12	4.14	3.60	4.61	0.190	<0.001	<0.001	0.001	0.836	0.304
Protein	3.19	3.14	3.09	3.44	3.45	3.39	0.069	0.703	0.152	<0.001	0.518	0.619
Lactose	4.70	4.76	4.70	4.55	4.55	4.55	0.033	0.417	0.978	<0.001	0.306	0.935
FPCM,[5] kg/d	26.0	24.3	27.1	29.2	28.2	30.8	1.31	0.027	0.021	<0.001	0.543	0.694
Milk urea, mg/dL	9.0	8.7	9.9	13.3	10.2	14.1	1.62	0.242	0.492	0.017	0.387	0.970

[1]Data are least squares means from the final 3 d of infusion.

[2]Low protein (LMP) or high protein (HMP) abomasal infusion treatments: LMP-C, 0.9% saline; LMP-GG, 1319 g/d glucose; LMP-LG, 676 g/d palm olein; HMP-C, 844 g/d of a complete EAA mixture in the same profile and amount as found in 1.5 kg casein; HMP-GG, 1319 g/d glucose + 844 g/d EAA; HMP-LG, 676 g/d palm olein + 844 g/d EAA. Infusions of LMP-GG, LMP-LG, and HMP-C supplied 20.5 MJ/d, and HMP-GG and HMP-LG supplied 41.0 MJ/d.

[3]GG = effect of energy from glucose; LG = effect of energy from fat; AA = effect of protein level.

[4]Diet only.

[5]Fat- and protein-corrected milk (FPCM; kg/d) = (0.337+ 0.116 × fat % + 0.06 × protein %) × milk yield (kg/d) (CVB, 2008).

Table 5.3. Apparent total-tract digestibility (%) of nutrients in lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d[1]

	Treatment[2]							*P*-value[3]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
DM	70.4	69.9	69.7	70.7	71.7	70.7	1.11	0.797	0.619	0.075	0.321	0.652
OM	72.0	71.4	71.2	72.2	73.2	71.9	1.17	0.808	0.511	0.105	0.305	0.718
CP	60.0	58.9	59.9	72.0	72.2	71.3	1.62	0.714	0.774	<0.001	0.628	0.804
NDF	59.7	58.9	61.0	59.4	62.6	61.1	2.22	0.426	0.311	0.195	0.180	0.897
Crude fat	63.9	57.7	75.2	65.6	57.6	77.8	2.03	<0.001	<0.001	0.384	0.494	0.768
Starch	98.2	98.0	98.6	98.4	98.3	98.5	0.19	0.200	0.100	0.356	0.754	0.291
Gross energy	69.8	70.5	69.9	71.7	73.7	72.5	1.16	0.097	0.571	0.001	0.408	0.650

[1]Data are least squares means calculated from feed and feces sampled during the final 3 d of infusion and were calculated considering the total nutrient inflow from the diet + infusions.

[2]Low protein (LMP) or high protein (HMP) abomasal infusion treatments: LMP-C, 0.9% saline; LMP-GG, 1319 g/d glucose; LMP-LG, 676 g/d palm olein; HMP-C, 844 g/d of a complete EAA mixture in the same profile and amount as found in 1.5 kg casein; HMP-GG, 1319 g/d glucose + 844 g/d EAA; HMP-LG, 676 g/d palm olein + 844 g/d EAA. Infusions of LMP-GG, LMP-LG, and HMP-C supplied 20.5 MJ/d, and HMP-GG and HMP-LG supplied 41.0 MJ/d.

[3]GG = effect of energy from glucose; LG = effect of energy from fat; AA = effect of protein level.

Milk FA Composition

Infusion of GG increased concentrations of total de novo (<16C) and mixed (16C) FA ($P < 0.001$) and decreased concentration of preformed (>16C) FA ($P < 0.001$; **Table 5.4**) in milk fat. Concentration of total SFA increased ($P < 0.001$), total MUFA decreased ($P < 0.001$), and total PUFA tended to decrease ($P = 0.068$) in response to GG. Individually, all FA ≤ C16:0 increased or tended to increase in response to GG ($P \leq 0.099$), with the exception of C4:0, *iso* C15:0, and *anteiso* C15:0, which decreased ($P \leq 0.011$) and *iso* C14:0, which was unaffected. The FA C18:3n-6 increased ($P = 0.023$) in response to GG. In addition, C15:0 was affected and C11:0, C18:3n-6, and C20:0 tended to be affected by a GG × AA interaction where their concentration increased further when GG was infused at the HMP level compared with the LMP level ($P \leq 0.086$). The FA 18:1 *trans*-10 + *trans*-11 tended to be affected by a GG × AA interaction where its concentration decreased further when GG was infused at the HMP level compared with the LMP level ($P = 0.096$). Infusion of GG decreased or tended to decrease ($P \leq 0.071$) concentration of C16:1 *cis*-9, C16:1 *trans*-9, C17:0, C17:1 *cis*-9, C18:0, C18:1 *cis*-9, C18:1 *cis*-12, C18:1 *cis*-13, C18:1 *trans*-15 + C18:1 *cis*-11, total CLA, C18:3n-3, and C20:4n-6.

Infusion of LG decreased concentrations of total de novo and mixed FA ($P < 0.001$) and increased concentration of preformed FA ($P < 0.001$) in milk fat. Concentration of total SFA decreased ($P < 0.001$), and concentrations of total MUFA and PUFA increased ($P < 0.001$) in response to LG. Individually, *iso* C17:0, C18:1 *cis*-9, C18:1 *trans*-15 + C18:1 *cis*-11, C18:2n-6, C18:3n-3, and C20:4n-3 increased or tended to increase in response to LG ($P \leq 0.082$). Concentrations of all FA ≤ C16:1 and C17:0, C17:1 *cis*-9, C18:0, C18:1 *cis*-13, C20:0, C20:2n-6, and C20:4n-6 decreased or tended to decrease in response to LG ($P \leq 0.069$). Total CLA was affected by a LG × AA interaction where its concentration increased when LG was infused at the LMP level but decreased at the HMP level ($P = 0.050$). The n-6 to n-3 ratio increased in response to LG ($P < 0.001$) and was unaffected by GG or protein level.

Infusions at the HMP level increased the concentration of total de novo FA ($P = 0.001$), decreased total mixed FA ($P = 0.015$), and had no effect on total preformed FA, SFA, MUFA, or PUFA concentrations in milk fat. Individually, all FA ≤ C12:0, C14:0, C14:1 *cis*-9, C15:0, C18:3n-6, and C20:0 increased ($P \leq 0.033$) and concentrations of C16:0, C18:0, C18:1 *trans*-10 + *trans*-11, C22:5n-3, and C24:0 decreased ($P \leq 0.044$) at the HMP level.

Table 5.4. Milk fatty acid (FA) composition of lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

	Treatment[1]							*P*-value[2]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
Concentration, g/100 g of FA												
C4:0	2.96	2.86	2.76	3.18	2.92	3.13	0.087	0.011	0.069	0.003	0.259	0.268
C6:0	2.06	2.15	1.79	2.19	2.23	2.01	0.068	0.099	<0.001	0.002	0.452	0.301
C8:0	1.23	1.42	1.03	1.34	1.47	1.19	0.057	<0.001	<0.001	0.007	0.408	0.500
C10:0	2.83	3.62	2.23	3.10	3.79	2.60	0.181	<0.001	<0.001	0.020	0.652	0.674
C11:0	0.19^{ab}	0.28^{c}	0.11^{d}	0.21^{a}	0.36^{e}	0.14^{bd}	0.022	<0.001	<0.001	0.001	0.061	0.733
C12:0	3.27	4.57	2.49	3.63	4.94	2.95	0.230	<0.001	<0.001	0.012	0.972	0.759
C14:0	12.0	13.0	9.6	12.5	13.7	10.1	0.53	0.001	<0.001	0.033	0.740	0.912
iso C14:0	0.13	0.12	0.08	0.11	0.12	0.08	0.012	0.870	<0.001	0.959	0.504	0.637
C14:1 *cis*-9	0.98	1.24	0.61	1.07	1.53	0.71	0.066	<0.001	<0.001	0.009	0.159	0.938
C15:0	1.24^{a}	1.36^{ab}	0.85^{c}	1.20^{a}	1.57^{b}	0.86^{c}	0.071	<0.001	<0.001	0.024	0.011	0.572
iso C15:0	0.28	0.23	0.18	0.26	0.22	0.19	0.009	<0.001	<0.001	0.755	0.234	0.200
anteiso C15:0	0.53	0.46	0.34	0.51	0.46	0.37	0.019	<0.001	<0.001	0.424	0.345	0.105
C16:0	34.1	39.9	32.1	32.7	38.2	30.6	1.03	<0.001	0.002	0.016	0.786	0.941
iso C16:0	0.29	0.26	0.22	0.28	0.27	0.21	0.016	0.113	<0.001	0.968	0.414	0.708
C16:1 *cis*-9	0.75	0.64	0.64	0.78	0.71	0.59	0.046	0.031	0.001	0.858	0.709	0.281
C16:1 *trans*-9	0.40	0.33	0.30	0.39	0.36	0.29	0.018	0.001	<0.001	0.523	0.253	0.989
C17:0	0.77	0.69	0.57	0.77	0.66	0.57	0.034	0.009	<0.001	0.672	0.699	0.875
iso C17:0	0.05	0.07	0.08	0.07	0.09	0.19	0.054	0.957	0.082	0.529	0.991	0.331
C17:1 *cis*-9	0.30	0.26	0.24	0.32	0.29	0.21	0.025	0.071	0.001	0.700	0.757	0.408
C18:0	9.82	6.83	8.45	9.05	5.95	7.71	0.402	<0.001	0.001	0.031	0.876	0.977
C18:1 *cis*-9[3]	19.8	15.0	26.9	20.5	15.5	27.4	1.24	<0.001	<0.001	0.592	0.890	0.883
C18:1 *cis*-12	0.30	0.25	0.29	0.29	0.26	0.27	0.022	0.002	0.171	0.511	0.728	0.474
C18:1 *cis*-13	0.41	0.30	0.35	0.42	0.31	0.33	0.034	<0.001	0.001	0.814	0.900	0.579
C18:1 *trans*-9	0.03	0.02	0.03	0.01	0.01	0.01	0.007	0.410	0.832	0.153	0.298	0.878
C18:1 *trans*-10 + *trans*-11	1.08^{a}	0.86^{ab}	1.08^{a}	1.04^{a}	0.55^{b}	0.86^{ab}	0.088	<0.001	0.248	0.002	0.096	0.242
C18:1 *trans*-15 + C18:1 *cis*-11	0.65	0.40	0.77	0.64	0.45	0.75	0.069	<0.001	0.005	0.816	0.400	0.971

Table 5.4 (continued). Milk fatty acid (FA) composition of lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

	Treatment[1]							*P*-value[2]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
Total CLA[4]	0.58^{ab}	0.49^{b}	0.64^{a}	0.65^{a}	0.56^{ab}	0.57^{ab}	0.042	0.005	0.738	0.894	0.913	0.050
C18:2n-6	1.74	1.53	4.10	1.73	1.43	4.08	0.164	0.149	<0.001	0.735	0.813	0.971
C18:3n-3	0.39	0.32	0.43	0.35	0.32	0.40	0.031	0.001	0.004	0.176	0.262	0.996
C18:3n-6	0.05^{a}	0.05^{ab}	0.05^{a}	0.06^{ab}	0.10^{b}	0.06^{ab}	0.010	0.023	0.928	0.006	0.086	0.841
C20:0	0.12^{abc}	0.11^{bc}	0.10^{c}	0.13^{ab}	0.14^{a}	0.11^{bc}	0.006	0.908	0.001	0.004	0.065	0.944
C20:2n-6	0.31	0.30	0.27	0.33	0.32	0.24	0.021	0.608	0.001	0.725	0.908	0.185
C20:3n-6	0.01	0.03	0.01	0.01	0.02	0.01	0.006	0.120	0.830	0.328	0.282	0.942
C20:4n-3	0.05	0.05	0.07	0.05	0.05	0.06	0.008	0.981	0.016	0.189	0.416	0.330
C20:4n-6	0.07	0.06	0.06	0.07	0.06	0.06	0.004	0.001	0.002	0.937	0.706	0.643
C20:5n-3	0.11	0.12	0.13	0.11	0.12	0.11	0.011	0.423	0.219	0.218	0.756	0.270
C22:0	0.03	0.02	0.02	0.03	0.03	0.03	0.007	0.656	0.544	0.167	0.578	0.532
C22:5n-3	0.04	0.08	0.05	0.04	0.04	0.04	0.012	0.126	0.563	0.044	0.140	0.859
C24:0	0.02	0.02	0.03	0.02	0.01	0.02	0.006	0.512	0.515	0.034	0.429	0.224
n-6 to n-3 ratio[5]	3.74	3.57	6.62	4.01	3.76	7.35	0.298	0.476	<0.001	0.123	0.883	0.444
Summation[6]												
De novo	27.7	31.0	22.0	29.3	33.3	24.4	1.09	<0.001	<0.001	0.001	0.575	0.585
Preformed	36.8	27.8	44.7	36.5	27.1	43.9	1.73	<0.001	<0.001	0.467	0.823	0.799
Mixed	35.5	41.2	33.2	34.2	39.6	31.7	1.01	<0.001	0.001	0.015	0.832	0.885
SFA	71.9	77.7	63.0	71.3	77.1	63.0	1.53	<0.001	<0.001	0.789	0.971	0.712
MUFA	24.7	19.3	31.2	25.3	19.9	31.3	1.34	<0.001	<0.001	0.662	0.996	0.770
PUFA	3.35	3.03	5.81	3.39	2.99	5.63	0.226	0.068	<0.001	0.574	0.839	0.563

[a-e]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Low protein (LMP) or high protein (HMP) abomasal infusion treatments: LMP-C, 0.9% saline; LMP-GG, 1319 g/d glucose; LMP-LG, 676 g/d palm olein; HMP-C, 844 g/d of a complete EAA mixture in the same profile and amount as found in 1.5 kg casein; HMP-GG, 1319 g/d glucose + 844 g/d EAA; HMP-LG, 676 g/d palm olein + 844 g/d EAA. Infusions of LMP-GG, LMP-LG, and HMP-C supplied 20.5 MJ/d, and HMP-GG and HMP-LG supplied 41.0 MJ/d.

[2]GG = effect of energy from glucose; LG = effect of energy from fat; AA = effect of protein level.

Table 5.4 (continued). Milk fatty acid (FA) composition of lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

[3]C18:1 cis-9 represents the sum of C18:1 cis-9 and C18:1 trans-12, as these 2 FA could not be separated in the analysis. The proportion of C18:1 trans-12 is considered negligible.

[4]Total CLA consists of mainly C18:2 cis-9,trans-11.

[5]Ratio between the sum of C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, and C20:4n-6 and the sum of C18:3n-3, C20:4n-3, C20:5n-3, and C22:5n-3.

[6]De novo-synthesized FA (<16 carbons) originate from mammary de novo synthesis, preformed FA (>16 carbons) originate from mammary plasma extraction, mixed FA (16 carbons) originate from both de novo-synthesized and preformed FA. Sum of the respective SFA, MUFA, and PUFA reported in this table.

Energy and N Balance

Metabolic BW tended (P = 0.071) to be greater with HMP than LMP treatments and was not affected by GG or LG infusion (**Table 5.5**). Daily CH_4 production expressed per unit metabolic BW tended to be affected by a GG × AA interaction where it decreased with GG but only at the LMP level (P = 0.079). The ratio of MEI to gross energy intake (GEI) tended to be affected by a GG × AA interaction where the ratio increased in response to GG infusion at the LMP level, but was unaffected by GG infusion at the HMP level (P = 0.073). A GG × AA interaction also affected heat production where it decreased with GG only at the LMP level (P = 0.027). Infusion of GG increased total ER (P = 0.034) and decreased milk energy output (P = 0.019). Infusion of GG decreased N intake (P = 0.011) and increased milk N efficiency (P = 0.004).

Infusion of LG decreased CH_4 production (P = 0.007), tended to increase MEI (P = 0.051), and increased the MEI: GEI ratio and energy output in milk ($P \leq 0.002$). A LG × AA interaction affected heat production where it decreased with LG only at the LMP level (P = 0.007). Infusion of LG decreased N intake (P = 0.023) but did not affect other N balance parameters. Infusions at the HMP level increased GEI, CH_4 production, MEI, the MEI: GEI ratio, heat production, and milk energy output ($P \leq 0.003$), and tended to increase ER in protein (P = 0.063). The HMP infusion level increased N intake and milk N output ($P < 0.001$), tended to increase N retention (P = 0.063), and decreased milk N efficiency (P = 0.001). The respiratory quotient (RQ) increased in response to GG (P = 0.005), decreased in response to LG (P = 0.002), and decreased at the HMP level (P = 0.040).

Plasma Constituents

Arterial plasma concentration of glucose and insulin increased in response to GG ($P \leq$ 0.044; **Table 5.6**) and concentration of BHB, non-esterified fatty acids (NEFA), and long-chain fatty acids (LCFA) decreased ($P \leq 0.030$). Plasma urea tended to be affected by a GG × AA interaction where its concentration decreased with GG only at the HMP level (P = 0.081). Infusion of LG increased triacylglyceride (TAG) and LCFA concentrations, and a LG × AA interaction tended to affect plasma urea concentration where LG tended to increase urea concentration at the LMP level, but tended to decrease urea concentration at the HMP level (P = 0.090). The HMP infusion level increased plasma BHB and urea concentrations ($P \leq$ 0.005).

Table 5.5. Energy and nitrogen balance of lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

	Treatment[1]							*P*-value[2]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
Metabolic BW,[3] $kg^{0.75}$	122	122	122	123	123	123	3.5	0.505	0.514	0.071	0.834	0.877
Energy balance, kJ/kg of $BW^{0.75}$ per d, unless otherwise stated												
GEI[4]	2598	2569	2591	2762	2795	2863	114.3	0.971	0.445	<0.001	0.610	0.381
Energy in manure	804	758	744	761	773	760	45.4	0.406	0.151	0.453	0.170	0.167
CH_4 production	181[a]	157[b]	159[b]	180[a]	173[ab]	174[ab]	8.1	0.003	0.007	0.003	0.079	0.105
MEI[5]	1613	1654	1687	1820	1849	1929	67.6	0.440	0.051	<0.001	0.884	0.710
MEI:GEI,[6] %	62.1[a]	64.5[b]	65.3[b]	66.0[bc]	66.2[bc]	67.4[c]	0.58	0.031	0.001	0.003	0.073	0.132
Heat production	904[ab]	861[bd]	857[d]	937[ac]	945[ac]	955[c]	21.5	0.121	0.197	<0.001	0.027	0.007
Energy in milk	660	621	698	735	709	787	27.6	0.019	0.002	<0.001	0.645	0.613
ER total[7]	49	173	133	148	196	187	32.8	0.034	0.123	0.326	0.322	0.559
ER protein[8]	-44	-33	7	34	78	62	30.4	0.523	0.360	0.063	0.705	0.796
ER fat[9]	93	206	126	115	118	125	37.8	0.317	0.711	0.439	0.343	0.842
RQ[10]	1.12	1.14	1.09	1.10	1.12	1.08	0.011	0.005	0.002	0.040	0.573	0.480
Nitrogen balance, mg/kg $BW^{0.75}$ per d												
N intake	3159	2921	2894	4151	3990	4064	142.4	0.011	0.023	<0.001	0.600	0.232
N manure	2345	2047	1784	2549	2053	2301	207.9	0.195	0.187	0.390	0.743	0.604
Fecal N[11]	1266	1196	1155	1164	1110	1174	77.8	0.268	0.361	0.547	0.879	0.271
Urine N[12]	1079	851	629	1385	942	1127	196.7	0.239	0.215	0.300	0.703	0.733
N milk	1022	1013	983	1270	1315	1248	51.6	0.513	0.278	<0.001	0.339	0.768
N condense + acid[13]	90	83	82	105	98	94	12.7	0.502	0.335	0.163	0.953	0.933
N retention[14]	-298	-223	45	227	524	421	205.2	0.523	0.360	0.063	0.705	0.796
Milk N efficiency,[15] %	32.4	34.8	34.2	30.7	32.9	30.7	1.00	0.004	0.217	0.001	0.980	0.228

[a-d]Means within a row with no common superscripts differ ($P < 0.05$).

Table 5.5 (continued). Energy and nitrogen balance of lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

[1]Low protein (LMP) or high protein (HMP) abomasal infusion treatments: LMP-C, 0.9% saline; LMP-GG, 1319 g/d glucose; LMP-LG, 676 g/d palm olein; HMP-C, 844 g/d of a complete EAA mixture in the same profile and amount as found in 1.5 kg casein; HMP-GG, 1319 g/d glucose + 844 g/d EAA; HMP-LG, 676 g/d palm olein + 844 g/d EAA. Infusions of LMP-GG, LMP-LG, and HMP-C supplied 20.5 MJ/d, and HMP-GG and HMP-LG supplied 41.0 MJ/d.

[2]AA = effect of protein level; GG = effect of energy from glucose; LG = effect of energy from fat.

[3]The mean BW per cow per balance period was used to calculate metabolic BW (BW0.75).

[4]GEI = gross energy intake (diet + infusions).

[5]Metabolizable energy intake = GEI - methane production - energy in manure.

[6]MEI:GEI = ratio between MEI and GEI.

[7]Energy retention total = MEI - heat production - energy in milk.

[8]Energy retention protein = protein gain (N × 6.25) × 23.6 kJ/g (energetic value of body protein).

[9]Energy retention fat = energy retention total - energy retention protein.

[10]Respiratory quotient.

[11]Fecal N = N intake × [1 – (CP digestibility/100)].

[12]Urine N = N manure – fecal N.

[13]N from condensate collected from heat exchanger + N trapped from outflowing air.

[14]N retention = N intake – N manure – N milk – N condense + acid.

[15]Milk N efficiency = (N milk/N intake) × 100.

Table 5.6. Arterial plasma concentrations of metabolites and insulin in lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

	Treatment[1]							*P*-value[2]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
Glucose, m*M*	3.45	4.00	3.60	3.57	4.02	3.57	0.107	<0.001	0.405	0.926	0.577	0.405
BHB, m*M*	0.65	0.36	0.58	0.71	0.49	0.71	0.050	<0.001	0.365	0.005	0.440	0.365
NEFA, µ*M*	162	62	152	110	65	180	36.5	0.030	0.351	0.620	0.392	0.217
TAG, µ*M*	48	47	87	57	50	95	4.0	0.302	<0.001	0.153	0.533	0.999
LCFA, µ*M*	307	202	412	280	215	465	38.0	0.016	<0.001	0.322	0.550	0.237
Urea, m*M*	2.07[a]	1.87[a]	2.33[ac]	3.10[b]	2.35[ac]	2.83[bc]	0.194	0.004	0.999	0.003	0.081	0.090
Insulin, mIU/L	12.9	17.2	11.4	14.5	18.9	14.4	2.47	0.044	0.678	0.263	0.987	0.732

[a-c]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Low protein (LMP) or high protein (HMP) abomasal infusion treatments: LMP-C, 0.9% saline; LMP-GG, 1319 g/d glucose; LMP-LG, 676 g/d palm olein; HMP-C, 844 g/d of a complete EAA mixture in the same profile and amount as found in 1.5 kg casein; HMP-GG, 1319 g/d glucose + 844 g/d EAA; HMP-LG, 676 g/d palm olein + 844 g/d EAA. Infusions of LMP-GG, LMP-LG, and HMP-C supplied 20.5 MJ/d, and HMP-GG and HMP-LG supplied 41.0 MJ/d.

[2]GG = effect of energy from glucose; LG = effect of energy from fat; AA = effect of protein level.

DISCUSSION

Energy and N Partitioning

This study tested energy and N balance in mid-lactation cows using 5-d abomasal infusions in a Latin square design. The findings of the current study, in particular with regard to energy and N partitioning, could appear to be relevant to the stage of lactation of the animals or to the duration of the abomasal infusions. First, we can assume these mid-lactation cows were not metabolically challenged with regard to energy balance, as the LMP-C infusion (the control treatment) resulted in positive ER and plasma NEFA concentrations indicative of cows in positive energy balance (<200 μ*M*; Adewuyi et al., 2005). Second, very short-term studies (<24 h) in non-ruminants have shown increases in protein synthesis in skeletal muscle during glucose and EAA infusions, as well as during insulin clamps (O'Connor et al., 2003; Wilson et al., 2010), and the cellular signaling cascades regulating these responses have been shown to respond within minutes to altered nutritional or hormonal stimuli. With this in mind, infusion periods of 5 d provide sufficient time to observe mobilization or deposition of energy metabolites and N from body pools to support a physiological response. The responses in milk production, DMI, and energy and N balance in the current study generally agree with other studies where starch was infused postruminally for 2 wk (Reynolds et al., 2001), and where glucogenic or lipogenic diets were fed for 28 d (Boerman et al., 2015) or for the first 9 wk of lactation (van Knegsel et al., 2007a,b), as discussed below.

Glucogenic Infusion. In contrast to our hypothesis, GG did not increase milk production, likely related to the decrease in DMI. Glucose infusion decreased DMI 0.7 kg/d compared with infusions with no GG, but total GEI from the diet plus infusion was not affected and supported the same total milk yield as LMP-C. Similarly, others have achieved equal milk production from control and glucose infusions (1000 to 1500 g/d) when maintaining the same level of total NE_L intake by adjusting DMI allowance (Lemosquet et al., 1997; Hurtaud et al., 1998, 2000). Abomasal infusion of GG increased the proportion of GEI recovered as ME and reduced CH_4 production, which can be attributed to lower diet DMI and minor contribution of hindgut fermentation to total enteric CH4 production (Ellis et al., 2008). In line with our hypothesis, GG increased body ER, associated with a 55 kJ/kg of $BW^{0.75}$ per d decrease in milk energy output compared with non-GG infusions, mainly because of a decrease in milk fat content. Arterial concentrations of glucose and insulin increased with GG, in agreement with several studies postruminally infusing glucose (Lemosquet et al., 1997; Rigout et al., 2002b; Nichols et al., 2016) or starch (Rius et al., 2010a). Through elevated circulating glucose concentration and the associated insulin release, 2 mechanisms may be contributing to the decreased DMI and increased energy balance observed with abomasal glucose infusion into mid-lactation cows. First, elevated insulin and glucose concentrations have been independently linked to the downregulation of hepatic gluconeogenesis, which

under steady-state feeding conditions and continuous glucose infusion may affect homeostatic control of liver tricarboxylic acid cycle intermediates contributing to regulation of glycemia through reduced feed intake (Lomax et al., 1979; Baird et al., 1980; McGuire et al., 1995). Insulin is also linked to the release of satiety-related gut peptides (Relling and Reynolds, 2007). Second, insulin stimulates lipogenesis in non-mammary tissues (Griinari et al., 1997), playing an important regulatory role in energy balance throughout lactation (Bauman and Elliot, 1983). This metabolic response is characterized by decreased plasma concentrations of acetate, BHB, and NEFA and is often observed when glucose absorption is increased through intake of glucogenic substrates or postruminal infusion of glucose (Rigout et al., 2002a; van Knegsel et al., 2007b; Nichols et al., 2016). Through this response, glucogenic diets fed to early and mid-lactation cows stimulate ER in body tissue and reduce milk fat synthesis by repartitioning energy-dense milk fat substrates toward adipose (McGuire et al., 1995; Griinari et al., 1997; van Knegsel et al., 2007a). Taken together, increased insulin concentration, decreased concentrations of BHB and NEFA, increased ER, and decreased milk energy output in response to GG support the findings of others who report positive energy balance and decreased milk energy in early and mid-lactation cows fed glucogenic diets (van Knegsel et al., 2007a,b; Boerman et al., 2015).

In line with reduced milk energy output, milk fat yield and concentration decreased in response to GG in agreement with other studies characterizing the effects of postruminal glucose or starch supply (Lemosquet et al., 1997; Hurtaud et al., 2000; Reynolds et al., 2001). Of milk fat produced, concentrations of ≤16C FA and SFA increased and that of preformed FA decreased with GG, which agrees with the observations of Lemosquet et al. (1997) and the lower intake of preformed LCFA. Reduced body fat mobilization, suggested by lower NEFA and increased ER with GG infusion, would also reduce the proportion of LCFA in milk fat (Hurtaud et al., 1998, 2000).

Nitrogen efficiency has been assessed at varying CP levels in response to altered energy levels incurred through changes in dietary NDF level (Broderick, 2003), supply of glucogenic energy from starch (Rius et al., 2010b; Cantalapiedra-Hijar et al., 2014), and postruminal glucose infusions (Clark et al., 1977). While we observed increased milk N efficiency in response to GG, this was driven by reduced N intake and not by increased milk N output, regardless of protein level of the infusion. We hypothesized that GG would stimulate milk protein yield at the LMP level by improving postabsorptive efficiency of N utilization through reduced splanchnic catabolism of AA, whereas at the HMP level GG would increase N retention. However, neither milk N output nor body N retention were affected by a GG × AA interaction. Nitrogen retention was negative on LMP-GG, but N intake decreased 112 mg/kg of $BW^{0.75}$ per d in response to GG and milk N was not affected. Low or negative N retention at the LMP level is not surprising considering only 75% of MP requirements were met. During

GG infusion, mobilization of endogenous AA from body protein pools may have been necessary to maintain milk protein synthesis during reduced N intake at the LMP level.

It is often hypothesized that extra energy will partition AA toward milk protein synthesis (Clark et al., 1977; Raggio et al., 2006; Rius et al., 2010b), but our results with GG infusion do not support this hypothesis. When combined with infusions of casein or AA mixtures, glucose or glucose precursors stimulate milk protein yield in some studies (Raggio et al., 2006; Rius et al., 2010a), but not in all (Clark et al., 1977; Nichols et al., 2016). However, when circulating AA levels are abundant, exogenous glucose appears to have an effect on AA storage in extra-mammary tissues rather than on the stimulation of milk protein synthesis (Clark et al., 1977; Nichols et al., 2016; Curtis et al., 2018), possibly through the anabolic action of insulin on skeletal muscle (Lobley, 1998) and adipose (Griinari et al., 1997). In agreement with this hypothesis, 13% of N intake was retained on HMP-GG, compared with 6% on HMP-C, with no appreciable change in milk N output (1% of N intake) between treatments. It appears that at the LMP level, GG infusion may support milk protein synthesis by stimulating turnover in labile protein pools, whereas at the HMP level, GG infusion may partition excess AA back into those pools. In support of body N retention, the numerical decrease in urine N output in response to GG at the HMP level (between HMP-C and HMP-GG) is nearly 2-fold the decrease at the LMP level (between LMP-C and LMP-GG), with no appreciable difference in fecal N output. Supplying GG at high MP levels apparently alters N utilization by lowering urinary N excretion, but does not partition extra N into milk. At a similar N intake as was achieved by our HMP level, Reynolds et al. (2001) also observed a decrease in urine N, no change in milk N, and an increase in tissue N retention with duodenal starch infusion compared with water.

The RQ was greater than 1.0 on all infusion treatments, likely resulting from the positive energy balance produced by the basal diet (Kuhla et al., 2015). De novo FA synthesis and ruminal anaerobic fermentation of dietary carbohydrates by ruminants can also result in a RQ larger than 1 (Gerrits et al., 2015). The increase in RQ in response to GG infusion agrees with the effect of GG on ER in body tissue, likely through adipogenesis.

Lipogenic Infusion. Infusion of palm olein decreased DMI 0.4 kg/d compared with infusions with no LG. Feeding supplemental LCFA commonly results in no change or a hypophagic effect where DMI is affected less as the degree of FA saturation increases (Pantoja et al., 1996; Harvatine and Allen, 2006a), and this relationship appears to hold in studies where fat is infused postruminally (Drackley et al., 1992; Bremmer et al., 1998). The hypophagic response to FA absorption in the lower gut may be mediated by gut peptides (Relling and Reynolds, 2007). The decrease in DMI in our experiment is less severe compared with others infusing fat sources to mid-lactation cows in comparable doses and in FA profile similar to palm olein (Bremmer et al., 1998; Drackley et al., 2007). This is possibly due to the feed intake and energy restriction used in the current study, whereas others fed ad libitum and diets formulated to meet energy requirements. Importantly, GEI from the diet plus LG

infusion was not affected, which supported the same total milk yield as LMP-C. Similar to GG, LG infusion increased the proportion of GEI recovered as ME and reduced CH_4 production, but also tended to increase MEI.

Feeding lipogenic diets or infusing fat increases total milk yield in some studies (Cant et al., 1991; Nichols et al., 2018) but not in others (Oldick et al., 1997; Harvatine and Allen, 2006b; Lock et al., 2013). Although LG infusion did not improve milk yield, extra MEI contributed to 61 kJ/kg of $BW^{0.75}$ per d increase in milk energy output compared with non-LG infusion treatments, mainly by an increase in milk fat content, which agrees with our hypothesis. Improved transfer of energy from feed to milk with FA supplementation in mid-lactation has been observed by others (Andrew et al., 1991; Lock et al., 2013; Boerman et al., 2015), and MEI partitioned toward milk energy agrees with previous reports of lipogenic diets partitioning energy into milk instead of body fat compared with glucogenic diets (van Knegsel et al., 2007a; Boerman et al., 2015). Increases in plasma TAG and LCFA confirm absorption of infused LCFA in the lower gut and are in agreement with previous abomasal infusions of saturated LCFA (Drackley et al., 1992; Bremmer et al., 1998) and studies where rumen-inert fat is fed (Hammon et al., 2008; Boerman et al., 2015). No change in plasma NEFA with LG infusion agrees with findings of Bremmer et al. (1998) and Harvatine and Allen (2006b), and suggests TAG hydrolysis or peripheral tissue uptake of FA was not affected by LG infusion. Many have reported positive effects on milk fat yield and concentration when FA supply to the small intestine is increased by feeding rumen-inert fat sources (van Knegsel et al., 2007a; Lock et al., 2013; Nichols et al., 2018). Infusion of LG increased the concentrations of total preformed FA and total MUFA in milk fat, and decreased the concentrations of total de novo, mixed, and saturated FA, which agrees with the apparent transfer of C18:0 and C18:1 *cis*-9 from palm olein infusion. The increase in PUFA and n-6 to n-3 ratio is supported by the transfer of C18:2n-6 from the infusion. Interestingly, C16:0 composed 43% of palm olein, but its concentration in milk fat decreased in response to LG, although the concentration of C16:0 in milk increased. Enjalbert et al. (2000) and Drackley et al. (2007) also reported increased milk C18:1 *cis*-9 content in milk fat at the expense of C16:0 during oleic acid infusion. This effect may be related to inhibition of de novo synthesis of ≤16C FA by C18:1 *cis*-9 through displacement of 14:0 and 16:0 by C18:1 at the *sn*-2 position of glycerol (Hansen and Knudsen, 1987; Loften et al., 2014). In addition, Enjalbert et al. (2000) reported high intestinal digestibility of C18:1 *cis*-9, but increased C16:0 content in feces during oleic acid infusion. Although we observed no effect of LG on manure energy output, if digestibility of C18:1 in the infusion was high and C16:0 digestibility was low, this could contribute to the decreased concentration of C16:0 in milk fat.

We hypothesized that LG would improve milk N efficiency at the LMP and at the HMP level. However, although N intake decreased with LG infusion, LG did not affect milk N efficiency, plasma urea concentration, or any other parameters related to N balance. Andrew

et al. (1991) and Cant et al. (1991) observed no difference in transfer efficiency of N intake into milk N when dietary fat was supplemented at high or low dietary CP protein levels or during AA infusions, respectively. In contrast, Nichols et al. (2018) report increased milk protein yield and increased milk N efficiency when saturated fat was supplemented to low- or high-protein diets achieved through rumen-protected protein supplementation. However, Nichols et al. (2018) reported a tendency for interaction between fat and protein supplementation on milk N efficiency, where the addition of fat numerically increased milk N efficiency to a larger extent at low protein level than at high protein level. In the present experiment, the same numerical trend occurred. At the LMP level, LG increased milk N efficiency 1.8% units, whereas at the HMP level LG produced the same milk N efficiency as HMP-C. Nitrogen intake decreased with LG, but N retention at the HMP level was numerically higher than at the LMP level. This may account for the numerically identical milk N efficiency between HMP-LG and HMP-C, despite lower N intake with LG. In contrast with the comparison between Nichols et al. (2018) (saturated fat) and the current work (mixture of saturated and monounsaturated fat), Bremmer et al. (1998) saw no difference in dietary CP intake or N components of milk between infusion of saturated LCFA and palm oil. The relationship between degree of saturation of postruminal FA supplements and milk N efficiency, with and without AA supplementation, remains poorly characterized.

The RQ decreased in response to LG infusion. Van Gastelen et al. (2017) observed a similar RQ of 1.10 when dairy cow diets were supplemented with linseed oil. Lower RQ during LG infusion, relative to infusions with no LG, suggests increased FA absorption and reduced FA synthesis during intermediary metabolism (Gerrits et al., 2015).

Protein Level. Effects of GG and LG proved to be largely independent of MP level in the current study. Similarly, the HMP level influenced energy and N partitioning independent of GG or LG due to the stimulation of DMI and increased N supply. The effect on DMI detected in the present study in response to AA might more strongly reflect the decrease in DMI when energy was added at the LMP level from GG or LG, because when the same amount of energy was provided with HMP-C, DMI was not numerically different from LMP-C. At the HMP level, DMI increased 0.1 kg/d without GG or LG and 0.9 kg/d with GG or LG. Because diet DMI increased at the HMP level, GEI increased 182 kJ/kg of BW0.75 per d on HMP-C compared with LMP-GG and LMP-LG, even though the infusions were isoenergetic. Gross energy intake was formulated to be highest on HMP-GG and HMP-LG because of the additive infusion doses of GG or LG and AA, which was achieved regardless of the independent effects of GG, LG, and AA on DMI.

Methane production increased at the HMP level from the increased DMI (Ellis et al., 2008), and the proportion of GEI recovered as MEI increased. Greater N supply at the HMP level did not affect total ER, but heat production increased only when GG or LG were added to the HMP level. A portion of this increase in heat production is attributed to rumen

microbial fermentation and O_2 consumption by the PDV and liver, related to greater DMI (Reynolds, 2002; Russell and Strobel, 2005). Supplying N in excess of requirement also produces heat during biological transformations of N molecules, such as AA oxidation, urea synthesis, and N recycling, before excretion (Reed et al., 2017). The form of dietary N affects the amount of energy required for its oxidative metabolism, which was found to be lower with RUP than with RDP per unit supplied (Reed et al., 2017). The increase in N intake between the LMP and HMP level represents an increase in essentially completely RUP (average increase of 13 g of N/d from the diet, which reflects a mixture of RDP and RUP, compared with 122 g of N/d from AA infusion, which is considered RUP). From estimations of Reed et al. (2017) that 3.3 MJ of heat is produced per kg of RUP, the HMP level produced 23 kJ/kg of $BW^{0.75}$ per d of heat from EAA infusion. Of the increase in heat production, 32% could have been achieved by metabolism of infused EAA. Taken together, this suggests the observed increase in heat production when AA is supplied together with GG or LG may have been more related to the overall increase in MEI at the HMP level than to increased catabolism of infused EAA. Lack of effect of AA on total ER agrees with Reed et al. (2017) but contrasts earlier suggestions that casein infusions stimulate milk production in part by releasing energy from adipose tissue stores (Ørskov et al., 1977; Whitelaw et al., 1986). In these studies where negative ER was observed, animals were in an earlier stage of lactation and total GEI was lower than in our study. In common with other reports of postruminal AA supplementation (Clark et al., 1977; Whitelaw et al., 1986; Wright et al., 1998), the HMP level increased milk N output, tended to increase N retention, increased plasma urea concentration, and decreased milk N efficiency. The N retention values reported here are in line with others who supply similar levels of postruminal AA to mid-lactation cows (Clark et al., 1977; Wright et al., 1998; Castillo et al., 2001). In general, milk urea concentration may serve as an on-farm indicator to monitor environmental N emission and milk N efficiency, but the relationship between milk urea and N excretion is variable (reviewed by Spek et al., 2013b). In our experiment, the decrease in milk N efficiency upon infusing EAA was reflected in increased milk urea levels, and milk N efficiency as well as milk urea were not affected by LG infusion. However, the improvement in milk N efficiency upon GG infusion independent of protein level did not coincide with lower milk urea levels, indicating that milk urea concentration is not necessarily a sound indicator of milk N efficiency.

Milk energy output increased at the HMP level, which agrees with the increased yield of milk fat, protein, and lactose when EAA were infused. The contribution of increased DMI to LCFA intake remained small (19 g/d increase in crude fat intake), suggesting intramammary FA synthesis supported the 71 g/d increase in milk fat yield stimulated by the HMP level. In agreement with intramammary FA synthesis, concentration of de novo FA in milk increased, and total 16C FA concentration decreased. Infusions of casein or AA mixtures usually increase milk protein but also lactose yield (Doepel and Lapierre, 2010; Galindo et al.,

2011; Nichols et al., 2016), likely through the effects of AA on whole-body glucose appearance, or through changes in mammary synthetic capacity associated with the stimulatory effect of AA on milk protein synthesis (Lemosquet et al., 2009; Lapierre et al., 2010). Interestingly, lactose content decreased at the HMP level. Depressed lactose content when mammary AA supply is high could be in response to the positive relationship between milk salt and milk casein content (Bijl et al., 2013) and mechanistically linked to the requirement to maintain milk osmolality when AA flux through Na^+-dependent transporters is elevated. An inverse relationship exists between milk lactose concentration and that of Na and K (Peaker, 1983), related to intracellular osmotic pressure, which equilibrates between milk and milk secretory cells in the udder (Shennan and Peaker, 2000). Under conditions of increased AA flux into mammary cells via Na^+-dependent transporters, Na^+/K^+ ATPase activity may be upregulated to balance intracellular Na^+ concentration and facilitate AA influx (McGivan and Pastor-Anglada, 1994). Increased intracellular K^+ concentration may thereby result in reduced lactose content to maintain constant osmotic pressure of milk.

The RQ decreased at the HMP level, which suggests AA were used for energy generation when EAA were infused, and agrees with increased AA catabolism observed in response to high levels of protein supply (Doepel et al., 2004; Lapierre et al., 2006). The average RQ at the HMP level of 1.11 is in line with the value of 1.08 reported by Whitelaw et al. (1986).

Digestibility

Minimal effects on ATTD of DM, OM, and NDF indicates that abomasal infusion of glucose, palm olein, or EAA did not disrupt normal digestive processes in the current study. Decreased ATTD of crude fat in response to GG can be attributed to the reduction in DMI and therefore crude fat intake, resulting in a relatively higher contribution of endogenous fat losses to fecal fat excretion (Kil et al., 2010). In response to LG, ATTD of crude fat increased more than expected based upon an assumed 80% digestibility of palm olein. Assuming crude fat digestibility on LMP-C represents the crude fat digestibility of the basal diet, and that this remains constant, the increase in fat digestibility with LG infusion suggests that digestibility of palm olein may have been higher (84–85%). Indeed, Drackley et al. (2007) and others (Bremmer et al., 1998; Enjalbert et al., 2000) have observed high intestinal digestibility of total LCFA, specifically oleic acid, reaching the small intestine during abomasal infusion of LCFA. Because almost half of palm olein is oleic acid, this could account for the high calculated apparent digestibility of infused fat. However, GE digestibility was not affected by LG, which would be expected if the digestibility of infused fat had an appreciable effect considering the high GE content of palm olein. The HMP level increased ATTD of DM (tendency only) and GE, which can be explained by the greater assumed digestibility of infused EAA (100%) compared with basal diet digestibility, as these effects disappeared when ATTD was calculated considering only inflows from the basal diet (data not shown). Similarly,

increased ATTD of CP at the HMP level can be attributed to high digestibility of the EAA infusion, which was also observed by Wright et al. (1998) when a highly digestible RUP source replaced a portion of their basal diet.

CONCLUSIONS

This study demonstrates that postruminal glucose and palm olein supported milk production and stimulated alterations in whole-body energy and N partitioning which were largely independent of protein-level. Glucose infusion promoted total body ER, reduced milk energy output, and improved milk N efficiency. Palm olein infusion partitioned extra energy intake toward milk fat production at the same level of total milk and protein production, and had no effect on milk N efficiency. Infusing EAA increased milk fat, protein, and lactose production without negatively affecting energy balance, but decreased milk N efficiency. Heat production increased at the high MP level, but only at the additive energy level where glucose or palm olein were infused with EAA. With regard to energy balance, palm olein allowed greater milk energy without affecting ER, whereas glucose promoted ER at low and high MP levels. Glucose infusion increased milk N efficiency at low and high MP levels but did not produce extra milk protein yield. The efficacy of lipogenic substrate on milk N efficiency should be further elucidated.

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Chapter 6

Mammary gland amino acid utilization in dairy cattle is affected more by exogenous glucose than by long-chain fatty acids at low and high metabolizable protein levels

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ABSTRACT. This study investigated mammary gland metabolism in lactating dairy cattle in response to energy from glucogenic (glucose; GG) or lipogenic (palm olein; LG) substrates at low (LMP) and high (HMP) metabolizable protein levels. Six rumen-fistulated, second lactation Holstein-Friesian dairy cows (97 ± 13 d in milk) were abomasally infused with saline (LMP-C), isoenergetic infusions (digestible energy basis) of 1319 g/d glucose (LMP-GG), 676 g/d palm olein (LMP-LG), or 844 g/d essential AA (HMP-C), or isoenergetic infusions of 1319 g/d glucose + 844 g/d essential AA (HMP-GG) or 676 g/d palm olein + 844 g/d essential AA (HMP-LG), according to a 6 × 6 Latin square design. Each experimental period consisted of 5 d of continuous infusion followed by 2 d of rest. A total mixed ration (consisting of 42% corn silage, 31% grass silage, and 27% concentrate on a dry matter basis) was formulated to meet 100 and 83% of net energy and metabolizable protein requirements, respectively, and was fed at 90% of ad libitum intake by individual cow. Arterial and venous blood samples were collected on d 5 of each period. Infusing GG or LG at the HMP level did not affect milk yield or composition differently than at the LMP level. Neither GG nor LG infusion stimulated milk protein or lactose yield, but fat yield tended to decrease with GG and tended to increase with LG. Infusion of GG increased arterial plasma concentrations of glucose and insulin, and decreased concentrations of β-hydroxybutyrate (BHB), non-esterified fatty acids, long-chain fatty acids (LCFA), and total AA, essential AA (EAA), and group 2 AA. Infusion of LG increased arterial triacylglycerides (TAG) and LCFA, and did not affect EAA concentrations. Compared with the LMP level, the HMP level increased arterial concentration of BHB, urea, and of all EAA groups, and decreased that of total non-EAA. Mammary plasma flow increased with GG and was not affected by LG or protein level. Uptake and clearance of total EAA and group 2 AA were affected or tended to be affected by GG × AA interactions, where their uptakes were lower and their clearances were higher with GG but only at the LMP level. Infusion of LG had no effect on uptake or clearance of any AA group. The HMP level increased uptake and decreased clearance of all EAA groups, and decreased non-EAA uptake. Infusion of GG tended to increase mammary glucose uptake, and tended to decrease BHB uptake only at the LMP level. Infusion of LG increased mammary uptake of TAG and LCFA, and increased or tended to increase clearance of TAG and LCFA. We suspect GG increased mammary plasma flow to maintain intramammary energy and AA balance, and stimulated lipogenesis in adipose which accounted for depressed arterial BHB and group 2 AA concentrations. Mammary net glucose uptake did not equilibrate with estimated requirements for milk lactose or fat synthesis, except during LMP-GG infusion. Results of this experiment illustrate flexibility in mammary metabolite utilization when absorptive supply of glucogenic, lipogenic, and aminogenic substrate is increased.

Key words: glucogenic, lipogenic, mammary gland, essential amino acid, milk synthesis

INTRODUCTION

Characterizing the effect of macronutrients on mammary gland metabolism of lactating dairy cows is important to achieve efficient and sustainable nutrient transfer from feed into milk components. Availability of metabolites for mammary uptake is affected by the form and amount of their precursor in the diet, their absorption from the gastrointestinal tract, and by splanchnic metabolism. Lactating mammary glands regulate local blood flow and transmembrane transport to extract substrates for milk component synthesis and to balance cellular ATP requirements when there is a deficiency or excess of energy metabolites (Cant et al., 2003). Postruminal supplementation of glucose or glucogenic substrates typically reduces whole-body AA catabolism and improves capture of MP in milk protein (Raggio et al., 2006a; Rius et al., 2010; Curtis et al., 2018), but produces variable milk protein yield responses (Reynolds et al., 2001; Raggio et al., 2006a; Nichols et al., 2016) and often reduces milk fat yield (Hurtaud et al., 2000; Rigout et al., 2002; Rigout et al., 2003). Hyperinsulinemia caused by elevated circulating glucose concentrations or insulin infusion stimulates milk protein synthesis (Mackle et al., 2000; Bequette et al., 2001; Rulquin et al., 2004); however, this endocrine response may also responsible for reduced arterial concentrations of EAA, acetate, BHB, and non-esterified fatty acids (NEFA) in mid- to late lactation cows when extra-mammary peripheral tissues are insulin sensitive (Mackle et al., 1999; Curtis et al., 2018).

Glucogenic substrates are important to lactating ruminant metabolism since mammary glucose use is high, but net glucose absorption from the gastrointestinal tract is low (Huntington et al., 2006). On the other hand, lipogenic substrates improve the transfer of dietary energy into milk energy (Van Knegsel et al., 2007; Boerman et al., 2015), but their metabolism does not provide a net supply of glucose. Lipogenic diets created through provision of high fibre forages, supplementation of rumen-inert long-chain fatty acids (LCFA), or both, may stimulate total milk, protein, and lactose production to the same or to a greater degree relative to glucogenic diets in early and mid-lactation cows (Van Knegsel et al., 2007; Hammon et al., 2008; Boerman et al., 2015), but lipogenic diets do not initiate the same insulin-stimulated endocrine cascade as glucogenic diets (Palmquist and Jenkins, 1980). Indeed, Van Hoeij et al. (2017) and Nichols et al. (2019a) reported reduced insulin concentrations with high fibre and fat-supplemented diets, respectively. Postruminal fat supplementation has received less attention relative to that of glucose with regard to its effect on mammary metabolite utilization. Early work described mammary metabolite use in response to largely unsaturated fat sources (Casper and Schingoethe, 1989; Cant et al., 1993a,b). Cant et al. (1993a) replaced a portion of cereal grains in the diet with a mix of saturated and unsaturated fats and observed increased mammary uptake of triacylglycerides (TAG) and output of LCFA into milk, higher lactose yield and increased mammary glucose utilization for lactose. In the same study, AA arteriovenous (AV) differences increased but mammary blood flow decreased on high fat diets (Cant et al., 1993b). Arieli et al. (2001)

observed no difference in mammary extraction efficiency of AA with abomasal infusion of corn oil. Recently, Nichols et al. (2019a) reported that supplementing rumen-inert saturated fat did not affect mammary AA metabolism.

In the same experiment as described in the current work, we found that at low and high MP levels, arterial insulin concentration in mid-lactation cows was increased by abomasal infusion of glucose but was not affected by infusion of palm olein (major FA constituents are palmitic, oleic, and linoleic acid; Nichols et al., 2019b). Furthermore, glucose infusion increased energy retention, reduced milk energy output, and improved milk N efficiency, whereas palm olein infusion increased milk energy output and had no effect on energy retention and milk N efficiency, and EAA infusion increased milk energy output and decreased milk N efficiency without affecting energy retention. In the current study we measured mammary gland metabolite utilization when glucose or palm olein are supplied at low and high MP levels. We hypothesized that the metabolic cascades induced by glucose or palm olein infusion, specifically with regards to insulin signalling, would differently stimulate mammary gland mechanisms to sequester AA and other metabolites. We expected the high MP level, achieved through EAA infusion, to increase mammary gland uptake of EAA, but also to increase whole-body and intramammary AA catabolism.

MATERIALS AND METHODS

Experimental Design

All experimental procedures were conducted under the Dutch Law on Animal Experiments in accordance with EU Directive 2010/63. The experimental design, animal housing, ration composition and preparation, and feed chemical analyses have been described in detail by Nichols et al. (2019b). Briefly, effects of energy from glucogenic (GG) or lipogenic (LG) substrates at low (LMP) and high (HMP) MP levels were tested using 6 rumen-fistulated, second lactation Holstein-Friesian dairy cows (97 ± 13 DIM) randomly assigned to a 6 × 6 Latin square design in which each experimental period consisted of 5 d of continuous abomasal infusion followed by 2 d of no infusion. Cows were housed individually in identical climate respiration chambers (CRC; described in detail by van Gastelen et al. (2015)), and were allowed 5 d of adaptation to the CRC environment before the first experimental period began. Cows were fed a TMR (14% CP) consisting of 42% corn silage, 31% grass silage, and 27% concentrate on a DM basis which was formulated to meet 100 and 83% of NEL and MP requirements (CVB, 2008), respectively, for cows consuming 20 kg DM/d and producing 30 kg/d of milk containing 40 g/kg fat and 34 g/kg protein. Daily feed intake for individual cows was restricted to 90% of individual daily ad libitum intake determined during a 10-d diet adaptation period in tie stalls before cows entered the CRC. Fresh feed was allocated twice daily during the entire experiment, with the exception of a 34-h window over d 4 and 5 of each period (from 0530 h on d 4 until 1530 h on d 5), where an automated

feeding system dispensed equal portions of feed every 2 h to promote metabolic steady-state conditions in preparation for the blood sampling protocol described below.

Infusion lines were placed in the abomasum via the rumen cannula 7 d before the first experimental period and were checked daily for patency and position. Abomasal infusion treatments were 1) 0.9% saline (**LMP-C**; 90% NE_L, 75% MP), 2) 1319 g/d glucose (**LMP-GG**; 100% NE_L, 75% MP), 3) 676 g/d palm olein (**LMP-LG**; 100% NE_L, 75% MP), 4) 844 g/d EAA (**HMP-C**; 100% NE_L, 120% MP), 5) 1319 g/d glucose + 844 g/d EAA (**HMP-GG**; 110% NE_L, 120% MP), and 6) 676 g/d palm olein + 844 g/d EAA (**HMP-LG**; 110% NE_L, 120% MP), where MP and NE_L reflect the proportion of requirements met by the restricted feeding level of the diet plus the infusate. Treatment solutions were administered in 10-L batches which were replenished daily and infused continuously via multi-channel peristaltic pumps at a rate of 6.95 mL/min to facilitate 120-h of continuous infusion. Daily infusion dosages (g/d) of LMP-GG, LMP-LG, and HMP-C were designed to be isoenergetic based on the gross energy content of the EAA infusion, which was calculated to be 24.3 MJ/kg based on the heat of combustion of individual EAA in the infusate, and assuming digestibility of palm olein, EAA and glucose to be 80%, 100%, and 100%, respectively. The EAA infusions delivered EAA in the same profile and amount as found in 1.5 kg casein according to Metcalf et al. (1996). Composition of the EAA mixture and palm olein FA composition are described by Nichols et al. (2019b).

Milk and Blood Collection and Analysis

Cows were milked twice daily at 0530 and 1530 h. Milk was collected, weighed, and sampled separately at each milking. Samples were stored at 4°C and analyzed within 4 d for protein, fat, lactose, and urea by mid-infrared spectroscopy (ISO 9622; ISO, 2013; VVB, Doetinchem, the Netherlands). At 0730, 0930, 1130, 1330, and 1530 h on d 5 of infusion, blood samples were collected into 10 mL sodium heparin and potassium EDTA Vacutainers (Becton Dickinson, Rutherford, NJ) from the coccygeal vessels and from the subcutaneous abdominal vein of each cow. Arteriovenous differences across the tail are assumed to be negligible and thus samples from the coccygeal vessels are representative of mammary arterial supply (Emery et al., 1965). Samples were collected from the left and right subcutaneous abdominal veins, alternating at each time point, to account for differences between sides. Collection tubes were immediately placed in ice and centrifuged at 3,000 × *g* for 15 min at room temperature. Plasma from each time point was collected and stored at -80°C pending analysis of AA, peptides, and AA metabolites. Plasma for analysis of other metabolites and insulin was pooled over sampling time points by cow and period and stored at -20°C until analysis. Plasma AA, peptides, and AA metabolite concentrations were determined using an ultra-performance liquid chromatography-mass spectrometry system (Waters Acquity Ultra Performance LC system, Waters Corp.) as described by Haque et al. (2012). Plasma concentrations of glucose, BHB, NEFA, TAG, urea, and insulin were analysed

by the Veterinary Diagnostic Laboratory (Utrecht University, the Netherlands) as described by van Knegsel et al. (2007).

Calculations and Statistical Analysis

Plasma concentrations of AA, peptides, and AA metabolites were averaged over the 5 sampling times. Milk crude protein was assumed to consist of 94.5% true protein (DePeters and Ferguson, 1992). All following calculations were based on this estimate of true protein yield. Long-chain fatty acid concentrations were calculated on a molar basis as 3 × TAG + NEFA. Mammary plasma flow (MPF) across the whole udder was estimated according to the Fick principle using Phe and Tyr as internal markers (Cant et al., 1993b), where MPF (L/h) = [milk Phe + Tyr output (μmol/h)] / [AV Phe + Tyr difference (μmol/L)], with an allowance for 3.37% contribution of blood-derived proteins to milk Phe + Tyr (Lapierre et al., 2012). Milk output of Phe + Tyr was estimated from the afternoon milk protein yield of d 5, corresponding to the blood samples taken that day, using mean Phe and Tyr contents reported by Mepham (1987) and Lapierre et al. (2012). Uptakes (mmol/h) of metabolites across the mammary glands were calculated as the product of their plasma AV differences and MPF. Positive uptakes indicate a net removal from plasma, whereas negative values indicate net release from the mammary glands. Mammary metabolite clearances were calculated from the model of Hanigan et al. (1998), where clearance (L/h) = (AV difference × MPF)/venous concentration. Mean milk protein AA composition reported by Mepham (1987) and Lapierre et al. (2012) was used to calculate AA uptake to output ratios (U:O). Mammary gland balance for glucose and LCFA were calculated according to estimations of Dijkstra et al. (1996) for glucose and LCFA, using milk protein, lactose, and fat yield from the afternoon milking on d 5 of infusion. The molecular weight of FA ≥16C in blood was calculated according to the molecular weight of ≥16C FA in milk (Nichols et al., 2019b), assuming that 50% of milk C16 originated from C16 FA in blood sequestered as preformed FA, and that 50% of milk C16 was synthesized de novo.

Variances in milk and milk component production, plasma constituent concentrations and AV differences, MPF, mammary metabolite uptakes and clearances, mammary metabolite uptake to output ratios, and mammary metabolite balances were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The model contained main and interaction effects of infusion treatment factors (GG, LG, and AA) and period as fixed effects and cow as a random effect. We observed no carryover effects between periods, assessed by testing for an effect of the previous treatment in the ANOVA. Differences were considered significant at $P \leq 0.05$ and tendencies were acknowledged at $0.05 < P \leq 0.10$. Multiple comparisons between treatment means were made using the Tukey-Kramer method when GG × AA or LG × AA interactions were detected at $P \leq 0.10$.

RESULTS

Milk Production

Daily lactation performance and DMI have been presented by Nichols et al. (2019b). The present paper reports milk production expressed on an hourly basis from the afternoon milking on d 5 of infusion (**Table 6.1**). Infusing GG or LG at the HMP level did not affect milk production or composition differently than at the LMP level (no GG × AA or LG × AA interactions; $P > 0.10$). Total milk yield was unaffected by GG and LG ($P > 0.64$) and increased at the HMP level ($P < 0.01$). Milk protein and lactose yield were unaffected by GG and LG ($P > 0.23$) and increased at the HMP level ($P < 0.01$). Infusion of GG and LG had no effect on protein or lactose content ($P > 0.11$), and the HMP level increased milk protein content ($P < 0.01$) and decreased lactose content ($P < 0.01$). Milk fat yield tended to decrease in response to GG ($P = 0.10$), tended to increase in response to LG ($P = 0.09$), and increased at the HMP level ($P < 0.01$). Milk fat content tended to decrease in response to GG ($P = 0.06$), increased in response to LG ($P = 0.02$), and was not affected by MP level ($P = 0.46$). Milk urea content tended to decrease in response to GG ($P = 0.06$), was not affected by LG ($P = 0.95$), and increased at the HMP level ($P = 0.01$).

Table 6.1. Milk and component production of lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d[1]

	Treatment[2]							*P*-value[3]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
Milk, kg/h	1.08	1.04	1.05	1.26	1.30	1.27	0.070	0.85	0.65	<0.01	0.16	0.47
Protein, g/h	34.3	32.2	32.2	43.1	44.6	42.6	2.20	0.79	0.24	<0.01	0.11	0.49
Protein, g/kg	31.9	31.2	30.8	34.2	34.4	33.5	0.61	0.65	0.12	<0.01	0.39	0.65
Fat, g/h	50.0	42.6	53.4	55.5	53.8	60.8	3.14	0.10	0.09	<0.01	0.29	0.76
Fat, g/kg	46.0	41.2	51.3	44.0	41.5	48.1	1.84	0.06	0.02	0.46	0.52	0.77
Lactose, g/h	51.0	49.6	49.6	57.4	58.9	57.9	3.28	0.96	0.77	<0.01	0.35	0.53
Lactose, g/kg	47.0	47.9	47.1	45.5	45.4	45.5	0.34	0.25	0.86	<0.01	0.19	0.98
Urea, mg/dL	9.5	8.8	9.3	15.3	10.3	15.5	1.94	0.06	0.95	0.01	0.14	0.91

[1]Data are least squares means from the afternoon milking on d 5 of infusion.

[2]Low protein (LMP) or high protein (HMP) abomasal infusion treatments: LMP-C, 0.9% saline; LMP-GG, 1319 g/d glucose; LMP-LG, 676 g/d palm olein; HMP-C, 844 g/d of a complete EAA mixture in the same profile and amount as found in 1.5 kg casein; HMP-GG, 1319 g/d glucose + 844 g/d EAA; HMP-LG, 676 g/d palm olein + 844 g/d EAA. Infusions of LMP-GG, LMP-LG, and HMP-C supplied 20.5 MJ/d, and HMP-GG and HMP-LG supplied 41.0 MJ/d.

[3]GG = effect of energy from glucose; LG = effect of energy from fat; AA = effect of protein level.

Arterial Metabolite and Insulin Concentrations and Metabolite AV Differences

Irrespective of MP level, arterial plasma concentration of glucose and insulin increased in response to GG ($P \leq 0.04$; **Table 6.2**) and concentration of BHB, NEFA, and LCFA decreased ($P \leq 0.03$). Plasma urea tended to be affected by a GG × AA interaction, where its concentration decreased with GG but only at the HMP level ($P = 0.08$). Regardless of MP level, infusion of GG decreased arterial plasma concentration of total EAA, group 2 AA, and BCAA ($P < 0.01$). Group 1 AA were not affected by GG ($P > 0.45$). A GG × AA interaction affected Leu concentration ($P = 0.05$), where it decreased with GG but only at the HMP level. Infusion of GG decreased Arg, Ile, Lys, and Val concentrations ($P < 0.01$), regardless of MP level. Infusion of GG did not affect arterial concentration of total NEAA. Individually, a GG × AA interaction affected Ala, Asp, and Glu ($P \leq 0.04$), where GG infusion decreased Ala concentration at the LMP level, and GG infusion resulted in numerically lower Asp and Glu at the LMP level compared with the HMP level. Irrespective of MP level, infusion of GG increased Gly and Ser ($P \leq 0.01$), decreased Orn ($P < 0.01$), and tended to decrease Cit and Pro ($P \leq 0.08$). Infusion of GG decreased carnosine concentration but only at the LMP level (GG × AA interaction; $P = 0.03$), and tended to decrease concentration of α-amino-n-butyric acid ($P = 0.09$) and α-amino-adipic acid ($P = 0.10$) irrespective of MP level.

Infusion of LG increased arterial plasma concentration of TAG and LCFA ($P < 0.01$) irrespective of MP level. Plasma urea concentration was numerically lower at the LMP level and numerically higher at the HMP level with LG infusion (tendency for LG × AA interaction; $P = 0.09$). Infusion of LG did not affect plasma concentration of any AA group or of any individual EAA ($P > 0.39$). A LG × AA interaction tended to affect Pro concentration, where it decreased only at the HMP level ($P = 0.09$). Infusion of LG tended to decrease concentrations of Asn (P = 0.06) and Tyr (P = 0.07). Concentration of 1 methyl-histidine (1M-His) was higher with LG infusion at the LMP level compared with the HMP level (LG × AA interaction; $P = 0.05$).

The HMP level increased arterial plasma concentrations of BHB, urea (dependent on GG and LG level), and concentrations of all EAA groups ($P \leq 0.01$). All individual EAA increased at the HMP level ($P < 0.01$). The HMP level decreased total NEAA concentration ($P < 0.01$), and individual concentrations of Ala (dependent on GG level), Asn, Gln, Gly, Pro (dependent on LG level), and Ser ($P \leq 0.05$). The HMP level increased Cit, Orn, and Tyr ($P \leq 0.01$), tended to increase Cys ($P = 0.08$). The HMP level increased the concentration of α-amino-n-butyric acid, α-amino-adipic acid, carnosine (dependent on GG level), cystathionine, and phosphoserine ($P \leq 0.01$), and decreased 1M-His (dependent on LG level), 3 methyl-histidine (3M-His), and hydroxyproline ($P \leq 0.04$).

Regardless of MP level, GG decreased the AV difference of BHB and TAG ($P < 0.01$), and tended to decreased that of LCFA ($P = 0.07$; **Table 6.3**). The AV differences of all EAA groups were affected by a GG × AA interaction where they decreased with GG only at the HMP level

($P \leq 0.05$). Individually, this interaction affected Met, Phe, Thr, Trp and Val ($P \leq 0.05$), and tended to affect His ($P = 0.09$), Ile ($P = 0.10$), and Leu ($P = 0.08$). Infusion of GG decreased AV differences of Arg and Lys, irrespective of MP level ($P < 0.01$). The AV difference of total NEAA tended to be affected by a GG × AA interaction where it decreased with GG only at the HMP level ($P = 0.06$). Individually, this interaction affected Cit, Gln, Gly, and Pro ($P \leq 0.05$), and tended to affect Ser ($P = 0.07$) and Tyr ($P = 0.09$). The AV difference of Glu was numerically decreased at the LMP level and increased at the HMP level (tendency for GG × AA interaction; $P = 0.06$). Regardless of MP level, GG decreased the AV differences of Asn and Orn ($P \leq 0.02$). Regardless of MP level, LG increased the AV difference of TAG and LCFA ($P < 0.01$). Infusion of LG did not affect the AV difference of any AA group or individual AA ($P > 0.10$), except that of His which tended to decrease ($P = 0.07$). The HMP level increased the AV difference of BHB ($P < 0.01$). The AV differences of all EAA groups increased at the HMP level (all dependent on GG level; $P \leq 0.01$). Individually, the AV differences of all EAA except Trp increased at the HMP level ($P \leq 0.03$), where the increases for His, Ile, Leu, Met, Phe, Thr, and Val depended on GG level. The AV difference of total NEAA was not affected by protein level ($P = 0.14$). Individually, the AV differences of Ala and Pro decreased (for Pro dependent on GG level; $P \leq 0.02$), and the AV difference of Gly tended to decrease (dependent on GG level; $P = 0.07$) at the HMP level.

Table 6.2. Arterial plasma concentrations of metabolites and insulin in lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

	Treatment[1]							*P*-value[2]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
Glucose, m*M*	3.45	4.00	3.60	3.57	4.02	3.57	0.107	<0.01	0.41	0.92	0.58	0.41
BHB, m*M*	0.65	0.36	0.58	0.71	0.49	0.71	0.050	<0.01	0.37	<0.01	0.44	0.37
NEFA, µ*M*	162	62	152	110	65	180	36.5	0.03	0.35	0.62	0.39	0.22
TAG, µ*M*	48	47	87	57	50	95	4.0	0.30	<0.01	0.15	0.53	0.99
LCFA, µ*M*	307	202	412	280	215	465	38.0	0.02	<0.01	0.32	0.55	0.24
Urea, m*M*	2.07[a]	1.87[a]	2.33[ac]	3.10[b]	2.35[ac]	2.83[bc]	0.194	<0.01	0.99	<0.01	0.08	0.09
Insulin, µIU/L	12.9	17.2	11.4	14.5	18.9	14.4	2.47	0.04	0.68	0.26	0.99	0.73
Amino acids, µ*M*												
EAA[3]	843	566	864	2036	1713	2007	112.8	<0.01	0.98	<0.01	0.53	0.84
Group 1[4]	182	161	174	463	459	438	26.4	0.46	0.57	<0.01	0.83	0.84
Group 2[5]	710	449	733	1634	1319	1622	93.8	<0.01	0.91	<0.01	0.45	0.85
BCAA[6]	448	251	480	1106	735	1139	70.1	<0.01	0.82	<0.01	0.14	0.79
NEAA[7]	1599	1570	1676	1292	1407	1143	84.4	0.42	0.69	<0.01	0.22	0.13
TAA[8]	2442	2136	2540	3328	3120	3151	161.2	0.05	0.86	<0.01	0.86	0.35
Arg	76	51	78	131	112	122	6.0	<0.01	0.41	<0.01	0.61	0.27
His	22	15	19	95	103	85	5.2	0.96	0.40	<0.01	0.22	0.73
Ile	130	66	141	231	148	231	13.9	<0.01	0.98	<0.01	0.45	0.48
Leu	110[a]	64[a]	117[ac]	312[b]	200[c]	317[b]	21.2	<0.01	0.93	<0.01	0.05	0.77
Lys	62	39	66	158	138	149	9.5	<0.01	0.60	<0.01	0.87	0.33
Met	21	17	21	95	99	92	5.5	0.81	0.95	<0.01	0.57	0.95
Phe	51	46	52	158	141	158	11.1	0.13	0.91	<0.01	0.34	0.96
Thr	123	108	110	239	334	212	26.2	0.33	0.97	<0.01	0.13	0.64
Trp	40	39	39	55	52	50	4.0	0.41	0.41	<0.01	0.63	0.50
Val	208	121	223	564	387	591	36.6	<0.01	0.71	<0.01	0.14	0.95
Ala	244[ab]	206[b]	273[a]	201[b]	228[ab]	196[b]	17.1	0.83	0.26	0.03	<0.01	0.14
Asn	57	47	55	50	49	40	3.7	0.14	0.06	0.05	0.11	0.21
Asp	10.5	6.4	10.4	7.5	8.5	7.1	1.44	0.22	0.88	0.67	0.04	0.96

Table 6.2 (continued). Arterial plasma concentrations of metabolites and insulin in lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

	Treatment[1]							*P*-value[2]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
Cit	75	63	75	97	86	97	11.6	0.07	0.88	<0.01	0.92	0.85
Cys	7.5	5.0	4.9	9.5	9.2	7.8	1.76	0.47	0.30	0.08	0.62	0.76
Gln	395	359	393	270	272	220	29.1	0.62	0.25	<0.01	0.31	0.30
Glu	54^{a}	45^{ab}	54^{a}	45^{ab}	49^{ab}	42^{b}	2.9	0.57	0.65	0.27	0.03	0.65
Gly	470	566	526	329	407	297	29.8	<0.01	0.56	<0.01	0.89	0.17
Orn	34	22	32	57	47	48	3.6	<0.01	0.42	<0.01	0.78	0.18
Pro	90^{a}	75^{ab}	91^{a}	78^{ab}	76^{ab}	64^{b}	6.0	0.08	0.15	0.01	0.11	0.09
Ser	114	132	120	90	111	73	5.8	0.01	0.74	<0.01	0.88	0.20
Tyr	49	44	43	60	65	52	3.8	0.89	0.07	<0.01	0.13	0.83
Other AA, peptides, and AA metabolites,[9] μ*M*												
1 Methyl-histidine	4.2^{ab}	4.5^{a}	5.2^{a}	3.3^{b}	3.4^{b}	3.2^{b}	0.36	0.55	0.09	<0.01	0.72	0.05
3 Methyl-histidine	5.4	4.8	5.8	4.8	4.1	4.1	0.39	0.15	0.59	0.01	0.93	0.13
α-Amino-*n*-butyric acid	29	18	27	46	40	45	6.0	0.09	0.74	<0.01	0.67	0.97
α-Amino-adipic acid	3.1	2.0	3.2	6.3	5.9	6.9	0.43	0.10	0.54	<0.01	0.53	0.71
β-Alanine	3.9	4.8	3.4	4.3	4.6	3.4	0.55	0.31	0.31	0.99	0.66	0.83
Carnosine	13^{abc}	10^{c}	11^{bc}	16^{ab}	17^{a}	15^{ab}	1.5	0.42	0.13	<0.01	0.03	0.69
Cystathionine	1.9	1.4	1.6	3.2	3.2	3.0	0.24	0.19	0.11	<0.01	0.14	0.96
Hydroxylysine	0.8	1.1	1.0	0.7	0.8	0.8	0.34	0.41	0.62	0.36	0.66	0.75
Hydroxyproline	15	16	15	12	15	11	1.1	0.17	0.59	0.04	0.38	0.68
Phosphoserine	2.3	1.8	2.3	3.4	4.7	3.5	0.67	0.82	0.42	0.01	0.60	0.44
Taurine	32	33	30	39	39	28	3.9	0.93	0.13	0.55	0.99	0.32

[a-c]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Low protein (LMP) or high protein (HMP) abomasal infusion treatments: LMP-C, 0.9% saline; LMP-GG, 1319 g/d glucose; LMP-LG, 676 g/d palm olein; HMP-C, 844 g/d of a complete EAA mixture in the same profile and amount as found in 1.5 kg casein; HMP-GG, 1319 g/d glucose + 844 g/d EAA; HMP-LG, 676 g/d palm olein + 844 g/d EAA. Infusions of LMP-GG, LMP-LG, and HMP-C supplied 20.5 MJ/d, and HMP-GG and HMP-LG supplied 41.0 MJ/d.

[2]GG = effect of energy from glucose; LG = effect of energy from fat; AA = effect of protein level.

Table 6.2 (continued). Arterial plasma concentrations of metabolites and insulin in lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

[3]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

[4]Group 1 = His, Met, Phe + Tyr, Trp.

[5]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.

[6]BCAA = Ile, Leu, Val.

[7]NEAA = Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Orn, Pro, Ser, Tyr.

[8]TAA = EAA + NEAA.

[9]Other N derivatives measured with ultra-performance liquid chromatography-mass spectrometry showing a plasma concentration higher than the limit of quantification.

Table 6.3. Mammary gland arteriovenous differences of metabolites in lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

	Treatment[1]							*P*-value[2]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
Glucose, m*M*	0.75	0.73	0.80	0.83	0.80	0.73	0.065	0.78	0.78	0.99	0.85	0.27
BHB, m*M*	0.20	0.10	0.16	0.27	0.18	0.25	0.023	<0.01	0.18	<0.01	0.87	0.77
NEFA, µ*M*	12	17	-2	23	12	40	20.5	0.87	0.93	0.36	0.68	0.45
TAG, µ*M*	30	22	62	37	20	68	3.7	<0.01	<0.01	0.46	0.22	0.99
LCFA, µ*M*	102	82	183	133	72	245	21.7	0.07	<0.01	0.24	0.34	0.49
Amino acids, µ*M*												
EAA[3]	304^{a}	239^{a}	303^{a}	607^{b}	394^{ac}	520^{bc}	44.9	<0.01	0.22	<0.01	0.03	0.24
Group 1[4]	73^{ab}	68^{b}	70^{b}	144^{c}	90^{ab}	113^{ac}	10.3	0.01	0.11	0.01	0.02	0.17
Group 2[5]	252^{a}	190^{a}	252^{a}	493^{b}	324^{ac}	431^{bc}	37.9	<0.01	0.29	<0.01	0.05	0.28
BCAA[6]	147^{a}	106^{a}	145^{a}	315^{b}	193^{ac}	269^{bc}	27.0	<0.01	0.24	<0.01	0.05	0.29
NEAA[7]	252	286	287	311	205	266	40.5	0.29	0.90	0.14	0.06	0.28
TAA[8]	556^{a}	525^{a}	590^{a}	918^{b}	599^{a}	786^{ab}	76.0	0.01	0.44	0.08	0.02	0.19
Arg	35	28	36	53	39	49	3.5	<0.01	0.47	<0.01	0.17	0.42
His	14^{ab}	10^{b}	12^{ab}	28^{c}	17^{ab}	20^{ac}	2.2	<0.01	0.07	0.01	0.09	0.18
Ile	41	28	39	78	51	68	6.2	<0.01	0.15	<0.01	0.10	0.35
Leu	55^{ab}	39^{b}	54^{ab}	114^{c}	75^{ad}	99^{cd}	8.6	<0.01	0.23	<0.01	0.08	0.30
Lys	39	26	41	69	55	66	4.9	<0.01	0.83	<0.01	0.72	0.33
Met	13^{ab}	10^{b}	13^{ab}	27^{c}	15^{ab}	21^{ac}	2.2	<0.01	0.17	0.01	0.05	0.18
Phe	21^{a}	21^{a}	21^{a}	49^{b}	31^{ac}	39^{bc}	3.4	0.01	0.16	<0.01	0.02	0.21
Thr	31^{a}	30^{a}	31^{a}	56^{b}	37^{ab}	48^{ab}	5.5	0.02	0.58	0.03	0.03	0.55
Trp	5	9	5	11	7	8	1.5	0.91	0.39	0.85	0.02	0.43
Val	51^{ab}	39^{b}	52^{ab}	123^{c}	67^{ab}	101^{ac}	12.9	<0.01	0.32	<0.01	0.04	0.29
Ala	29	41	37	18	11	19	9.4	0.88	0.55	<0.01	0.22	0.70
Asn	14	12	13	19	13	17	1.9	0.02	0.38	0.29	0.15	0.43
Asp	0.5	0.2	0.8	0.2	1.0	0.9	0.55	0.82	0.26	0.40	0.44	0.63
Cit	4	8	5	11	3	8	2.6	0.17	0.64	0.42	0.01	0.42
Cys	0.7	1.3	0.6	1.6	0.7	1.2	0.52	0.85	0.48	0.99	0.18	0.65

Table 6.3 (continued). Mammary gland arteriovenous differences of metabolites in lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

	Treatment[1]							*P*-value[2]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
Gln	76	77	84	101	62	81	12.0	0.04	0.54	0.29	0.04	0.18
Glu	37	31	37	32	36	30	3.0	0.77	0.69	0.72	0.06	0.72
Gly	26	49	35	39	19	29	10.3	0.99	0.95	0.07	0.04	0.38
Orn	17	12	17	20	14	19	2.0	<0.01	0.52	0.33	0.90	0.58
Pro	15	16	16	16	9	13	2.4	0.12	0.65	0.02	0.05	0.30
Ser	12	21	22	24	18	25	4.2	0.72	0.18	0.93	0.07	0.26
Tyr	20[a]	19[a]	20[a]	30[b]	21[a]	24[ab]	2.5	0.02	0.11	0.21	0.09	0.19

[a-d]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Low protein (LMP) or high protein (HMP) abomasal infusion treatments: LMP-C, 0.9% saline; LMP-GG, 1319 g/d glucose; LMP-LG, 676 g/d palm olein; HMP-C, 844 g/d of a complete EAA mixture in the same profile and amount as found in 1.5 kg casein; HMP-GG, 1319 g/d glucose + 844 g/d EAA; HMP-LG, 676 g/d palm olein + 844 g/d EAA. Infusions of LMP-GG, LMP-LG, and HMP-C supplied 20.5 MJ/d, and HMP-GG and HMP-LG supplied 41.0 MJ/d.

[2]GG = effect of energy from glucose; LG = effect of energy from fat; AA = effect of protein level.

[3]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

[4]Group 1 = His, Met, Phe + Tyr, Trp.

[5]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.

[6]BCAA = Ile, Leu, Val.

[7]NEAA = Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Orn, Pro, Ser, Tyr.

[8]TAA = EAA + NEAA.

Mammary Plasma Flow and Metabolite Kinetics

Irrespective of MP level, infusion of GG increased MPF ($P = 0.02$; **Table 6.4**), and tended to increase mammary net glucose uptake ($P = 0.07$; **Table 6.4**). A GG × AA interaction tended to affect BHB uptake where it was numerically lower with GG only at the LMP level ($P = 0.10$). A GG × AA interaction tended to affect uptake of total EAA ($P = 0.09$) and group 2 AA ($P = 0.08$) where their uptake was numerically lower at the LMP level and not affected at the HMP level. Regardless of MP level, infusion of GG did not affect mammary gland net uptake of group 1 AA or BCAA ($P > 0.12$). Individually, a GG × AA interaction affected Arg and Lys ($P \leq 0.03$), and tended to affect Ile and Leu ($P \leq 0.08$), where their uptake was decreased with GG at the LMP level. Uptake of Trp was higher with GG at the LMP level (GG × AA interaction; $P = 0.03$). Irrespective of MP level, infusion of GG decreased uptake of His and Met ($P = 0.05$). Mammary net uptake of total NEAA was not affected by GG ($P = 0.48$). A GG × AA interaction affected uptake of Glu, Orn, and Tyr, where Glu uptake increased with GG only at the HMP level ($P = 0.02$), Orn uptake decreased with GG only at the LMP level ($P = 0.04$), and Tyr uptake was numerically higher with GG at the HMP level ($P = 0.03$). A GG × AA interaction tended to affect uptake of Cit which was numerically lower at the HMP level ($P = 0.07$).

Irrespective of MP level, LG infusion increased mammary net uptake of TAG and LCFA ($P < 0.01$), and had no effect on uptake of any AA group or individual AA, except Ser uptake which tended to increase ($P = 0.07$). Mammary net uptake of BHB was affected by MP level, but only in the presence of GG ($P = 0.02$). The HMP level increased mammary uptake of all EAA groups ($P < 0.01$). The HMP level increased individual uptake of all EAA ($P < 0.01$), with the exception of Trp which was unaffected, and where uptakes of Arg and Lys were dependent on GG level. Mammary net uptake of total NEAA decreased at the HMP level ($P = 0.03$). Individually, the HMP level increased Tyr uptake ($P = 0.01$; dependent on GG), tended to increase Asn uptake ($P = 0.08$), and decreased uptake of Ala, Gly, and Pro ($P \leq 0.04$).

Infusion of GG had no effect on mammary clearance of glucose, BHB, NEFA, TAG, or LCFA, irrespective of MP level ($P \geq 0.11$; **Table 6.5**). Total EAA clearance was affected ($P = 0.04$) and group 2 AA clearance tended to be affected ($P = 0.10$) by a GG × AA interaction, where it increased in response to GG only at the LMP level. Clearance of total BCAA increased in response to GG regardless of MP level ($P < 0.01$). Individually, a GG × AA interaction affected Leu and Trp ($P \leq 0.05$), and tended to affect Arg and Val ($P \leq 0.08$), where clearances increased in response to GG only at the LMP level. Clearance of Ile increased ($P < 0.01$) and clearance of Lys tended to increase ($P = 0.06$) in response to GG, regardless of MP level. Total NEAA clearance was unaffected by GG ($P = 0.92$). Individually, a GG × AA interaction affected Cit ($P = 0.05$) and tended to affect Cys ($P = 0.07$), where their clearances increased in response to GG only at the LMP level. Infusion of GG increased Glu clearance regardless of MP level ($P = 0.04$).

Infusion of LG increased clearance of TAG ($P = 0.01$) and tended to increase clearance of LCFA ($P = 0.06$), regardless of MP level. Irrespective of MP level, LG did not affect clearance of any AA group or of any individual EAA. A LG × AA interaction affected Asp ($P = 0.04$) and tended to affect Asn ($P = 0.06$) where clearances increased with LG only at the HMP level. Clearance of Ser increased in response to LG regardless of MP level ($P < 0.01$). The HMP level tended to increase mammary clearance of BHB ($P = 0.10$). Clearance of all EAA groups decreased at the HMP level ($P < 0.01$). Clearance of total EAA was affected by a GG × AA interaction ($P = 0.04$) where it decreased more at the HMP level relative to the LMP level in the presence of GG. Clearances of all individual EAA decreased at the HMP level ($P \leq 0.05$), except clearances of Trp and Val which were affected by MP level in the presence of GG ($P \leq 0.07$), and Arg and Leu which decreased more at the HMP level relative to the LMP level in the presence of GG ($P \leq 0.08$). Total NEAA clearance was unaffected by MP level ($P = 0.41$). Individually, the HMP level increased clearance of Asn (dependent on LG), Gln, and Ser ($P < 0.01$), tended to increase clearance of Asp ($P = 0.08$; dependent on LG), and decreased clearance of Ala, Orn, and Pro ($P \leq 0.05$).

Table 6.4. Whole-mammary gland plasma flow and net uptakes of metabolites in lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

	Treatment[1]							*P*-value[2]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
Plasma flow, L/h	489	520	468	337	507	394	56.0	0.02	0.66	0.31	0.11	0.35
Net mammary uptake, mmol/h												
Glucose	366.9	369.6	370.6	276.3	411.4	292.9	43.51	0.07	0.68	0.64	0.14	0.99
BHB	90.8	51.7	75.0	87.3	87.3	94.2	10.55	0.10	0.70	0.02	0.10	0.33
NEFA	8.3	8.2	-3.9	11.9	7.6	12.1	9.84	0.84	0.51	0.39	0.85	0.50
TAG	14.4	10.8	28.8	12.1	12.4	27.1	2.65	0.49	<0.01	0.96	0.37	0.88
LCFA	51.6	40.4	82.4	48.3	45.2	93.5	10.58	0.42	<0.01	0.29	0.56	0.36
Amino acids												
EAA[3]	141.8^{a}	114.9^{a}	135.0^{a}	191.5^{b}	197.5^{b}	199.4^{b}	14.43	0.28	0.96	<0.01	0.09	0.44
Group 1[4]	33.9	31.6	31.6	44.1	43.8	42.8	2.40	0.33	0.19	<0.01	0.44	0.68
Group 2[5]	117.3^{a}	91.8^{a}	112.1^{a}	156.7^{b}	164.0^{b}	165.8^{b}	12.87	0.31	0.83	<0.01	0.08	0.43
BCAA[6]	68.4	51.3	63.9	99.8	97.7	102.0	8.08	0.13	0.85	<0.01	0.24	0.59
NEAA[7]	118.8	127.7	128.0	96.1	102.4	106.1	13.90	0.48	0.37	0.03	0.91	0.97
TAA[8]	260.7	242.5	263.0	287.6	299.9	305.5	26.11	0.86	0.54	0.01	0.36	0.64
Arg	16.4^{abc}	13.6^{c}	15.9^{bc}	16.9^{abd}	19.7^{d}	19.0^{ad}	1.55	0.98	0.41	<0.01	0.01	0.18
His	6.4	4.9	5.6	8.3	8.1	7.6	0.59	0.05	0.11	<0.01	0.16	0.82
Ile	19.0^{a}	13.5^{a}	16.9^{a}	25.0^{b}	25.8^{b}	25.9^{b}	1.95	0.16	0.72	<0.01	0.06	0.36
Leu	25.7^{a}	19.3^{a}	24.1^{a}	36.6^{b}	38.0^{b}	37.9^{b}	2.86	0.26	0.95	<0.01	0.08	0.51
Lys	18.4^{ab}	13.4^{b}	18.5^{ab}	22.6^{ac}	28.1^{c}	26.2^{c}	2.62	0.89	0.36	<0.01	0.01	0.39
Met	5.9	4.7	5.7	8.2	7.6	8.0	0.62	0.05	0.63	<0.01	0.52	0.96
Phe	9.9	9.6	9.4	15.0	14.9	14.6	0.77	0.68	0.40	<0.01	0.89	0.88
Thr	14.1	13.5	13.8	17.4	18.5	18.6	1.87	0.86	0.74	<0.01	0.56	0.60
Trp	2.4	3.8	2.3	3.3	3.0	3.2	0.37	0.12	0.75	0.83	0.03	0.98
Val	23.8	18.6	22.9	38.3	33.8	38.2	3.63	0.11	0.87	<0.01	0.90	0.89
Ala	14.1	18.8	16.9	5.6	5.6	8.8	3.76	0.44	0.33	<0.01	0.44	0.96
Asn	6.3	5.1	5.9	6.1	6.3	6.7	0.71	0.38	0.91	0.08	0.20	0.39

Table 6.4 (continued). Whole-mammary gland plasma flow and net uptakes of metabolites in lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

	Treatment[1]							*P*-value[2]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
Asp	0.3	0.3	0.3	0.1	0.5	0.4	0.24	0.31	0.41	0.46	0.28	0.53
Cit	1.8	3.0	2.1	2.8	1.1	2.7	0.85	0.70	0.94	0.44	0.07	0.81
Cys	0.3	0.5	0.2	0.5	0.3	0.5	0.17	0.87	0.73	0.74	0.27	0.99
Gln	34.8	33.9	37.7	30.8	31.2	31.9	3.87	0.94	0.51	0.17	0.83	0.76
Glu	17.9^{a}	16.4^{ab}	16.9^{ab}	11.0^{c}	17.8^{a}	12.1^{bc}	2.11	0.11	0.98	0.29	0.02	0.51
Gly	12.5	20.1	15.0	10.8	8.6	11.3	3.65	0.44	0.66	0.04	0.17	0.77
Orn	8.4^{a}	6.0^{b}	7.7^{ab}	6.6^{ab}	7.4^{ab}	7.4^{ab}	0.83	0.31	0.89	0.47	0.04	0.32
Pro	7.0	7.1	7.0	4.9	4.4	5.1	0.94	0.76	0.88	<0.01	0.66	0.89
Ser	6.2	8.2	9.5	7.7	8.9	10.1	1.86	0.30	0.07	0.67	0.78	0.77
Tyr	9.4	8.5	8.8	9.2	10.2	9.3	0.67	0.91	0.47	0.01	0.03	0.39

[a-d]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Low protein (LMP) or high protein (HMP) abomasal infusion treatments: LMP-C, 0.9% saline; LMP-GG, 1319 g/d glucose; LMP-LG, 676 g/d palm olein; HMP-C, 844 g/d of a complete EAA mixture in the same profile and amount as found in 1.5 kg casein; HMP-GG, 1319 g/d glucose + 844 g/d EAA; HMP-LG, 676 g/d palm olein + 844 g/d EAA. Infusions of LMP-GG, LMP-LG, and HMP-C supplied 20.5 MJ/d, and HMP-GG and HMP-LG supplied 41.0 MJ/d.

[2]GG = effect of energy from glucose; LG = effect of energy from fat; AA = effect of protein level.

[3]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

[4]Group 1 = His, Met, Phe + Tyr, Trp.

[5]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.

[6]BCAA = Ile, Leu, Val.

[7]NEAA = Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Orn, Pro, Ser, Tyr.

[8]TAA = EAA + NEAA.

Table 6.5. Mammary clearances (L/h) of metabolites in lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

	Treatment[1]							*P*-value[2]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
Glucose	132	113	136	101	130	103	14.8	0.65	0.72	0.55	0.12	0.84
BHB	205	197	190	202	273	211	26.3	0.28	0.91	0.10	0.18	0.68
NEFA	36	148	-44	82	184	94	108.1	0.27	0.72	0.37	0.98	0.64
TAG	828	498	1282	641	440	1095	154.1	0.11	0.01	0.47	0.64	0.99
LCFA	287	343	384	298	345	426	56.3	0.37	0.06	0.68	0.98	0.79
Amino acids												
EAA[3]	268^{a}	360^{b}	247^{a}	134^{c}	156^{c}	138^{c}	20.3	<0.01	0.59	<0.01	0.04	0.45
Group 1[4]	322	345	306	145	127	137	19.8	0.85	0.40	<0.01	0.14	0.74
Group 2[5]	265^{a}	369^{c}	243^{ad}	137^{b}	171^{bd}	144^{b}	25.0	<0.01	0.72	<0.01	0.10	0.49
BCAA[6]	238	377	202	126	184	122	29.9	<0.01	0.42	<0.01	0.11	0.50
NEAA[7]	90	101	92	98	89	123	13.9	0.92	0.24	0.41	0.38	0.29
TAA[8]	140	152	134	119	122	132	13.9	0.47	0.74	0.13	0.68	0.34
Arg	422^{a}	620^{c}	393^{a}	216^{b}	283^{ab}	276^{ab}	52.5	<0.01	0.66	<0.01	0.08	0.23
His	995	1183	1024	127	100	125	177.3	0.56	0.92	<0.01	0.43	0.91
Ile	231	398	183	163	268	164	43.6	<0.01	0.52	0.05	0.39	0.50
Leu	500^{a}	824^{c}	407^{a}	188^{b}	318^{ab}	182^{b}	55.8	<0.01	0.30	<0.01	0.05	0.36
Lys	862	1079	805	251	361	330	108.4	0.06	0.90	<0.01	0.53	0.43
Met	672	757	669	120	98	115	53.7	0.55	0.94	<0.01	0.31	0.98
Phe	351	383	309	151	147	129	24.5	0.47	0.11	<0.01	0.37	0.60
Thr	164	200	259	97	67	117	38.8	0.94	0.14	<0.01	0.38	0.33
Trp	69^{a}	129^{b}	68^{a}	78^{a}	74^{a}	79^{ab}	11.7	0.02	0.99	0.07	0.01	0.92
Val	157^{a}	242^{b}	139^{a}	86^{a}	108^{a}	82^{a}	20.6	<0.01	0.52	<0.01	0.07	0.68
Ala	70	115	79	33	31	50	20.5	0.18	0.40	<0.01	0.14	0.78
Asn	147^{a}	153^{a}	142^{a}	208^{ab}	186^{a}	291^{b}	26.4	0.72	0.09	<0.01	0.53	0.06
Asp	67.9	38.0	28.3	-3.2	80.5	167.0	61.15	0.59	0.20	0.08	0.26	0.04
Cit	23	52	29	31	17	30	10.5	0.47	0.84	0.11	0.05	0.73
Cys	37	119	65	69	70	59	21.2	0.06	0.67	0.22	0.07	0.39

Table 6.5 (continued). Mammary clearances (L/h) of metabolites in lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

	Treatment[1]							*P*-value[2]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
Gln	109	130	121	197	168	240	27.1	0.87	0.23	<0.01	0.28	0.50
Glu	1136	1201	1031	908	1313	1086	182.3	0.04	0.73	0.44	0.12	0.19
Gly	29	40	30	38	24	43	9.0	0.80	0.77	0.85	0.16	0.81
Orn	535	607	547	179	238	260	63.9	0.14	0.29	<0.01	0.89	0.43
Pro	97	126	95	79	67	98	17.1	0.53	0.52	0.05	0.14	0.45
Ser	72	77	101	118	106	219	28.2	0.88	0.01	<0.01	0.69	0.12
Tyr	356	358	383	335	243	397	54.8	0.36	0.37	0.31	0.35	0.72

[a-d]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Low protein (LMP) or high protein (HMP) abomasal infusion treatments: LMP-C, 0.9% saline; LMP-GG, 1319 g/d glucose; LMP-LG, 676 g/d palm olein; HMP-C, 844 g/d of a complete EAA mixture in the same profile and amount as found in 1.5 kg casein; HMP-GG, 1319 g/d glucose + 844 g/d EAA; HMP-LG, 676 g/d palm olein + 844 g/d EAA. Infusions of LMP-GG, LMP-LG, and HMP-C supplied 20.5 MJ/d, and HMP-GG and HMP-LG supplied 41.0 MJ/d.

[2]GG = effect of energy from glucose; LG = effect of energy from fat; AA = effect of protein level.

[3]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

[4]Group 1 = His, Met, Phe + Tyr, Trp.

[5]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.

[6]BCAA = Ile, Leu, Val.

[7]NEAA = Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Orn, Pro, Ser, Tyr.

[8]TAA = EAA + NEAA.

Mammary Gland AA U:O and Metabolite Balance

Irrespective of MP level, infusion of GG did not affect the mammary gland uptake to milk protein output ratio (U:O) of total EAA, group 1 AA or group 2 AA, but tended to decrease the U:O of total BCAA ($P = 0.09$; **Table 6.6**). A GG × AA interaction affected U:O of Arg and Lys which numerically decreased with GG at the LMP level and increased with GG at the HMP level ($P \leq 0.03$), affected U:O of Trp where it increased with GG but only at the LMP level ($P = 0.01$), and tended to affect U:O of Ile where it decreased with GG but only at the LMP level ($P = 0.08$). The U:O of His and Met decreased ($P \leq 0.03$) and U:O of Val tended to decrease ($P = 0.08$) in response to GG, regardless of MP level. Infusion of GG did not affect U:O of total NEAA ($P = 0.27$). A GG × AA interaction tended to affect Glu ($P = 0.08$) and Gly ($P = 0.09$), where GG numerically increased U:O of Glu at the HMP level, and numerically increased U:O of Gly at the LMP level and decreased it at the HMP level.

Regardless of MP level, infusion of LG had no effect on U:O of any AA group or individual AA, except for U:O of Ser which increased ($P = 0.04$). The HMP level increased U:O of total EAA, group 2 AA and BCAA ($P < 0.01$), and U:O of group 1 AA was not affected by MP level. In the presence of GG, the U:O of Ile and Lys increased at the HMP level compared with the LMP level ($P \leq 0.02$). The U:O of Leu, Phe, and Val increased ($P \leq 0.01$) and U:O of His ($P = 0.07$) and Met ($P = 0.08$) tended to increase at the HMP level. In the presence of GG, the U:O of Trp decreased at the HMP level compared with the LMP level ($P = 0.03$). The HMP level decreased the U:O of total NEAA ($P < 0.01$). Individually, U:O of all NEAA decreased at the HMP level ($P < 0.01$), except Asn which tended to decrease ($P = 0.07$), and Asp, Cys, and Ser which were unaffected ($P > 0.10$).

Lactose output as a proportion of mammary glucose uptake decreased in response to GG regardless of MP level ($P = 0.03$), and increased at the HMP level ($P = 0.03$; **Table 6.7**). The calculated amount of glucose required for milk fat synthesis tended to decrease in response to GG ($P = 0.10$) and tended to increase in response to LG ($P = 0.09$). The calculated glucose required for lactose and milk fat synthesis increased at the HMP level ($P \leq 0.01$). Net glucose uptake by the mammary gland did not cover calculated glucose requirements for lactose and fat synthesis on any treatment, with the exception of LMP-GG. Infusion of GG reduced the deficit ($P = 0.03$), while the HMP level increased it ($P = 0.02$). Output of LCFA in milk decreased in response to GG and increased in response to LG ($P < 0.01$). Net mammary LCFA uptake did not equilibrate with output in milk on any treatment, but infusion of LG reduced the deficit ($P = 0.04$).

Table 6.6. Mammary gland AA uptake to milk output ratios in lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

	Treatment[1]							*P*-value[2]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
EAA[3]	1.19	1.02	1.21	1.27	1.27	1.35	0.061	0.18	0.44	<0.01	0.17	0.63
Group 1[4]	1.01	1.00	1.00	1.04	1.00	1.03	0.018	0.16	0.58	0.46	0.32	0.81
Group 2[5]	1.24	1.02	1.26	1.30	1.32	1.40	0.075	0.21	0.41	0.01	0.13	0.60
BCAA[6]	1.21	0.96	1.20	1.38	1.32	1.45	0.087	0.09	0.74	<0.01	0.32	0.72
NEAA[7]	0.67	0.78	0.77	0.42	0.44	0.47	0.058	0.27	0.20	<0.01	0.38	0.62
TAA[8]	0.89	0.88	0.96	0.78	0.79	0.84	0.049	0.99	0.18	0.03	0.86	0.94
TAA-N[9]	0.94	0.90	1.00	0.85	0.86	0.92	0.049	0.79	0.20	0.19	0.66	0.97
Arg	2.53^{ab}	2.21^{bc}	2.59^{a}	2.04^{c}	2.32^{abc}	2.34^{abc}	0.116	0.87	0.11	0.54	0.01	0.29
His	1.11	0.91	1.04	1.16	1.08	1.09	0.061	0.03	0.26	0.07	0.31	0.99
Ile	1.33^{ab}	1.00^{b}	1.27^{ab}	1.37^{a}	1.38^{a}	1.45^{a}	0.086	0.09	0.91	0.01	0.08	0.47
Leu	1.07	0.84	1.07	1.19	1.21	1.26	0.068	0.18	0.62	<0.01	0.13	0.64
Lys	0.98^{ab}	0.75^{b}	1.06^{ab}	0.95^{ab}	1.16^{a}	1.11^{a}	0.090	0.93	0.20	0.02	0.03	0.66
Met	0.98	0.83	1.00	1.07	0.96	1.07	0.053	0.02	0.84	0.08	0.74	0.85
Phe	1.03	1.07	1.04	1.25	1.19	1.24	0.031	0.74	0.98	<0.01	0.13	0.71
Thr	1.08	1.10	1.12	1.05	1.07	1.15	0.092	0.86	0.44	0.99	0.97	0.72
Trp	0.93^{a}	1.62^{b}	0.96^{a}	1.05^{ab}	0.93^{a}	1.02^{ab}	0.139	0.05	0.99	0.03	0.01	0.81
Val	1.30	1.07	1.33	1.64	1.41	1.68	0.135	0.08	0.79	0.01	0.97	0.99
Ala	1.12	1.64	1.41	0.35	0.31	0.53	0.262	0.30	0.31	<0.01	0.23	0.82
Asn	0.62	0.54	0.61	0.47	0.47	0.52	0.044	0.31	0.63	0.07	0.33	0.49
Asp	0.018	0.016	0.023	0.003	0.028	0.022	0.0162	0.47	0.47	0.74	0.40	0.64
Cys	0.12	0.21	0.11	0.17	0.11	0.17	0.064	0.86	0.84	0.76	0.26	0.92
Gln	1.67	1.72	1.91	1.17	1.13	1.21	0.147	0.97	0.33	<0.01	0.76	0.48
Glu	0.49^{a}	0.47^{a}	0.50^{a}	0.23^{b}	0.37^{ab}	0.26^{ab}	0.047	0.19	0.65	<0.01	0.08	0.82
Gly	1.54	2.68	1.94	1.08	0.79	1.11	0.427	0.30	0.60	<0.01	0.09	0.66
Pro	0.24	0.26	0.26	0.13	0.11	0.14	0.026	0.99	0.59	<0.01	0.35	0.72
Ser	0.33	0.47	0.54	0.32	0.35	0.43	0.084	0.24	0.04	0.11	0.44	0.48
Tyr	0.97	0.93	0.96	0.76	0.81	0.77	0.030	0.74	0.98	<0.01	0.13	0.71

Table 6.6 (continued). Mammary gland AA uptake to milk output ratios in lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

[a-c]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Low protein (LMP) or high protein (HMP) abomasal infusion treatments: LMP-C, 0.9% saline; LMP-GG, 1319 g/d glucose; LMP-LG, 676 g/d palm olein; HMP-C, 844 g/d of a complete EAA mixture in the same profile and amount as found in 1.5 kg casein; HMP-GG, 1319 g/d glucose + 844 g/d EAA; HMP-LG, 676 g/d palm olein + 844 g/d EAA. Infusions of LMP-GG, LMP-LG, and HMP-C supplied 20.5 MJ/d, and HMP-GG and HMP-LG supplied 41.0 MJ/d.

[2]GG = effect of energy from glucose; LG = effect of energy from fat; AA = effect of protein level.

[3]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

[4]Group 1 = His, Met, Phe + Tyr, Trp.

[5]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.

[6]BCAA = Ile, Leu, Val.

[7]NEAA = Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser, Tyr.

[8]TAA = EAA + NEAA.

[9]TAA on a N basis.

Table 6.7. Calculated mammary gland glucose and long-chain fatty acid balance in lactating dairy cows (n = 6) receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d

	Treatment[1]							*P*-value[2]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
Glucose												
Uptake, g/h	66	67	67	50	74	53	7.8	0.07	0.68	0.64	0.14	0.99
Lactose output:glucose uptake	0.80	0.79	0.80	1.26	0.88	1.16	0.095	0.03	0.54	0.03	0.32	0.81
Required for lactose,[3] g/h	54	52	52	60	62	61	3.5	0.96	0.77	<0.01	0.35	0.53
Required for fat, g/h	15	13	17	17	17	19	1.0	0.10	0.09	<0.01	0.29	0.76
Excess,[4] g/h	-3.4	1.2	-1.9	-27.7	-4.5	-26.9	6.10	0.03	0.78	0.02	0.18	0.88
LCFA uptake,[5] g/h	15	12	24	14	13	27	3.0	0.42	<0.01	0.29	0.56	0.36
LCFA output,[6] g/h	23	18	29	24	19	30	1.3	<0.01	<0.01	0.24	0.84	0.96
LCFA balance, g/h	-8.5	-6.7	-5.8	-10.5	-5.8	-3.7	2.95	0.17	0.04	0.52	0.54	0.36

[1]Low protein (LMP) or high protein (HMP) abomasal infusion treatments: LMP-C, 0.9% saline; LMP-GG, 1319 g/d glucose; LMP-LG, 676 g/d palm olein; HMP-C, 844 g/d of a complete EAA mixture in the same profile and amount as found in 1.5 kg casein; HMP-GG, 1319 g/d glucose + 844 g/d EAA; HMP-LG, 676 g/d palm olein + 844 g/d EAA. Infusions of LMP-GG, LMP-LG, and HMP-C supplied 20.5 MJ/d, and HMP-GG and HMP-LG supplied 41.0 MJ/d.

[2]GG = effect of energy from glucose; LG = effect of energy from fat; AA = effect of protein level.

[3]Requirements for lactose and fat estimated based on calculations of Dijkstra et al. (1996).

[4]Excess = Uptake – required for lactose – required for fat.

[5]Molecular weight of FA ≥16C in blood was calculated according to the molecular weight of ≥16C FA in milk (Nichols et al., 2019b), assuming 50% of milk C16 originated from C16 FA in blood sequestered as preformed FA and that 50% of milk C16 was synthesized de novo.

[6]Preformed milk FA (>16C) and 50% of milk C16 FA Nichols et al. (2019b).

DISCUSSION

The current results complement previously reported daily lactation performance, milk FA composition, and energy and N balance from the same study (Nichols et al., 2019b). Notably, GG reduced milk energy output and increased body energy retention, whereas LG increased milk energy output and did not affect body energy retention. There was body N mobilization on LMP-GG but N retention on HMP-GG, and GG improved milk N efficiency regardless of protein level. Body N retention was positive with LG infusion, and LG had no effect on milk N efficiency (Nichols et al., 2019b). Considering the milk output and whole-body energy and N balance responses observed in this experiment, in the present work we investigated mammary gland metabolism in response to glucose and palm olein supplied at low and high MP levels.

Milk protein yield and AA catabolism increased at the high MP level

The objective of EAA infusion in the current study was to create a low and high MP level over which to compare the effects of GG and LG. We expected EAA infusion to independently increase arterial EAA concentrations and milk protein yield, in line with previous reports of postruminal EAA infusion (Doepel and Lapierre, 2010; Doelman et al., 2015; Nichols et al., 2016). Indeed, at the HMP level arterial concentration of total EAA was 2.5 times that of the LMP level. In agreement with our hypothesis, mammary net uptake of all EAA groups increased at the HMP level, but MPF was not affected and mammary clearance decreased. Decreased mammary clearance of EAA is consistent with surplus EAA supply for milk protein synthesis and elevated arterial concentrations at the HMP level. Excess EAA become available for extra-mammary metabolism, which is in line with increased body N retention at the HMP level (Nichols et al., 2019b). The U:O of group 1 AA (His, Met, Phe+Tyr, Trp) in this study agrees with their canonical 1:1 net U:O in milk protein and was not affected by MP level, while the U:O of group 2 AA (Arg, Ile, Leu, Lys, Val, Thr) and NEAA increased and decreased, respectively, at the HMP level. Arterial concentration and mammary uptake of total NEAA decreased which suggests more extensive hepatic catabolism of NEAA at the HMP level. This dynamic of mammary gland AA utilization during EAA supplementation, where group 2 AA mammary uptake and intramammary catabolism increases while NEAA uptake decreases, agrees with the observations of Nichols et al. (2016) during abomasal infusion of the same EAA profile and dose.

Regardless of MP level, U:O of total group 1 AA was not different from 1. However, the average U:O of Phe at the HMP level was 1.23, which is relatively greater than the average U:O of the other individual group 1 AA at the HMP level (His – 1.11; Met – 1.03; Trp – 1.00). At the LMP level, average Phe U:O is 1.05 and in line with previous ranges observed from cows fed low (11.8%) and high (16.5%) CP contents (Crompton et al., 2014). The individual U:O of Tyr decreased from 0.95 on average at the LMP level, to 0.78 on average at the HMP level, the latter of which is low compared with the range reported by Crompton et al. (2014).

It is possible that the high level of EAA infusion in the current study caused a relative shortage of Tyr, and that intramammary hydroxylation of Phe to Tyr increased at the HMP level. If the excess uptake relative to milk output of Phe was converted to Tyr, according to our assumption for the Fick Principle, approximately 22% of milk Tyr would have come from mammary-sequestered Phe. Verbeke et al. (1972) suggested that hydroxylation of Phe to Tyr is influenced by Phe concentration, and Jorgensen and Larson (1968) showed that this conversion is affected by presence or absence of Tyr in mammary cells. Both observations would support a high level of conversion of Phe to Tyr when mammary gland Phe uptake increased 54% at the HMP level relative to the LMP level. However, the extent to which intramammary Phe contributes to Tyr synthesis is still not well established, and significant amounts of intracellular Tyr may originate from mammary tissue breakdown or may arise not only from plasma free AA but also from mammary uptake of peptides (Lemosquet et al., 2010; Crompton et al., 2014).

The HMP level decreased net U:O of TAA. Our EAA infusion rate of 844 g/d is high relative to others infusing EAA mixtures in a casein profile (Doepel and Lapierre, 2010; Lapierre et al., 2013; Doelman et al., 2015). When exogenous EAA supply is high and imbalanced relative to NEAA for casein synthesis and intramammary metabolism increases, net U:O might not accurately represent TAA transfer. Mammary EAA uptake increased, likely in response to secretory cell demand to support milk protein synthesis, but mammary uptake of total NEAA decreased. Therefore, of the EAA sequestered by the gland, more were used for de novo NEAA synthesis to compensate for their increased requirements for milk protein synthesis, evidenced by the increased U:O of total group 2 AA and BCAA at the HMP level. Although EAA (except group 1 AA) were extracted in excess of their requirement, intramammary NEAA uptake was reduced relative to their increased output, resulting in net U:O of TAA of < 1. When calculated on an N basis, the U:O of TAA is closer to unity, which supports contribution of N from group 2 AA to N required for de novo NEAA synthesis (Lapierre et al., 2012). At infusion doses similar to ours, but when mixtures of EAA+NEAA or when casein is infused, U:O of TAA are ≥ 1 (Guinard and Rulquin, 1994; Raggio et al., 2006b; Doepel and Lapierre, 2010). It is possible that under the condition of high EAA supply that is also imbalanced relative to NEAA required for casein synthesis, peptide-bound AA are extracted by the gland to compensate for the deficiency. Evidence of this contribution at low or high protein supplies is equivocal, but has been suggested in scenarios where AA supply for milk protein synthesis is lacking (Backwell et al., 1994; Bequette et al., 1999; Bequette et al., 2001).

Glucogenic infusion reduced group 2 AA concentration but milk protein yield was maintained

Mammary plasma flow increased 22% in response to 1.3 kg/d GG infusion, which agrees with increases in MPF of 24 and 36% in cows infused for 14 d with 1 kg/d duodenal glucose or ruminal propionate (Rulquin et al., 2004; Raggio et al., 2006b). Mammary plasma

flow will increase or decrease to maintain intramammary energy balance via 2C compounds (acetate and BHB; Cant et al., 2003). Glucogenic infusions typically reduce circulating acetate and BHB concentrations (Rigout et al., 2002; Lemosquet et al., 2009; Curtis et al., 2018), which likely stimulates the concomitantly observed hyperaemia. Arterial BHB concentration decreased 36% with GG infusion in the current study, which is in line with proposed regulation of MPF and in agreement with Curtis et al. (2018) where arterial concentration of acetate + BHB decreased 31% and MPF increased 36% with intravenous glucose infusion. Elevated glucose and insulin concentrations observed in response to GG may have reduced arterial BHB through stimulatory effects on lipogenesis in adipose (Vernon et al., 1985), anti-ketogenic effects in the liver, and BHB oxidation by peripheral tissues (Ørskov et al., 1999).

Arterial plasma concentrations of all group 2 AA, with the exception of Thr, decreased in response to GG, independent of MP level. Others have reported reduced concentrations of group 2 AA upon postruminal infusion of propionate, starch, and glucose (Raggio et al., 2006a; Rius et al., 2010; Nichols et al., 2016), often with no concomitant increase in their net mammary uptake (Raggio et al., 2006b; Nichols et al., 2016; Curtis et al., 2018). In the current study, a GG × AA interaction affected mammary uptake of Arg, Lys, Ile (tendency), and Leu (tendency), and tended to affect mammary uptake of total group 2 AA, indicating lower uptake of these AA at the LMP level and no effect at the HMP level. Considering that GG infusion reduced circulating group 2 AA, increased MPF, and did not decrease milk protein yield, it follows that efficiency of group 2 AA uptake increased with GG. This increase was detected in the mammary clearance parameter for Ile and Lys, but was more significant at the LMP level than at the HMP level for Arg, Leu, Val, and total group 2 AA (GG × AA interactions). Intramammary group 2 AA catabolism was reduced on LMP-GG, as evidenced by their numerically decreased U:O, and by the GG × AA interactions on this parameter for Arg, Ile, and Lys, where their U:O was lower with GG at the LMP level and not affected at the HMP level.

Group 2 AA are typically extracted by the udder in a greater amount relative to their output in milk, and are used for de novo synthesis of NEAA and as a supply of glycolytic and tricarboxylic acid cycle intermediates (Mepham, 1987; Lapierre et al., 2012). This canonical pattern was observed on all treatments except LMP-GG, where U:O of the group 2 AA was not different from 1. Milk protein yield did not decrease on LMP-GG relative to LMP-C or LMP-LG; therefore, at the LMP level, intramammary compensation must have occurred to yield AA-N and carbon required to synthesize NEAA and generate energy. Of the group 2 AA, individual U:O of Arg, Thr, and Val remained > 1 and thus could still contribute AA-N and carbon. The U:O of NEAA was numerically highest on LMP-GG. Of the NEAA, U:O of Ala and Gly increased most (numerically) on LMP-GG relative to LMP-C and LMP-LG, possibly to offset lower de novo synthesis of these AA. The U:O of Ala appears to be particularly responsive under conditions of low protein intake (Raggio et al., 2006b; Doepel and Lapierre, 2010;

Haque et al., 2015) and during insulin infusion (Bequette et al., 2001). During insulin stimulation and when protein supply is limited, the gland retains more AA from arterial influx, reduces intramammary catabolism, and reduces AA exit via venous drainage (Mackle et al., 2000; Bequette et al., 2001). Decreased intramammary group 2 AA catabolism would result in lower U:O but no change in milk protein yield, as was observed in response to LMP-GG. In addition, mammary extraction of circulating peptides may contribute relatively more to milk protein synthesis on LMP-GG (Backwell et al., 1994; Bequette et al., 1999; Lapierre et al., 2012), but were not quantified in the present AV measurements.

Glucose infusion directed a portion of circulating group 2 AA towards utilization in non-mammary tissues, as evidenced by their lower arterial concentration at low and high MP levels whilst mammary uptake of group 2 AA was not increased. Metabolism of BCAA and Lys in ruminants occurs predominantly in non-hepatic tissues (Lapierre et al., 2002), and non-mammary sinks for group 2 AA are oxidative catabolism, skeletal muscle protein synthesis, or adipose TAG synthesis (Brosnan and Brosnan, 2006; Raggio et al., 2006a). The fact that a portion of the group 2 AA were partitioned to extra-mammary peripheral tissues in response to GG agrees with numerical reductions in urinary N output and increase in body N retention (observed by Nichols et al., 2019b), and with the reduced plasma urea concentration at the HMP level with GG (tendency for a GG × AA interaction). Decreased plasma urea concentration with GG, in particular at the HMP level, is in accordance with overall reduced oxidative catabolism of AA, and agrees with the observations of Raggio et al. (2006a) where whole-body oxidation of Leu was not affected during propionate infusion, and was reduced when propionate and casein were infused together. Arterial concentrations of 1 and 3M-His were not affected by GG, in agreement with the observation of Nichols et al. (2016) during abomasal infusion of 1 kg of glucose, indicating that muscle protein degradation was not increased which is in line with the anti-proteolytic effect of insulin on muscle (Lobley, 1998). However, GG increased the arterial concentration of Gly, which is released from skeletal muscle at high levels relative to other AA when body protein is mobilized (Doepel et al., 2002). These indicators suggest that turnover in the skeletal muscle pool during GG infusion could have sequestered some of the group 2 AA. Considering specifically the BCAA, recent work suggests a role for adipose tissue in BCAA metabolism in ruminants when glucose increases insulin concentration (Nichols et al., 2016; Curtis et al., 2018), linked to the same mechanism through which it stimulates lipogenesis and decreases arterial acetate, BHB, and NEFA concentrations. Alpha-keto acids produced from BCAA can serve as primers for de novo FA synthesis (Brosnan and Brosnan, 2006). Taken together, the increase in insulin and decrease in BHB and NEFA concentrations, the reduction in milk fat yield, and the increase in body energy retention (observed by Nichols et al., 2019b) in response to GG suggests contribution to lipogenesis in adipose was a likely sink for BCAA during glucogenic infusion.

In summary, regardless of MP level, GG decreased the arterial concentration of group 2 AA. At the LMP level, GG infusion resulted in numerically lower mammary net uptake of group 2 AA, numerically reduced intramammary group 2 AA catabolism (lower U:O) and did not affect milk protein yield. During GG infusion at the HMP level, mammary net uptake of group 2 AA was not affected, milk protein yield was not affected, but intramammary catabolism of group 2 AA numerically increased (higher U:O). Anabolic effects of insulin on peripheral tissues during GG infusion likely accounted for the reduced arterial concentrations of BHB and group 2 AA, and the reduced intramammary catabolism of group 2 AA to maintain milk protein synthesis at the LMP level.

Lipogenic infusion did not affect mammary AA utilization

Infusion of LG did not affect MPF or mammary net uptake of any AA group. This agrees with the findings of Nichols et al. (2019a) in response to dietary supplementation with rumen-inert saturated fat. However, those authors did report a tendency for milk protein yield to increase 45 g/d with fat supplementation, whereas in the current study milk protein yield was not affected by LG. Interestingly, Nichols et al. (2019a) report Ser as the only AA to be affected by fat feeding at the level of mammary uptake and clearance, which was also observed in the current study. Data characterising mammary gland kinetics in response to fat supplementation is limited. Previous work suggested that feeding LCFA might improve mammary AA extraction efficiency, but that this improvement is counteracted by depressed MPF if intramammary energy requirements are reduced when short-chain FA and glucose are spared by a reduced de novo FA synthesis (reviewed by DePeters and Cant, 1992). Reduced FA synthesis from 2C compounds decreases ATP utilization, but subsequent stimulation of lactose synthesis from glucose increases ATP utilization (Cant et al., 2003). Therefore, according to the hypothesis that MPF is regulated to maintain intramammary energy balance, it follows that LG did not affect MPF in the current study because LG did not affect mammary BHB uptake, increased TAG and LCFA uptake, and decreased de novo milk FA synthesis (Nichols et al., 2019b).

Insulin concentration was not affected by LG, and thus the anabolic effects of insulin on peripheral tissues did not initiate partitioning of EAA towards extra-mammary tissues in response to LG, in contrast with the effect on GG infusion and in line with our hypothesis. Therefore, a greater portion of circulating AA were available for mammary AA uptake with LG infusion, but this was not observed, and milk protein output was not affected. Plasma urea concentration tended to increase with LG at the LMP level, tended to decrease at the HMP level, but was numerically higher on HMP-LG than on LMP-LG which is consistent with greater AA catabolism as protein supply increases. Nichols et al. (2019a) reported no effect on plasma urea concentration when saturated fat was supplemented in a high protein diet. The fact that LG did not alter mammary gland AA utilization supports the absence of an effect on milk N efficiency observed in this study (Nichols et al., 2019b).

Mammary glucose and LCFA balance

Assuming 1.05 and 0.31 g of glucose is required to synthesize 1 g of lactose and fat, respectively (Dijkstra et al., 1996), net mammary glucose uptake was insufficient to cover estimated requirements for lactose and fat synthesis on all treatments except LMP-GG. Nevertheless, lactose output expressed relative to net glucose uptake averaged 0.80 at the LMP level which is in line with others (Bickerstaffe et al., 1974; Lemosquet et al., 2009; Galindo et al., 2011). Net glucose uptake tended to increase and milk fat yield decreased with GG, which mitigated the intramammary glucose deficit for lactose and fat synthesis. The 192 g/d increase in lactose yield stimulated by the HMP level agrees with others who have reported increases in total milk and lactose yield in response to postruminal AA supplementation (Doepel and Lapierre, 2010; Galindo et al., 2011; Nichols et al., 2016). However, this increase in lactose synthesis did not coincide with increased mammary glucose uptake in response to EAA infusion. Similarly, Lemosquet et al. (2009) observed no increase in glucose uptake, but lactose yield increased in response to casein infusion. In fact, in the current study, the numerically lower MPF on HMP-C and HMP-LG relative to HMP-GG and LMP infusions resulted in numerically lower mammary glucose uptake on these treatments. It is estimated that 100% of glucose in lactose but only 60 to 80% of galactose in lactose is derived from plasma glucose (Bickerstaffe et al., 1974; Sunehag et al., 2002; Bequette et al., 2006). Amino acids could contribute to carbon substrates required for galactose synthesis by the gland, thereby contributing to lactose synthesis (Bickerstaffe et al., 1974; Bequette et al., 2006), and this contribution could be affected by absorptive EAA level (Lapierre et al., 2013; Maxin et al., 2013). Upon infusion of labelled glucose, Lapierre et al. (2013) demonstrated that 18% of milk galactose did not originate from glucose in lactating mammary glands, but the ratio of enriched galactose to enriched glucose in lactose was not affected by EAA infusion. In contrast, Maxin et al. (2013) observed lower contribution of labelled glucose in milk galactose during abomasal AA infusion, indicating that a larger portion of non-glucose carbon (up to 50%) was used for galactose synthesis in the mammary gland when AA were infused. Bequette et al. (2006) estimated that 12% of galactose synthesised by mammary cells in vitro was derived from EAA catabolism. In the current study, the U:O of TAA was the farthest from unity at the HMP level. If a portion of AA extracted by the gland contributed to galactose synthesis, this would not be accounted for in the TAA balance or in the glucose balance, but would reduce the apparent glucose deficit for lactose synthesis. In response to abomasal infusion of the same EAA profile and dose, the reported lactose yield expressed relative to reported mammary glucose uptake by Nichols et al. (2016) was 1.12, comparable with the values of 1.26 and 1.16 on HMP-C and HMP-LG in the current study. Furthermore, mammary mechanisms such as glucose phosphorylation and concentrations of metabolites glucose-6-P and glucose-1-P regulate lactose synthesis independent of glucose supply (Xiao

and Cant, 2005), and the synthesis and secretion of milk protein and fat may play a role in regulation of lactose synthesis (Lapierre et al., 2010).

Lipogenic infusion did not affect circulating glucose or net mammary glucose uptake, but produced the same level of lactose yield as GG, independent of MP level. Lactose production with lipogenic diets may be equal to or greater than that with glucogenic diets (Van Knegsel et al., 2007; Hammon et al., 2008; Lohrenz et al., 2010), or during isoenergetic supplementation of fat and protein (Nichols et al., 2019a). Absorption of LCFA into circulation promotes their direct incorporation into milk fat, thereby reducing de novo FA synthesis and glucose requirements for oxidative catabolism, which in turn may spare some intramammary glucose for lactose synthesis (Chilliard, 1993; Hammon et al., 2008). In agreement, mammary net uptake of TAG and LCFA (≥16C) increased with LG, and milk LCFA (≥16C) output increased (Nichols et al., 2019b). However, net LCFA uptake did not equilibrate with LCFA output in milk on any treatment. A larger portion than the assumed 50% of C16 may have been synthesized de novo. A larger synthesis rate would mitigate some or all of the calculated shortfall in net mammary supply of LCFA from blood relative to milk LCFA. With the assumption of more 16C FA being synthesized de novo in the gland, intramammary glucose requirement would become increasingly deficient to support glycerol synthesis and yield NADPH. The concentration of FA <16C, assumed to be 100% synthesized de novo, decreased in milk fat with LG infusion (Nichols et al., 2019b) which would allow some glucose sparing. At the HMP level, arterial concentration and mammary uptake of BHB increased. This is consistent with increased concentration of <16C FA in milk fat (Nichols et al., 2019b). Contribution of BHB to de novo milk FA synthesis would partly offset the apparent deficit in LCFA balance at the HMP level.

CONCLUSIONS

Increased absorptive supply of glucose and palm olein differently affected mammary gland metabolite utilization, irrespective of MP level. Anabolic effects of insulin on extra-mammary peripheral tissues during glucose infusion likely accounted for the reduced arterial concentrations of BHB and group 2 AA and increased MPF, regardless of MP level, and the reduced intramammary catabolism of group 2 AA at the LMP level. We suspect glucose stimulated lipogenesis in adipose and increased MPF to maintain intramammary ATP balance. Regardless of protein level, palm olein did not promote an insulin response and did not affect arterial AA concentrations or mammary AA utilization. The HMP level increased milk protein yield, increased uptake of all EAA groups, increased intramammary catabolism of group 2 AA and BCAA but decreased that of NEAA. Mammary net glucose uptake did not equilibrate with estimated requirements for milk lactose or fat synthesis, except during glucose infusion at the LMP level. This deficit could have been mitigated through shifts in intramammary metabolite partitioning such as contribution of EAA carbon to galactose, and

decreased de novo 16C FA synthesis. These results suggest that lactose secretion is not solely dependent on mammary glucose supply, and illustrate flexibility of mammary metabolite utilization.

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Chapter 7

Energy and nitrogen balance of dairy cattle as affected by provision of different essential amino acid profiles at the same metabolizable protein supply

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ABSTRACT. Amino acid composition of metabolizable protein (MP) is important in dairy cattle diets, but impacts of AA imbalances on energy and N utilisation are unclear. This study determined the effect of different AA profiles within a constant supplemental MP level on whole-body energy and N partitioning in dairy cattle. Five rumen-fistulated Holstein-Friesian dairy cows (2.8 ± 0.4 lactations; 81 ± 11 d in milk) were randomly assigned to a 5 × 5 Latin square design in which each experimental period consisted of 5 d of continuous abomasal infusion followed by 2 d of rest. A total mixed ration consisting of 58% corn silage, 16% alfalfa hay, and 26% concentrate (dry matter basis) was formulated to meet 100 and 83% of net energy and MP requirements, respectively, and was fed at 90% of ad libitum intake by individual cow. Abomasal infusion treatments were saline (SAL) or 562 g/d of essential AA delivered in 4 profiles where individual AA content corresponded to their relative content in casein. The profiles were 1) a complete essential AA mixture (EAAC), 2) Ile, Leu, and Val (BCAA), 3) His, Ile, Leu, Met, Phe, Trp, Val (GR1+ILV), and 4) Arg, His, Lys, Met, Phe, Thr, Trp (GR1+ALT). The experiment was conducted in climate respiration chambers to determine energy and N balance in conjunction with milk production and composition, digestibility, and plasma constituents. Compared with SAL, infusion of EAAC increased milk, protein, and lactose yield, increased energy retained as body protein, and did not affect milk N efficiency. Total N intake and urine N output was higher with all AA infusions relative to SAL. Compared with EAAC, infusions of GR1+ILV and GR1+ALT produced the same milk yield and the same yield and content of milk fat, protein and lactose, and had the same energy and N retention. Milk N efficiency was not different between EAAC and GR1+ILV, but was lower with GR1+ALT compared with EAAC, and tended to be lower with GR1+ALT compared with GR1+ILV. Infusion of BCAA tended to decrease dry matter intake compared with the other AA infusions. Milk production and composition was not different between BCAA and SAL. Infusion of BCAA decreased or tended to decrease milk, protein, and lactose yields and milk protein content, and increased milk fat and lactose content, compared with EAAC. Milk N efficiency decreased with BCAA compared with SAL, EAAC, and GR1+ILV. Milk urea concentration was not affected by EAA infusions. Plasma urea concentration did not differ between EAAC and SAL, tended to increase with BCAA and GR1+ILV over SAL, and increased with GR1+ALT compared with EAAC and SAL. In conclusion, removing Ile, Leu, and Val or removing Arg, Lys, and Thr from infusions when the total amount of EAA infused remained constant did not impair milk production compared with a complete EAA profile, but milk N efficiency decreased when Ile, Leu, and Val were absent. Infusion of only Ile, Leu, and Val decreased milk protein yield and content and reduced milk N efficiency compared with a complete EAA profile.

Key words: energy balance, nitrogen balance, milk nitrogen efficiency, amino acid imbalance, metabolizable protein

INTRODUCTION

Dietary protein for lactating dairy cattle must allow a quantitatively and qualitatively optimal absorptive AA supply to support maintenance, reproduction, and milk protein synthesis. Simply increasing total absorptive AA supply does not guarantee positive responses, because as AA supply increases, transfer efficiency of absorbed AA into milk protein generally decreases (Doepel et al., 2004; Huhtanen and Hristov, 2010; Nichols et al., 2016) and AA catabolism and N excretion increases (Bach et al., 2000; Castillo et al., 2001; Raggio et al., 2004). However, when extra N intake comes from MP with a desirable EAA profile for milk protein synthesis, milk N efficiency can be improved (Haque et al., 2012; Haque et al., 2015). Splanchnic regulation of whole-body N exchange plays a role in the balance between whole-body energy requirements, catabolism of excess AA, and peripheral tissue anabolism (Blouin et al., 2002; Raggio et al., 2004; Lapierre et al., 2005). With regards to mammary gland metabolism, transfer of AA-N into milk from group 1 AA (His, Met, Phe+Tyr, Trp) canonically occurs in a 1:1 ratio with their uptake from the arterial supply (Mepham, 1982; Lapierre et al., 2012). When their duodenal supply is reduced, these EAA often decrease milk protein synthesis (Weekes et al., 2006; Doelman et al., 2015a; Doepel et al., 2016) but their hepatic catabolism is flexible and may decrease in an effort to support mammary protein synthesis (Raggio et al., 2004; Lapierre et al., 2005). The mammary gland obtains substantial N and carbon for de novo NEAA synthesis and for glycolytic and tricarboxylic acid (TCA) cycle intermediates from excess uptake of AA-N from group 2 AA (Arg, Ile, Leu, Lys, Thr, and Val) relative to their output in milk (Mepham, 1982; Lapierre et al., 2012). The branched-chain AA (Ile, Leu, and Val) and Lys are unique because they are preferentially catabolized in extra-hepatic tissues (Brosnan and Brosnan, 2006; Lapierre et al., 2005), and Leu has an acute stimulatory effect on protein synthesis in skeletal muscle and mammary glands (Wilson et al., 2010; Appuhamy et al., 2011).

Milk protein synthesis in bovine mammary glands does not function according to the expectations of a limiting AA system (Cant et al., 2003), and responses to postruminally infused AA mixtures are not attributable to only one AA within the mix (Schwab et al. 1976; Kim et al. 2000; Robinson et al., 2000). Detrimental effects of single AA supplementation in high-producing dairy cows can arise from reduced net transport of individual AA into mammary alveolar cells resulting from increased competition for limited transport capacity via countertransport systems (Maas et al., 1998), and from induced metabolic imbalances resulting in energy-costly functions for catabolism and excretion of excess AA (Weekes et al., 2006; Calsamiglia et al., 2010; Reed et al., 2017). Therefore, under practical feeding conditions, focus should be placed on formulating rations with a wider profile of EAA in MP, rather than focusing on supplementation of single AA. Several studies have investigated milk production and N metabolism in response to MP supplements and dietary ingredients providing wider EAA profiles (Bach et al., 2000; Haque et al., 2012; Maxin et al., 2013), but

quantification of the impact of EAA profile of MP on whole-body energy and N partitioning requires further refinement.

Previous studies have evaluated the effects of EAA deficiencies in duodenal supply, where single AA or groups of AA were subtracted from postruminal infusions of complete AA profiles (Weekes et al., 2006; Doelman et al., 2015a,b). The design in the present experiment differs, in that AA groups were absent relative to a complete EAA infusion, but the total infused AA supply was maintained through compensation with the other EAA in the infusion. Therefore, the present experiment examines the effects of EAA profile within a constant supply of MP on whole-body energy and N metabolism of lactating dairy cattle. We hypothesized that, due to the unique aspects of branched-chain AA metabolism, their absence from a complete EAA profile would illicit different effects on N partitioning compared with the removal of Arg, Lys, and Thr, and that an infusion lacking Arg, Lys, and Thr would result in similar milk protein yield and whole-body N balance compared with a complete EAA profile. We expected the largest differences in N balance and milk production with infusion of branched-chain AA alone when compared with the complete EAA profile.

MATERIALS AND METHODS

Experimental Design and Respiration Chamber Housing

The following experimental procedures were conducted under the Dutch Law on Animal Experiments in accordance with EU Directive 2010/63. Five rumen-fistulated, Holstein-Friesian dairy cows were randomly assigned to a 5 × 5 Latin square design where each experimental period consisted of 5 d of continuous abomasal infusion followed by 2 d of rest (**Figure 7.1**). Cows were in second (n = 1) or third (n = 4) lactation with an average milk production of 33.1 ± 2.28 kg/d at 81 ± 11 DIM and 631 ± 71.1 kg BW. Cows were adapted to the experimental conditions for 19 d prior to the first experimental period. For the first 14 d of adaptation, cows were housed individually in tie stalls for acclimatization to the diet and the restriction in movement. From d 15 of the adaptation period, cows were housed individually in identical climate respiration chambers (CRC) for 5 d of adaptation before the first experimental period began. Cows were housed in CRC for the entire experiment to facilitate determination of gaseous exchange, energy and N balance, and apparent total-tract nutrient digestibility (ATTD). Detailed descriptions of the CRC design and gas measurements are given by Heetkamp et al. (2015) and van Gastelen et al. (2015). Briefly, each CRC compartment measured 11.8 m^2 and had a volume of 34.5 m^3. The ventilation rate was 43 m^3/h, relative humidity was maintained at 65%, and temperature at 16°C inside each compartment. The CRC were designed with thin walls equipped with windows to allow audio and visual contact between cows and minimize the effects of social isolation on behavior and performance. Cows were exposed to 17.5 h of light per d (0530 to 2300 h).

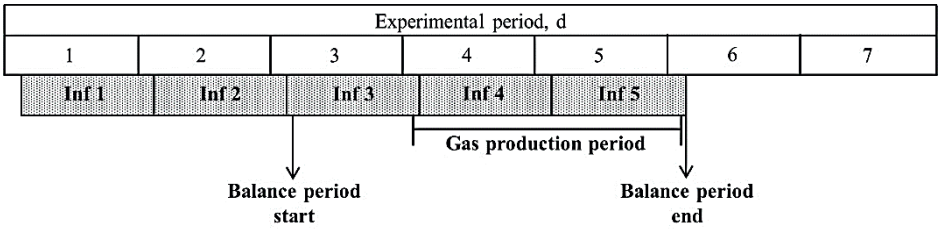

Figure 7.1. Design of a single 7-d experimental period. Inf 1 – Inf 5 = 120-h infusion period beginning at 0900 h on d 1 and ending at 0900 h on d 6 of each experimental period. The infusion period was followed by a 48-h wash-out period. Balance period = 71-h period of total manure collection with milk and feces samples from 1000 h on d 3 until 0900 h on d 6. Gas production period = 48-h period of gas production and consumption measured from 0800 h on d 4 until 0800 h on d 6.

Gas concentrations and ventilation rates were corrected for pressure, temperature, and relative humidity to arrive at standard temperature and pressure dew point volumes of inlet and exhaust air. Consumption of O_2 and production of CO_2 and CH_4 inside each chamber was calculated from the difference between inlet and exhaust gas volumes. Gas analysis measurement in this experiment was performed as described by van Gastelen et al. (2015), where 4 CRC compartments shared a single gas analyser, but with the addition of a second gas analyser measuring gas from the 1 additional CRC compartment in 6-min intervals. Calibration gases were sampled once daily instead of the inlet air. The analyzed and actual values of these calibration gases were used to correct the analyzed gas concentrations from the inlet and exhaust air of the 5 chambers. Before the experiment started, CO_2 recovery was checked by releasing known amounts of CO_2 into each chamber and comparing the known values with data from the gas analysis system to calculate the recovery. The recovered amounts of CO_2 were between 99 and 100% (99.3 ± 0.41%). Gas measurements during time points when staff entered the CRC compartments (maximum 30 minutes for milking, feeding, checking abomasal infusion lines) were discarded from the data analysis. Consumption of O_2 and production of CO_2 and CH_4 was assumed to be linear between the last data point before opening and the first data point after closing the CRC.

Diet, Feeding, and Treatment Infusions

Cows were fed a TMR consisting of 58% corn silage, 16% alfalfa hay, and 26% concentrate on a DM basis (**Table 7.1**), formulated to meet 100 and 83% of NE_L and MP requirements (CVB, 2008), respectively, for cows consuming 21 kg DM/d and producing 33 kg/d of milk containing 4.1% fat and 3.4% protein. Cows had individual and free access to drinking water throughout the entire experiment. Cows were fed ad libitum for the first 10 d of the 19-d adaptation period. Intake during the final 5 d of this 10-d ad libitum intake period was used to calculate a 10% daily intake restriction for individual cows. Cows were fed this fixed amount from d 11 of adaptation for the remainder of the adaptation and experimental

periods described above. Fresh feed was allocated twice daily at 0530 and 1530 h by manually mixing the roughage and concentrate portions into a TMR for individual cows. The roughage portion (corn silage + alfalfa hay) of the diet was mixed twice weekly and stored at 4°C for no longer than 4 d before feeding. The concentrate contained 0.25% titanium dioxide as an inert marker for estimation of ATTD. Feed refusals at each feeding time point were collected and weighed to determine daily feed intake. For a 58-h period over d 3 to d 5 of each experimental period (0530 h on d 3 until 1530 h on d 5), cows were fed using an automated feeding system which dispensed equal portions of feed every 2 h to promote metabolic steady-state conditions in preparation for the blood sampling protocol described below.

Table 7.1. Ingredient composition of TMR and analyzed and calculated chemical composition of ingredients (corn silage, alfalfa hay, and concentrate) and complete TMR (g/kg DM, unless otherwise noted)

	Ingredient			
Item	Corn silage	Alfalfa hay	Concentrate[1]	TMR[2]
Inclusion	580	157	263	-
Chemical composition				
DM, g/kg	334	896	898	456
Gross energy, MJ/kg DM	18.8	18.2	17.0	18.2
Crude ash	37	92	115	66
CP	77	120	252	130
Crude fat	30	13	35	28
NDF	372	549	167	346
ADF	210	435	84	212
ADL	10	86	15	23
Starch	347	NA[3]	287	277
Sugar	NA	46	79	28
DVE[4]	55	54	135	76
OEB[5]	-45	2	106	2
NE_L,[6] MJ/kg DM	6.99	4.55	8.07	6.89

[1]Contained (g/kg DM): ground corn 8% CP, 406; solvent-extracted rapeseed meal 34% CP, 182; beet pulp 19% sugar, 164; soybean meal 49% CP, 127; urea, 23; limestone 37% Ca, 19; sodium bicarbonate, 18; magnesium sulphate, 16; magnesium oxide, 13; Hidropalm, 12; monocalcium phosphate, 9; trace mineral and vitamin premix, 7; NaCl, 6; TiO2 was included at 0.25% of concentrate DM.

[2]Values for TMR were calculated based on ration composition and analyzed and calculated values obtained for roughages and concentrate.

[3]NA = not analyzed.

[4]Intestinal digestible protein (CVB, 2008).

[5]Rumen degradable protein balance (CVB, 2008).

[6]NE_L calculated with the VEM system (CVB, 2008).

Infusion lines were placed in the abomasum via the rumen cannula 2 d before the first experimental period and were checked daily for patency and position. The abomasal infusion device has been described by Nichols et al. (2019). Infusion treatments were 0.9% saline (**SAL**) or 562 g/d of AA delivered in 4 different profiles (**Table 7.2**) consisting of 1) a complete EAA mixture (**EAAC**), 2) Ile, Leu, and Val (**BCAA**), 3) His, Ile, Leu, Met, Phe, Trp, Val (**GR1+ILV**), and 4) Arg, His, Lys, Met, Phe, Thr, Trp (**GR1+ALT**). Within each AA infusion, EAA were infused in amounts relative to their content in 1 kg casein, according to Metcalf et al. (1996). Including intake from the restricted feeding level of the basal diet plus the infusions, target requirements for NE_L and MP were formulated to be met to 90 and 75%, respectively, for SAL, and 95 and 104%, respectively, for AA infusions. All AA were provided by Ajinomoto Animal Nutrition Europe (Paris, France) and Ajinomoto Omnichem (Wetteren, Belgium) with the exception of DL-Met which was provided by Adisseo France (Malicorne, France). Treatment solutions were administered in 15-L batches which were replenished daily and infused via multi-channel peristaltic pumps at a rate of 10.4 mL/min to facilitate 120-h of continuous infusion (0900 h on d 1 until 0900 h on d 6 of each experimental period; Figure 7.1).

Table 7.2. Composition of abomasal AA infusions[1]

	Treatment[2]			
Item	EAAC	BCAA	GR1+ILV	GR1+ALT
AA, g/d				
L-Arg	39	0	0	64
L-His	32	0	47	52
L- Ile	57	150	84	0
L-Leu	94	245	138	0
L-Lys	98	0	0	159
DL- Met	27	0	40	44
L-Phe	94	0	138	152
L-Thr	42	0	0	68
L-Trp	14	0	21	23
L-Val	64	167	94	0
Total	562	562	562	562
Gross energy,[3] MJ/d	13.6	15.0	14.6	12.8
N,[4] g/d	81	62	66	93

[1]0.9% saline infusion was the negative control treatment and supplied no AA, gross energy, or N.

[2]EAAC, Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; BCAA, Ile, Leu, Val; GR1+ILV, His, Ile, Leu, Met, Phe, Trp, Val; GR1+ALT, Arg, His, Lys, Met, Phe, Thr, Trp. All AA treatments supplied 562 g/d metabolizable protein where AA were infused in amounts relative to their content in casein.

[3]Calculated based on the heat of combustion of individual AA in the infusate.

[4]Calculated based on the molar weight of N in individual AA in the infusate.

Measurements and Sample Collection

Energy and N balance and ATTD were based on manure and feces collection from d 3 (1000 h) through d 6 (0900 h) (balance period; Figure 7.1), whereas O_2 consumption and CO_2 and CH_4 production were based on data recorded from d 4 (0800 h) through d 6 (0800 h) of each experimental period. Each CRC compartment was cleaned at 0900 h on d 3 (taking approximately 60 min) to remove all manure collected from the end of the previous period to facilitate a fresh total collection period. At the end of each balance period (which corresponded with the end of the infusion period), cows were weighed. The manure from each compartment produced during the 71-h balance period was separately and quantitatively collected, weighed, and mixed. Manure samples were collected and stored at -20°C until analysis. In addition, to quantify contribution of N from volatilized ammonia appearing from excreted and mixed urine and feces, samples of condensed water from the chamber heat exchanger and from 25% sulphuric acid solution (wt/wt), through which outflowing air was led to trap aerial ammonia, were collected from each CRC compartment. These samples were stored at 4°C until analysis. During the balance period, feces was collected by rectal grab sampling at 0530 and 1530 h (6 samples) and immediately pooled into a composite sample by cow which was stored at -20°C until analysis. Feed refusals, when present, were collected during the balance period and stored at 4°C. After each balance period they were pooled by cow, sampled, and stored at -20°C until analysis.

Cows were milked twice daily at 0530 and 1530 h during the adaptation and experimental periods. Milk weight was recorded at each milking, and samples were collected at each milking into tubes containing sodium azide and stored at 4°C until analysis within 4 d. An additional milk sample (5 g/kg milk) was collected separately and pooled by cow at each milking during the balance period (6 milkings) and stored at -20°C until GE and N analyses. Samples of corn silage, alfalfa hay, and concentrate were collected twice weekly during feed preparation. These samples were pooled per experimental period and stored at -20°C until analysis. On d 4 of each experimental period, blood samples were collected by venipuncture from the coccygeal vessels into 10 mL sodium heparin and potassium EDTA Vacutainers (Becton Dickinson, Rutherford, NJ) at 0730, 0930, 1130, 1330, and 1530 h. After each sampling point, collection tubes were immediately placed in ice and centrifuged at 3,000 × *g* at room temperature for 15 min. Plasma was pooled over sampling time points by cow and period and stored at -20°C until analysis.

Analytical Procedures

Samples of corn silage, alfalfa hay, concentrate, manure, and feces were thawed at room temperature, oven-dried at 60°C until a constant weight was reached, and ground to pass a 1-mm screen using a Wiley mill (Peppink 100AN, Olst, the Netherlands). Wet chemical analysis for DM, ash, N, NH_3, crude fat, starch, sugars, NDF, ADF, ADL, and titanium was performed as described by Nichols et al. (2018). Crude protein content was calculated as

total analyzed N × 6.25. An adiabatic bomb calorimeter (IKA-C700, Janke and Kunkel, Heitersheim, Germany) was used for determination of GE content (ISO 9831; ISO, 1998). Corn silage, alfalfa hay, and concentrate samples were analyzed for DM, ash, N, crude fat, starch (except alfalfa hay), sugars (except corn silage), NDF, ADF, ADL, GE, and titanium (concentrate only). Samples of refused feed were analyzed for DM. Manure samples were analyzed for DM, N, and GE. Feces samples were analyzed for DM, ash, N, crude fat, starch, NDF, GE, and titanium. In addition, samples of condensed water and the sulphuric acid solution were analyzed for N. Reported values for nutrient content of the TMR were calculated from ration composition and analyzed values obtained for the roughage and concentrate. The NE_L was calculated with the VEM (feed unit lactation) system according to Van Es (1978). Reported DVE (intestinal digestible protein), OEB (rumen degradable protein balance), and NE_L were obtained by near-infrared spectroscopy analysis for corn silage and alfalfa hay (Eurofins Agro, Wageningen, the Netherlands). For the concentrate, DVE, OEB, and NE_L were calculated based on table values for composition of the ingredients (CVB, 2008). For the TMR, these were calculated from ration composition of all roughage and concentrate ingredients.

Milk samples from the morning and afternoon milkings were analyzed separately for protein, fat, lactose, and urea by mid-infrared spectroscopy (ISO 9622; ISO, 2013; VVB, Doetinchem, the Netherlands). Pooled milk samples were analyzed for GE and N in fresh material as described above. Blood plasma was analysed by the Veterinary Diagnostic Laboratory (Utrecht University, the Netherlands) as described by van Knegsel et al. (2007).

Calculations and Statistical Analysis

Heat production (kJ/d) was calculated as $16.175 \times VO_2$ (L/d) + $5.021 \times VCO_2$ (L/d) where VO_2 and VCO_2 are volumes of O_2 consumed and CO_2 produced, respectively (Gerrits et al., 2015). Apparent total tract digestibility was calculated considering the nutrient inflow from the diet and the treatment infusions. The infusion treatments contributed DM, ash, OM, CP and GE. Dry matter of the infusions was comprised of the infusion ingredients (assumed to the 100% DM), ash from the saline (99 g/d NaCl) and EAA infusions, and hydroxide from mixing the AA solutions (77 g/d NaOH and 50 g/d HCl were used to facilitate EAA mixing). Nitrogen content of the infusions was calculated based on the molar weight of N in individual AA in the infusate. The GE content of the AA infusions were calculated based on the heat of combustion of individual AA in the infusate, and digestibility of all infusions was assumed to be 100%.

Milk yield, milk composition and DMI were averaged over the 3-d balance period. One cow did not receive the correct treatment in period 1 and was thus removed from the statistical analysis for this period (n = 4 for GR1+ALT; n = 5 for all other treatments). Variances in lactation performance, energy and N balance, digestibility, and plasma constituents were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The model

contained treatment and period as fixed effects and cow as a random effect. We observed no carryover effects between periods, assessed by testing for an effect of the previous treatment in the ANOVA. Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. Multiple comparisons between treatment means were made using the Tukey-Kramer method.

RESULTS

Dry Matter Intake, Milk Production, and Digestibility

Infusion of BCAA tended to decrease DMI compared with EAAC, GR1+ILV, and GR1+ALT ($P = 0.10$; **Table 7.3**). Infusion of EAAC increased milk yield and FPCM yield 5.0 and 3.4 kg/d, respectively, over SAL ($P \leq 0.03$). Similarly, GR1+ILV increased milk yield and FPCM yield 5.0 and 3.6 kg/d, respectively, over SAL ($P \leq 0.03$). Milk fat yield was not affected by treatment ($P = 0.52$), but BCAA increased milk fat concentration over EAAC ($P = 0.04$). Infusion of BCAA produced the same level of milk protein yield as SAL ($P > 0.10$) and decreased it compared with EAAC and GR1+ILV ($P \leq 0.02$), while infusion of EAAC and GR1+ILV increased milk protein yield 224 and 187 g/d, respectively, over SAL ($P \leq 0.02$). Infusion of BCAA decreased milk protein concentration 10% compared with EAAC ($P = 0.03$). The ratio between protein and fat content in milk was not different between SAL and BCAA ($P = 0.87$), and increased with EAAC compared with SAL ($P = 0.03$). The ratio decreased with BCAA infusion compared with EAAC ($P = 0.01$), and tended to decrease with BCAA infusion compared with GR1+ILV ($P = 0.06$). Milk lactose yield was not different between SAL and BCAA ($P > 0.10$), but increased on average 188 g/d over SAL when EAAC, GR1+ILV, or GR1+ALT were infused ($P = 0.05$). Infusion of BCAA increased lactose concentration 0.16 units compared with EAAC and GR1+ILV ($P \leq 0.02$). Milk urea concentration was not affected by treatment ($P = 0.41$).

Apparent total-tract digestibility of DM and OM differed between BCAA and GR1+ALT ($P = 0.04$; **Table 7.4**). All AA infusions increased CP digestibility compared with SAL (average 12% increase; $P < 0.01$). Starch digestibility tended to be higher with BCAA compared with SAL ($P = 0.06$) and GR1+ALT ($P = 0.09$). Gross energy digestibility was higher with BCAA compared with SAL ($P = 0.03$) and GR1+ALT ($P = 0.04$). Digestibility of NDF and crude fat were not affected by treatment ($P > 0.27$).

Table 7.3. Performance of lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d[1]

	Treatment[2]						
Item	SAL	EAAC	BCAA	GR1+ILV	GR1+ALT	SEM	*P*-value
DMI,[3] kg/d	19.2	19.8	17.9	19.8	19.9	1.02	0.06
Yield							
Milk, kg/d	29.4[a]	34.4[b]	30.8[ab]	34.4[b]	33.3[ab]	2.00	0.01
Fat, g/d	1247	1281	1315	1321	1273	62.3	0.52
Protein, g/d	902[a]	1126[b]	907[a]	1089[b]	1034[ab]	51.8	<0.01
Lactose, g/d	1385[a]	1581[b]	1463[ab]	1575[b]	1563[b]	89.2	0.03
Composition, %							
Fat	4.33[ab]	3.84[b]	4.45[a]	3.98[ab]	4.00[ab]	0.307	0.03
Protein	3.08[ab]	3.28[a]	2.95[b]	3.18[ab]	3.14[ab]	0.099	0.04
Lactose	4.70[ab]	4.60[b]	4.75[a]	4.59[b]	4.69[ab]	0.038	0.01
Protein:fat[4]	0.72[a]	0.88[b]	0.68[a]	0.81[ab]	0.80[ab]	0.055	0.01
FPCM,[5] kg/d	30.1[a]	33.5[b]	31.3[ab]	33.7[b]	32.5[ab]	1.29	0.02
Milk urea, mg/dL	11.2	11.2	11.2	12.8	13.5	2.03	0.41

[a,b]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Data are least squares means from the final 3 d of infusion.

[2]SAL, 0.9% saline; EAAC, Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; BCAA, Ile, Leu, Val; GR1+ILV, His, Ile, Leu, Met, Phe, Trp, Val; GR1+ALT, Arg, His, Lys, Met, Phe, Thr, Trp. All AA treatments supplied 562 g/d metabolizable protein where AA were infused in amounts relative to their content in casein.

[3]Diet only.

[4]Protein % ÷ fat %.

[5]Fat- and protein-corrected milk (FPCM; kg/d) = (0.337+ 0.116 × fat % + 0.06 × protein %) × milk yield (kg/d) (CVB, 2008).

Table 7.4. Apparent total-tract digestibility (%) of nutrients in lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d[1]

	Treatment[2]						
Item	SAL	EAAC	BCAA	GR1+ILV	GR1+ALT	SEM	*P*-value
DM	67.8[ab]	67.3[ab]	68.9[a]	68.3[ab]	66.4[b]	0.97	0.05
OM	69.3[ab]	68.8[ab]	70.4[a]	69.9[ab]	67.9[b]	0.98	0.05
CP	62.0[a]	70.8[b]	69.9[b]	68.4[b]	69.8[b]	0.90	<0.01
NDF	43.1	42.3	45.2	44.4	40.3	2.28	0.27
Crude fat	71.2	72.7	71.6	70.6	71.9	0.98	0.59
Starch	97.4	97.5	98.3	97.5	97.4	0.38	0.05
Gross energy	66.7[a]	67.4[ab]	69.3[b]	68.5[ab]	66.6[a]	0.88	0.02

[a,b]Means within a row with no common superscripts differ ($P < 0.05$).

[1]Data are least squares means calculated from feed and feces sampled during the final 3 d of infusion and were calculated considering the total nutrient inflow from the diet + infusions.

[2]SAL, 0.9% saline; EAAC, Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; BCAA, Ile, Leu, Val; GR1+ILV, His, Ile, Leu, Met, Phe, Trp, Val; GR1+ALT, Arg, His, Lys, Met, Phe, Thr, Trp. All AA treatments supplied 562 g/d metabolizable protein where AA were infused in amounts relative to their content in casein.

Energy and Nitrogen Balance

Metabolic BW was not affected by treatment ($P = 0.24$; **Table 7.5**). Total GE intake (GEI) was not different between AA infusions and SAL ($P \geq 0.30$), but tended to be decreased with BCAA compared with EAAC ($P = 0.07$) and GR1+ILV ($P = 0.10$). Energy output in manure and daily CH_4 production were not affected by treatment ($P \geq 0.50$). Metabolizable energy intake (MEI) was lower with BCAA compared with EAAC ($P = 0.03$), and tended to be lower with BCAA compared with GR1+ILV ($P = 0.08$) and GR1+ALT ($P = 0.10$). The ratio of MEI to GEI was not affected by treatment ($P = 0.42$). All AA infusions tended to increase heat production compared with SAL ($P = 0.07$). Compared with SAL, energy output in milk increased with EAAC ($P = 0.02$) and tended to increase with GR1+ILV ($P = 0.06$). Infusion of BCAA tended to decrease milk energy output compared with EAAC ($P = 0.10$). Total energy retention (ER) and ER in fat decreased with BCAA compared with SAL ($P \leq 0.05$). Infusion of EAAC increased and tended to increase ER in protein compared with SAL ($P = 0.03$) and BCAA ($P = 0.09$), respectively. The respiratory quotient (RQ) decreased with BCAA compared with SAL ($P = 0.04$), but did not differ across AA infusions ($P > 0.60$).

Nitrogen intake increased with all AA infusions compared with SAL ($P \leq 0.02$), but was lower with BCAA infusion compared with EAAC, GR1+ILV, and GR1+ALT ($P \leq 0.04$). Manure N output was higher with BCAA, GR1+ILV, and GR1+ALT infusions compared with SAL ($P \leq 0.02$), and was higher with GR1+ALT infusion compared with EAAC ($P = 0.02$). Infusion of BCAA decreased fecal N output compared with SAL ($P = 0.05$), and tended to decrease it compared with GR1+ILV ($P = 0.06$) and GR1+ALT ($P = 0.07$). The proportion of N intake excreted in feces decreased with all AA infusions compared with SAL ($P < 0.01$). Urine N output was higher with BCAA, GR1+ILV, and GR1+ALT ($P \leq 0.03$), and tended to be higher with EAAC ($P = 0.06$), compared with SAL. The proportion of N intake excreted in urine increased over SAL and EAAC with BCAA infusion ($P \leq 0.04$), and tended to increase over SAL with GR1+ALT ($P = 0.10$). Milk N output increased with EAAC and GR1+ILV ($P \leq 0.01$) and tended to increase with GR1+ALT ($P = 0.10$) compared with SAL. Milk N output on BCAA infusion was lower compared with EAAC and GR1+ILV ($P \leq 0.01$) and tended to be lower than GR1+ALT ($P = 0.08$). Nitrogen trapped in condensed water and air increased with BCAA compared with SAL ($P = 0.02$). Nitrogen retention increased with EAAC compared with SAL ($P = 0.03$), and tended to decrease with BCAA compared with EAAC ($P = 0.09$). Milk N efficiency was not different between SAL, EAAC, and GR1+ILV ($P > 0.70$). Milk N efficiency decreased with BCAA infusion compared with SAL, EAAC, and GR1+ILV ($P \leq 0.03$). Infusion of GR1+ALT decreased milk N efficiency compared with EAAC ($P = 0.02$) and tended to decrease it compared with GR1+ILV ($P = 0.08$).

Table 7.5. Energy and nitrogen balance of lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d

	Treatment[1]						
Item	SAL	EAAC	BCAA	GR1+ILV	GR1+ALT	SEM	*P*
Metabolic BW[2], $kg^{0.75}$	123	123	123	124	124	5.2	0.24
Energy balance, kJ/kg $BW^{0.75}$/d, unless otherwise stated							
GEI[3]	2846	3048	2749	3023	3036	107.8	0.04
Energy in manure	913	1003	975	1018	1029	59.2	0.50
CH_4 production	189	187	186	192	185	9.1	0.81
MEI[4]	1744^{ab}	1857^{a}	1588^{b}	1813^{ab}	1823^{ab}	72.7	0.03
MEI:GEI[5], %	61.1	61.0	57.8	59.9	60.5	1.40	0.42
Heat production	950	1005	979	1000	1005	25.7	0.07
Energy in milk	747^{a}	838^{b}	771^{ab}	821^{ab}	806^{ab}	26.3	0.02
ER total[6]	47^{a}	15^{ab}	-162^{b}	-8^{ab}	10^{ab}	66.5	0.06
ER protein[7]	11^{a}	46^{b}	18^{ab}	23^{ab}	38^{ab}	7.7	0.03
ER fat[8]	36^{a}	-31^{ab}	-180^{b}	-31^{ab}	-26^{ab}	62.7	0.06
RQ[9]	1.17^{a}	1.14^{ab}	1.13^{b}	1.14^{ab}	1.14^{ab}	0.015	0.07
Nitrogen balance, mg/kg $BW^{0.75}$/d							
N intake[10]	3295^{a}	4034^{b}	3619^{c}	3913^{b}	4105^{b}	122.6	<0.01
N manure	2001^{a}	2188^{ab}	2268^{bc}	2289^{bc}	2464^{c}	85.0	<0.01
Fecal N[11]	1246^{a}	1180^{ab}	1090^{b}	1240^{ab}	1249^{ab}	66.0	0.04
Fecal N/N intake	0.40^{a}	0.29^{b}	0.30^{b}	0.32^{b}	0.30^{b}	0.009	<0.01
Urine N[12]	755^{a}	1008^{ab}	1179^{b}	1049^{b}	1219^{b}	58.4	<0.01
Urine N/N intake	0.23^{a}	0.25^{a}	0.32^{b}	0.27^{ab}	0.30^{ab}	0.017	0.01
N milk	1135^{a}	1445^{b}	1124^{a}	1366^{b}	1303^{ab}	42.1	<0.01
N condense + acid[13]	84^{a}	94^{ab}	109^{b}	102^{ab}	100^{ab}	15.5	0.03
N retention[14]	75^{a}	309^{b}	118^{ab}	156^{ab}	255^{ab}	52.0	0.03
Milk N efficiency[14], %	34.5^{ab}	35.8^{a}	31.1^{c}	34.9^{ab}	31.7^{bc}	0.89	<0.01

[a-c]Means within a row with no common superscripts differ ($P < 0.05$).

[2]SAL, 0.9% saline; EAAC, Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; BCAA, Ile, Leu, Val; GR1+ILV, His, Ile, Leu, Met, Phe, Trp, Val; GR1+ALT, Arg, His, Lys, Met, Phe, Thr, Trp. All AA treatments supplied 562 g/d metabolizable protein where AA were infused in amounts relative to their content in casein.

[2]The mean BW per cow per balance period was used to calculate metabolic BW ($BW^{0.75}$).

[3]GEI = gross energy intake (diet + infusions).

[4]Metabolizable energy intake = GEI - methane production - energy in manure.

[5]MEI:GEI = ratio between MEI and GEI.

[6]Energy retention total = MEI - heat production - energy in milk.

[7]Energy retention protein = protein gain (N × 6.25) × 23.6 kJ/g (energetic value of body protein).

[8]Energy retention fat = energy retention total - energy retention protein.

[9]Respiratory quotient.

[10]Diet + infusions.

[11]Fecal N = N intake × [1 – (CP digestibility/100)].

[12]Urine N = N manure – fecal N.

[13]N from condense collected from heat exchanger + N trapped from outflowing air.

[14]N retention = N intake (including infusate N) – N manure – N milk – N condense + acid.

[15]Milk N efficiency = (N milk/N intake) × 100.

Plasma Constituents

Arterial plasma concentrations of glucose, BHB, non-esterified fatty acids (NEFA), and triacylglycerides (TAG) were not affected by AA infusions ($P \geq 0.49$; **Table 7.6**). Plasma urea concentration increased with GR1+ALT compared with SAL and EAAC ($P \leq 0.05$), and tended to increase over SAL with BCAA ($P = 0.08$) and GR1+ILV ($P = 0.06$). Plasma insulin concentration tended to decreased with EAAC compared with SAL ($P = 0.06$), and decreased with EAAC compared with GR1+ILV ($P = 0.05$). Infusion of BCAA decreased plasma insulin concentration compared with SAL and GR1+ILV ($P < 0.01$).

Table 7.6. Arterial plasma concentrations of metabolites and insulin in lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d

	Treatment[1]						
Item[2]	SAL	EAAC	BCAA	GR1+ILV	GR1+ALT	SEM	*P*-value
Glucose, m*M*	3.64	3.64	3.58	3.36	3.71	0.140	0.49
BHB, m*M*	0.76	0.90	0.84	0.95	0.78	0.122	0.74
NEFA, µ*M*	64	74	86	68	80	16.1	0.78
TAG, µ*M*	66	66	58	66	65	4.1	0.51
Urea, m*M*	2.26^{a}	2.50^{a}	2.78^{ab}	2.80^{ab}	3.12^{b}	0.239	0.01
Insulin, mIU/L	20.5^{ab}	16.3^{bc}	13.8^{c}	20.6^{a}	16.9^{abc}	3.00	<0.01

$^{a-c}$Means within a row with no common superscripts differ ($P < 0.05$).

[1]SAL, 0.9% saline; EAAC, Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; BCAA, Ile, Leu, Val; GR1+ILV, His, Ile, Leu, Met, Phe, Trp, Val; GR1+ALT, Arg, His, Lys, Met, Phe, Thr, Trp. All AA treatments supplied 562 g/d metabolizable protein where AA were infused in amounts relative to their content in casein.

[2]NEFA = nonesterified fatty acids; TAG = triacylglycerol.

DISCUSSION

The aim of this study was to determine the effect of AA profile within a constant MP supply on milk production and whole-body energy and N partitioning. Notably, infusion of a complete EAA profile increased milk protein yield 25% compared with SAL, while milk N efficiency did not decrease. Removing Ile, Leu, and Val, or Arg, Lys, and Thr, from infusions did not impair milk production compared with a complete EAA profile, but milk N efficiency decreased when Ile, Leu, and Val were removed. Further, infusion of only Ile, Leu, and Val offered no improvement in milk production and reduced milk N efficiency. The fact that the infused MP level was kept constant across AA treatments in this study is an important difference compared with others testing subtractions of individual AA or groups of AA (Weekes et al., 2006; Doelman et al., 2015a,b; Lapierre et al., 2009). Because the AA infusions were iso-MP, they were consequently not isoenergetic or isonitrogenous, and the absolute amount of each individual AA infused differed with each treatment (Table 7.2). Metabolizable protein intake from the diet plus infusion of EAAC, GR1+ILV, or GR1+ALT supplied 107, 111, and 117% of calculated MP requirements based on observed DMI and milk production (CVB, 2008). These AA infusions increased total MP supply 138% over SAL. Based on MP intake

from the infusion and the observed DMI and milk production on BCAA infusion, this treatment supplied 125% of MP requirements and increased MP supply 132% over SAL.

All AA infusions increased ATTD of CP, which can be attributed to the greater digestibility of infused AA compared with basal diet digestibility, as this effect disappeared when ATTD was calculated considering only intake of the basal diet (data not shown). If the CP digestibility of 62% observed on SAL represents CP digestibility of the basal diet during AA infusions, calculated ATTD of the infused AA was 111, 103, 98, and 106% on EAAC, BCAA, GR1+ILV, and GR1+ALT, respectively. It is expected that dilution with endogenous protein would result in slight increases over the true value. Therefore, these values support our assumption that infused AA would be 100% digestible, and suggests that the capacity for intestinal AA absorption was not limited by high levels of individual AA in the incomplete EAA infusions.

Complete EAA infusion increased milk protein synthesis without decreasing milk N efficiency

The complete EAA profile in this experiment was intended as a positive control in order to compare the responses stimulated by the incomplete EAA profiles in the BCAA, GR1+ILV, and GR1+ALT infusions. In previous experiments feeding low CP diets to cows (11-14%), postruminal infusion of 562 g/d of EAA in the profile of casein stimulated an average increase of 3.2 kg/d of total milk and 164 g/d of milk protein compared with a saline control (Doelman et al., 2015a,b). Infusion of 1.5-times this level stimulated an average of 3.7 kg/d of total milk and 221 g/d of milk protein compared with a saline control (Nichols et al., 2016; Nichols et al., 2019). In the current experiment, the 562 g/d dose was used to illicit a clear response, while avoiding potentially too-high levels of individual AA in the incomplete infusions. As expected, EAAC increased total milk yield, protein yield, and lactose yield. Milk protein yield was increased 224 g/d with EAAC over SAL, resulting in 35% marginal use efficiency of infused EAA. This is greater than the predicted 25% marginal efficiency of casein if infused in the same 562 g/d dose (Huhtanen and Hristov, 2010), and the marginal use of the same EAA profile infused at 1.5-times this dose calculated from reports by Nichols et al. (2016) and Nichols et al. (2019), which ranged from 22 to 30%. Compared with casein, marginal efficiency is expected to be greater with EAA infusion, as NEAA in casein offer little towards increases in milk protein synthesis (Metcalf et al., 1996; Doepel and Lapierre, 2010). It also appears that within supplementation of EAA in a casein profile, their use can become more efficient at a lower dose than at a higher dose.

Compared with SAL, infusion of EAAC numerically increased GEI and MEI 202 and 113 kJ/kg $BW^{0.75}$/d, respectively, and the proportion of GEI recovered as MEI did not differ. Infusion of EAA, regardless of profile, tended to increase heat production, partly related to the rise in MEI. Supplying N in excess of requirement produces heat during biological transformations of N molecules, mainly from energy required for ureagenesis (Martin and Blaxter, 1965). Energy required for oxidative metabolism of RUP was found to be lower than

with RDP per unit supplied (Reed et al., 2017). The increase in N intake with abomasally infused AA represents 100% RUP (average increase of 76 g N/d across treatments). Based on the estimation of Reed et al. (2017) that 3.3 MJ of heat is produced per kg of RUP, metabolism of 562 g/d of infused AA would produce 15 kJ/kg $BW^{0.75}$/d of heat. This accounts for 34% of the average increase in heat production with EAA infusions over SAL. The lower RQ relative to SAL during EAA infusions suggests a portion of AA were catabolised for energy generation. These findings are in line with Nichols et al. (2019) who also reported a contribution to the incremental increase in heat production of 32% from metabolism of 844 g/d of abomasally infused EAA, and a drop in RQ from on average 1.12 without EAA infusion to 1.10 with EAA infusion.

Milk N output increased with the extra N intake on EAAC, resulting in the same milk N efficiency between EAAC and SAL. Manure N output was not affected, but N excretion tended to shift towards urine, which is in line with generally higher output of N in urine compared with faeces as N intake increases (Dijkstra et al., 2013) and can be explained by the high intestinal digestibility of infused EAA. A larger portion of N intake was retained in the body during EAAC infusion compared with SAL, and consequently energy deposited as body protein also increased. Body N retention reported here is in line with Nichols et al. (2019) in response to EAA infusion in the same profile, and with others who supplied postruminal AA to mid-lactation cows (Clark et al., 1977; Wright et al., 1998; Castillo et al., 2001).

Overall, EAAC infusion illustrated the positive effects of EAA-balanced MP with regards to milk production and milk N efficiency. Postruminal EAA supplementation in a complete casein profile increased milk protein yield, and extra N intake from EAA infusion was transferred into milk protein with the same efficiency as that on SAL. It therefore serves, as planned, as a positive control when reflecting on the efficacy of incomplete EAA profiles at the same level of MP supply.

BCAA infusion induced an AA imbalance and reduced milk N efficiency

In line with our hypothesis, milk production and whole-body energy and N balance differed most dramatically in response to BCAA infusion compared with that on EAAC. Compared with the other AA infusions, yield of total milk, protein, and lactose were, on average, 3.2 kg/d, 176 g/d, and 110 g/d lower with BCAA, and DMI tended to decrease by 1.8 kg/d. Under conditions of imbalanced AA intake, especially in low protein diets, feed intake depression is a homeostatic adaptation against a diet that is incompatible with maintenance of protein synthesis and regulation of AA concentrations in body fluids (Harper et al., 1970; Gietzen et al., 2007). In animals infused with imbalanced AA profiles, this response would be reflected in reduced intake of the basal diet. The severity of AA imbalance in a diet influences the magnitude and duration of observed hypophagic effects (Harper et al., 1970; Gietzen et al., 1993). With BCAA infusion, Ile, Leu, and Val were delivered at levels 2.6-times those in EAAC, and, to our knowledge, these infusion amounts in g/d are higher

than any reported previously in lactating ruminants. In the GR1+ILV and GR1+ALT infusions, the imbalances were relatively less severe, with AA present in amounts 1.5 and 1.6-times those in EAAC, respectively. Therefore, the severity of the imbalance may explain the observed hypophagic effect of BCAA infusion and why no difference in DMI in response to GR1+ILV and GR1+ALT infusions was observed.

The higher GE content of the BCAA infusion did not compensate fully for the lower DMI, and GEI from the diet plus infusion tended to be lower than EAAC. Gross energy digestibility was higher and starch digestibility tended to be higher than SAL, likely due to the lower DMI (Colucci et al., 1982; Huhtanen et al., 2006). Despite the 1.3 kg/d lower DMI compared with SAL, infusion of BCAA produced the same yield of total milk, protein, and lactose. Accordingly, milk energy output was not different from SAL with BCAA infusion, but energy balance was negative due to lower MEI. Decreased plasma insulin concentration in response to BCAA can be attributed to the depressed DMI, in favour of increased hepatic gluconeogenesis and AA release from skeletal muscle (Brockman and Laarveld, 1986; Lobley, 1998). Maintained arterial plasma glucose concentration between SAL and BCAA suggests increased glucose production by the liver or decreased glucose oxidation. Considering the lower DMI, decreased plasma insulin concentration, and the negative energy balance on BCAA infusion, TAG mobilization from adipose might be expected. However, arterial plasma concentrations of BHB, NEFA and TAG were not affected, suggesting substrates for gluconeogenesis and inputs to the TCA cycle arose from catabolism of infused branched-chain AA. The ruminant liver principally does not catabolize branched-chain AA (Lapierre et al., 2002; Raggio et al., 2004; Berthiaume et al., 2006) due to low tissue abundance of branched-chain aminotransferase, the enzyme required for transamination of branched-chain AA to their respective α-ketoacids (Brosnan and Brosnan, 2006). Instead, extensive metabolism of branched-chain AA in lactating ruminants occurs in the mammary gland (Bequette et al., 2001; Thivierge et al., 2002; Raggio et al., 2004) and in the gut (MacRae et al., 1997; El-Kadi et al. 2006), but also in skeletal muscle and adipose tissue (Vernon et al., 1985; Bequette et al., 2001). Glutamate produced during branched-chain AA transamination contributes to the synthesis of other NEAA, which can be mobilized from skeletal muscle or transported from the gut and used for gluconeogenesis, or can be used in support of milk protein synthesis in the mammary gland. Oxidation of branched-chain α-ketoacid intermediates, either in the liver or in peripheral tissues, yields acetyl-CoA (Leu and Ile) or succinyl-CoA (Ile and Val) which contribute anapleurotically to the TCA cycle. In agreement with increased AA catabolism on BCAA, a greater proportion of N intake was excreted via urine compared with SAL and EAAC, and plasma urea concentration tended to increase compared with SAL. Catabolism of AA for energy with BCAA infusion also agrees with the lower RQ compared with SAL.

Total N intake was higher on BCAA compared with SAL, but milk protein yield and N output in milk did not differ. Increased intramammary de novo NEAA synthesis from

branched-chain AA during BCAA infusion would support milk protein yield, partition circulating NEAA towards gluconeogenesis, and may spare some EAA from hepatic catabolism in favour of milk protein synthesis (Lapierre et al., 2005; Doepel and Lapierre, 2010; Nichols et al., 2016). Milk lactose yield was maintained between BCAA and SAL, in line with stimulated gluconeogenesis and repartitioning of glucogenic AA. Milk fat yield was not different across infusions, but milk fat content was higher on BCAA compared with EAAC. This higher milk fat content did not coincide with changes in circulating BHB, TAG, or NEFA concentrations. Maintained milk fat synthesis agrees with increased intramammary branched-chain AA catabolism, as decarboxylated branched-chain α-ketoacids can serve as primers for de novo synthesis of FA (Vernon et al., 1985; Crown et al., 2015). The larger increase in milk fat relative to milk protein with BCAA infusion compared with SAL adds credence to the AA imbalance on BCAA infusion, because in contrast to protein synthesis, FA can be produced from branched-chain AA as a product of their catabolism without requiring other AA. This is also reflected in the lower milk protein-to-fat content ratio on BCAA compared with EAAC and numerically lower protein-to-fat content ratio compared with SAL, a measure which is considered to be an indicator of dietary AA imbalance (Cant et al., 2001; Weekes et al., 2006). Milk N efficiency was highest with SAL and EAAC and lowest with BCAA, but milk urea concentration was 11.2 mg/dL with all 3 treatments. The relationship between milk urea and urinary N excretion is highly variable (Spek et al., 2013). In agreement with previous work where postruminal AA supply was increased (Nichols et al., 2018, 2019), milk urea concentration was not a good measure of overall N efficiency in the current study. There are clear differences in milk N efficiency, manure N excretion, and plasma urea concentration across SAL and AA infusions, but these differences were not detected in milk urea concentration.

In summary, supplementing 562 g/d of MP comprised of only Ile, Leu, and Val in the BCAA infusion reduced DMI, increased catabolism of infused AA, and decreased milk N efficiency compared with SAL and EAAC. Total milk yield and yield of milk protein, fat, and lactose was maintained at the same level as SAL, suggesting catabolism of infused branched-chain AA contributed to whole-body and mammary gland energy requirements in support of milk production. Compared with a complete EAA profile, supplementing a profile of only branched-chain AA induced an AA imbalance that was inhibitory to efficient milk protein synthesis, resulted in negative energy balance, and increased N excretion.

Similar milk production between EAAC, GR1+ILV, and GR1+ALT

Dry matter intake was not different between EAAC, GR1+ILV, and GR1+ALT. On average, infusion of GR1+ILV and GR1+ALT numerically increased GEI and MEI by 184 and 74 kJ/kg $BW^{0.75}$/d, respectively, over SAL, and neither GEI or MEI were appreciably different than that on EAAC. Infusion of GR1+ILV increased yield of total milk, protein, and lactose 5.0 kg/d, 187 g/d, and 190 g/d, respectively, over SAL, and milk fat yield did not differ. No difference

in milk or component production between EAAC and GR1+ILV indicates that Arg, Lys, and Thr were not required to stimulate the same level of milk production as a complete EAA profile if group 1 AA, Ile, Leu, and Val are increased to such an extent that supplemented MP level is not changed. This finding supports our hypothesis that the absence of Arg, Lys, and Thr from the infusion would have minimal effects on milk protein yield. Several studies have assessed subtractions of Lys on milk production, far fewer have assessed subtractions of Arg or Thr, and no study exists where these 3 AA are exclusively removed as a group. Subtraction of Arg from abomasal EAA infusions had no effect on total milk, protein, lactose, or fat production (Doepel and Lapierre, 2011; Haque et al., 2013). Doepel et al. (2016) observed no difference in milk or milk component production when Thr was removed from a complete AA infusion. The effect of postruminal Lys supplementation on milk and component production is variable (Robinson et al., 2010; Lee et al., 2015; Giallongo et al., 2016), but subtracting only Lys from a complete total AA or EAA profile, when its removal is not compensated in MP supply, consistently has negative effects on milk protein yield (Fraser et al., 1991; Weekes et al., 2006; Doelman et al. 2015b). The current results suggest that the negative effects of an apparent Lys shortage can be prevented by increasing other supplemented EAA to an equal level to compensate for Lys deletion. This suggests that when Lys is subtracted from AA supplements, the negative effects observed on milk protein synthesis are not necessarily due to Lys being the single limiting AA as such, but due to the fact that total AA supply becomes lower when Lys is subtracted.

Increases in total milk and protein yield on GR1+ALT compared with SAL were not significant, but were numerically considerable at 3.9 kg/d and 132 g/d, respectively, and were not different from their respective yields on EAAC. Similar to EAAC and GR1+ILV, milk lactose yield tended to increase (178 g/d) with GR1+ALT over SAL, and milk fat yield was not affected. These findings are in agreement with some previous studies testing branched-chain AA subtraction, individually or as a group. Weekes et al. (2006) observed no different in milk yield or composition when branched-chain AA were removed from a complete AA infusion. Subtractions of Ile or Val did not affect milk component production by cows (Haque et al., 2013), and absence of Leu from a mixture of 18 AA infused i.v. did not affect milk protein yield from goats (Bequette et al., 1996). In contrast, milk protein yield decreased 123 g/d while total milk and lactose yield were not affected when branched-chain AA were removed from an EAA infusion (Doelman et al. 2015b).

Milk protein synthesis in the absence of Arg, Lys, and Thr or in the absence of Ile, Leu and Val on GR1+ILV and GR1+ALT, respectively, may have been maintained through intramammary compensation between the group 2 AA to meet requirements for NEAA synthesis and energy. Relative to EAAC, the GR1+ILV profile increased the supply of Ile, Leu, and Val by 47%, and similarly Arg, Lys, and Thr supply increased 62% in the GR1+ALT profile. These substitutions in group 2 AA-N could have provided intramammary N and carbon in

support of milk component synthesis (Lapierre et al., 2012). Indeed, when infusions deficient in Arg, Ile, or Val were kept isonitrogenous with a complete EAA infusion using NEAA, there was no difference in milk protein yield or milk N efficiency (Haque et al., 2013).

While group 2 AA may compensate for each other with respect to intramammary N and carbon requirements, those EAA absent from GR1+ILV and GR1+ALT infusions cannot be synthesized de novo. An exception is Arg, which can be produced endogenously but not in adequate amounts to meet requirements of high-producing cows (Doepel et al., 2004). In order to support the increase in milk protein synthesis when GR1+ILV or GR1+ALT were infused compared with SAL, either metabolism of the EAA absent from the infusions was downregulated in splanchnic and peripheral tissues, their oxidation in the mammary gland was reduced, their mobilization from endogenous sources was stimulated, or a combination of these processes occurred. Amino acid fluxes through splanchnic tissues are sensitive to changes in feeding and MP level (Lapierre et al., 2000; Blouin et al., 2002; Raggio et al., 2004), but are less defined in lactating cows under conditions of altered duodenal supply of individual AA or to AA profiles (Bach et al., 2000; Berthiaume et al., 2006; Tagari et al., 2008). Net Lys removal by the liver in dairy cows is negligible, particularly at low MP supplies and under conditions of apparent Lys deficiency (Raggio et al., 2004; Lapierre et al., 2009), so with GR1+ILV infusion, Lys catabolism was likely decreased in peripheral tissues. Furthermore, hepatic Thr catabolism is flexible, and may have decreased in response to the reduced availability (Lapierre et al., 2005), and contribution from de novo Arg synthesis could have increased or its catabolism decreased. With GR1+ALT infusion, Ile, Leu, and Val catabolism in the mammary gland could have decreased, as a portion of their intramammary catabolism may be non-obligate (Bequette et al., 1996; Bequette et al., 2001). Branched-chain AA also could have been mobilized from skeletal muscle, supported by the numerically lower plasma insulin concentration on GR1+ALT compared with SAL.

In summary, whether Arg, Lys, and Thr, or Ile, Leu, and Val, are absent from a complete EAA profile, the same level of total milk, protein, fat, and lactose yield can be achieved as on a complete EAA profile if the other 7 EAA are present and together deliver the same MP supply. Due to their similar dynamics with regard to mammary gland utilization, group 2 AA may reciprocally compensate N and carbon for milk component synthesis when, as a group, their supply in MP is relatively low.

The GR1+ALT profile achieved lower milk N efficiency than GR1+ILV and EAAC

The most notable difference between GR1+ILV and GR1+ALT when compared with EAAC is the resulting milk N efficiency. The numerical differences in N intake across EAAC, GR1+ILV, and GR1+ALT are due to the difference in N supplied in the infusions. Transfer of N from the diet plus the infusion into milk was achieved with the same efficiency between EAAC and GR1+ILV, in line with our hypothesis. Manure N output with GR1+ILV increased over SAL, but was not different from that on EAAC. Overall, N partitioning did not majorly differ in the

absence of Arg, Lys, and Thr relative to the complete EAA profile. We hypothesised that N partitioning would be affected differently in the absence of Ile, Leu, and Val compared with the complete EAA profile, and this was confirmed. Milk N efficiency was 4.1 and 3.2 units lower on GR1+ALT compared with EAAC and GR1+ILV, respectively, and there was a numerical 142 mg/kg $BW^{0.75}$/d difference in milk N output between EAAC and GR1+ALT. The absence of Ile, Leu, and Val in the GR1+ALT infusion stimulated quantitatively less milk protein synthesis than on EAAC, and marginal use efficiency of infused EAA on GR1+ALT (18%) was lower than that on EAAC (35%), contributing to the greater proportion of N intake excreted in urine compared with that on EAAC. The marginal EAA use efficiency in the absence of Arg, Lys and Thr on GR1+ILV infusion was 28%, which is closer to the marginal efficiency obtained with EAAC when compared with that the absence of Ile, Leu, and Val on GR1+ALT infusion. Indeed, infusion of GR1+ALT tended to decrease milk N efficiency (31.7%) compared with GR1+ILV (34.9%), and GR1+ALT resulted in greater N excretion in manure compared with EAAC.

Differences in N partitioning between GR1+ILV and GR1+ALT infusions could have been due to differences in metabolism of the group 2 AA present in the respective infusions, and to the higher supply of group 1 AA in the GR1+ALT infusion compared with GR1+ILV. Hepatic removal of His, Met, and Phe influences efficiency of transfer of absorbed AA into milk AA. The net flux of these EAA across the liver is generally equal to their mammary net flux and milk output (Lapierre et al., 2005). Metabolism of His, Met, and Phe at hepatic first-pass could have been altered by the absence of the respective group 2 AA in the infusions, but this is unlikely because of the distinction in affinity for hepatic or peripheral catabolism between these AA groups (Lobley and Lapierre, 2003; Lapierre et al., 2005). Because there is almost no net branched-chain AA uptake by the liver (Lapierre et al., 2002; Raggio et al., 2004), a relatively larger proportion of exogenous branched-chain AA on GR1+ILV would have been available for mammary gland uptake, as they composed 56% of the AA in this infusion. In contrast, when the GR1+ALT profile was infused, although liver removal of Lys is usually negligible (Raggio et al., 2004; Lapierre et al., 2009), Arg and Thr may have undergone relatively more hepatic catabolism at first-pass (Blouin et al., 2002; Tagari et al., 2008) compared with Ile, Leu, and Val in the GR1+ILV infusion. Furthermore, considering the fact that, relative to GR1+ILV, GR1+ALT supplied 10% more group 1 AA but produced 5% less milk protein, a greater amount of His, Met, and Phe could have been catabolised in the liver after mammary gland first-pass during GR1+ALT infusion compared with GR1+ILV. The suggestion of greater hepatic catabolism of these AA is supported by the higher plasma urea concentration on GR1+ALT compared with EAAC. This is also in line with the 28% marginal transfer efficiency of infused EAA on GR1+ILV being more comparable with the 35% on EAAC than the 18% on GR1+ALT.

In summary, supplementing the same level of MP in an EAA profile where Arg, Lys, and Thr are absent compared with a complete EAA profile achieved the same level of milk protein yield and milk N efficiency, and manure N excretion and body N retention did not differ. In contrast, supplementing an EAA profile where Ile, Leu, and Val are absent resulted in increased plasma urea concentration and manure N output and lower milk N efficiency compared with a complete EAA profile.

CONCLUSIONS

The same level of total milk, protein, fat, and lactose yield can be achieved as on a complete EAA profile whether Arg, Lys, and Thr, or Ile, Leu, and Val are absent from a postruminal EAA supplement, if the other 7 EAA are present to compensate the MP supply. Supplementing an EAA profile where Arg, Lys, and Thr were absent achieved the same level of milk N efficiency and N excretion in manure as a complete EAA profile. Absence of Ile, Leu, and Val resulted in lower milk N efficiency and higher manure N excretion compared with a complete EAA profile, and tended to result in lower milk N efficiency compared with the EAA profile without Arg, Lys and Thr. Compared with a complete EAA profile, supplementing only Ile, Leu, and Val reduced feed intake, was inhibitory to efficient milk protein synthesis, increased the proportion of N intake excreted in urine, and resulted in negative energy balance. Taking into account the interactions between individual AA and the total EAA profile when supplementing EAA into diets for dairy cattle may allow optimization of N utilization through repartitioning of AA between catabolism and anabolism.

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Chapter 8

Mammary gland amino acid metabolism of dairy cattle is flexible in response to postruminal infusion of different essential amino acid profiles

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ABSTRACT. This study investigated mammary gland metabolism in dairy cattle in response to supplemental metabolizable protein (MP) composed of different essential AA (EAA) profiles. Five multiparous, rumen-fistulated, Holstein-Friesian dairy cows (2.8 ± 0.4 lactations; 81 ± 11 d in milk) were abomasally infused according to a 5 × 5 Latin square design with saline (SAL) or 562 g/d of essential AA delivered in different profiles where individual AA content corresponded to their relative content in casein. The profiles consisted of 1) a complete essential AA mixture (EAAC), 2) Ile, Leu, and Val (BCAA), 3) His, Ile, Leu, Met, Phe, Trp, Val (GR1+ILV), and 4) Arg, His, Lys, Met, Phe, Thr, Trp (GR1+ALT). A total mixed ration (58% corn silage, 16% alfalfa hay, and 26% concentrate on a dry matter basis) was formulated to meet 100 and 83% of net energy and MP requirements, respectively, and was fed at 90% of ad libitum intake on an individual cow basis. Each experimental period consisted of 5 d of continuous abomasal infusion followed by 2 d of no infusion. Arterial and venous blood samples with respect to the mammary gland were collected on d 4 of each period. Milk protein yield did not differ between EAAC, GR1+ILV, and GR1+ALT, or between SAL and BCAA, and increased over SAL with EAAC and GR1+ILV. Arterial plasma concentrations and mammary net uptake of glucose, β-hydroxybutyrate, non-esterified fatty acids, triacylglycerides, and long-chain fatty acids were not affected by AA infusions. In general, increases in arterial AA concentrations reflected their inclusion levels in the infusates when compared with SAL, or when compared with EAAC. Mammary plasma flow increased with BCAA infusion compared with EAAC and GR1+ILV. Infusion of EAAC tended to increase mammary gland net uptake of total EAA, in particular that of group 2 AA, and decreased the mammary uptake to milk protein output ratio (U:O) of non-EAA compared with SAL. Infusion of BCAA increased mammary uptake of the branched-chain AA (Ile, Leu, and Val) and increased U:O of total EAA and of the branched-chain AA over all treatments. Uptake of branched-chain AA tended to be higher on GR1+ILV compared with GR1+ALT, and uptake of non-branched-chain group 2 AA (Arg, Lys and Thr) was higher on GR1+ALT compared with GR1+ILV. The U:O of non-branched-chain group 2 AA tended to decrease with GR1+ILV infusion compared with EAAC, and this arose primarily from a decrease in the U:O of Lys. During GR1+ALT infusion, U:O of non-branched-chain group 2 AA was greater than that during EAAC infusion, whereas U:O of individual branched-chain AA were not different from EAAC. In conclusion, when Ile, Leu, and Val or Arg, Lys, and Thr were absent from abomasal EAA infusions, intramammary catabolism of the present group 2 AA compensated in support of milk protein synthesis, particularly when Lys uptake was depressed. Intramammary catabolism of Ile, Leu, and Val supported milk protein synthesis during BCAA infusion, and likely contributed significantly to the synthesis of milk fat and lactose over their use for NEAA synthesis.

Key Words: essential amino acid, metabolizable protein, mammary gland, protein synthesis, amino acid imbalance

INTRODUCTION

In lactating cattle, mammary gland sequestration of AA into milk protein plays a key role in the efficiency of transfer of dietary N into milk N. The majority of AA uptake in the splanchnic bed over that required for obligate processes arises from AA delivered through the arterial supply and not during absorption or first-pass metabolism (Lobley and Lapierre, 2003). Therefore, AA sequestration in peripheral tissues, including the mammary gland, impacts the profile and supply of AA entering the portal-drained viscera (PDV) and the liver from peripheral circulation. The incremental efficiency of absorbed AA use will be determined primarily by the response of milk protein synthesis and secretion to the supply of EAA (Haque et al., 2012; Haque et al., 2015). In order for increased mammary EAA supply to effect uptake by the gland, accompanying increases in milk protein synthesis, tissue protein accretion, or intramammary AA catabolism must occur (Cant et al., 2018). Of the EAA, mammary gland net transfer of group 1 AA (His, Met, Phe+Tyr, and Trp) into milk canonically occurs in a 1:1 ratio with their uptake from arterial circulation. The defining characteristic of group 2 AA (Arg, Ile, Leu, Lys, Thr, and Val) is their excess mammary net uptake relative to their output in milk (Mepham, 1982; Lapierre et al., 2012). Intramammary metabolism of the excess group 2 AA, particularly the branched-chain AA (Ile, Leu, and Val; Roets et al., 1979a; Roets et al., 1983; Bequette et al., 2006), and Arg and Lys (Mepham and Linzell, 1967; Mabjeesh et al., 2000; Lapierre et al., 2009), provides substantial N and carbon for de novo NEAA synthesis and for glycolytic and tricarboxylic acid cycle intermediates. The mammary gland may adapt to deficiencies of single EAA by altering the rate of blood flow to the tissue (Bequette et al., 1996, 2000; Doepel et al., 2016), cellular AA transporter activity (Baumrucker, 1985; Maas et al., 1998; Bequette et al., 2000), the level of intramammary AA catabolism (Lapierre et al., 2009), the rate of protein synthetic activities (Doelman et al., 2015a,b), or through a combination of these, in an effort to maintain milk protein synthesis.

Previous studies have evaluated effects of EAA deficiencies in duodenal supply, where single AA or groups of AA were subtracted from postruminal infusions of complete AA profiles (Weekes et al., 2006; Doelman et al., 2015a,b), but fewer have examined the effects on mammary gland metabolism (Doepel and Lapierre, 2011; Haque et al., 2015; Doepel et al., 2016). The current experiment examined mammary gland responses to incomplete EAA profiles, with the general hypothesis that when supplemental MP level is constant but arises from incomplete EAA profiles, intramammary catabolism of the supplemented EAA would increase in support of milk protein synthesis. This hypothesis arose from previously reported results of the same study (Nichols et al., 2019a), where cows postruminally supplemented with 562 g/d of EAA in profiles where Arg, Lys, and Thr, or Ile, Leu, and Val were absent produced the same level of total milk, protein, fat, and lactose as compared with infusion of a complete EAA profile at the same dose. Furthermore, infusion of only Ile, Leu, and Val resulted in lower milk protein yield compared with the complete EAA profile, but supported

the same level of milk protein production as the negative control (saline), despite lower feed intake. Based on the similar milk protein yield produced with supplementation of a complete EAA profile and those lacking Arg, Lys, and Thr or Ile, Leu, and Val, we hypothesized that intramammary catabolism of those EAA would decrease when they were absent from the infusion, and would increase when they were present. We expected intramammary catabolism of the branched-chain AA to increase with infusion of only Ile, Leu, and Val.

MATERIALS AND METHODS

Experimental Design

All experimental procedures were conducted under the Dutch Law on Animal Experiments in accordance with EU Directive 2010/63. The experimental design, animal housing, ration composition and preparation, and feed chemical analyses have been described in detail by Nichols et al. (2019a). Briefly, the effects of EAA profiles within a constant supplemented MP level were tested using 5 rumen-fistulated, Holstein-Friesian dairy cows (2.8 ± 0.4 lactations; 81 ± 11 DIM) randomly assigned to a 5 × 5 Latin square design, in which each experimental period consisted of 5 d of continuous abomasal infusion followed by 2 d of no infusion. Cows were housed individually in identical climate respiration chambers (CRC; described in detail by van Gastelen et al. (2015)), and were allowed 5 d of adaptation to the CRC environment before the first experimental period began. Cows were fed a TMR (13% CP) consisting of 58% corn silage, 16% alfalfa hay, and 26% concentrate on a DM basis which was formulated to meet 100 and 83% of NEL and MP requirements (CVB, 2008), respectively, for cows consuming 21 kg DM/d and producing 33 kg/d of milk containing 41 g/kg fat and 34 g/kg protein. Daily feed intake for individual cows was restricted to 90% of individual daily ad libitum intake determined during a 10-d diet-adaptation period in tie stalls before cows entered the CRC. Fresh feed was allocated twice daily during the entire experiment, with the exception of a 58-h window over d 3 to d 5 of each period (from 0530 h on d 3 until 1530 h on d 5), where an automated feeding system dispensed equal portions of feed every 2 h to promote metabolic steady-state conditions in preparation for the blood sampling protocol described below. Cows had individual and free access to drinking water throughout the entire experiment.

Infusion lines were placed in the abomasum via the rumen cannula 2 d before the first experimental period and were checked daily for patency and position. Abomasal infusion treatments were 0.9% saline (**SAL**) and 4 different AA profiles (562 g/d of AA; **Table 8.1**) consisting of 1) a complete EAA mixture (**EAAC**), 2) Ile, Leu, and Val (**BCAA**), 3) His, Ile, Leu, Met, Phe, Trp, Val (**GR1+ILV**), and 4) Arg, His, Lys, Met, Phe, Thr, Trp (**GR1+ALT**). Within each AA infusion, EAA were infused in amounts relative to their content in 1 kg casein, according to Metcalf et al. (1996). Including intake from the restricted feeding level of the basal diet plus the infusions, target requirements for NE_L and MP were formulated to be met to 90 and

75%, respectively, for SAL, and 95 and 104%, respectively, for AA infusions. Treatment solutions were administered in 15-L batches which were replenished daily and infused via multi-channel peristaltic pumps at a rate of 10.4 mL/min to facilitate 120-h of continuous infusion.

Table 8.1. Rate of abomasal AA infusion (g/d)

	Treatment[1]			
Item	EAAC	BCAA	GR1+ILV	GR1+ALT
AA				
L-Arg	39	0	0	64
L-His	32	0	47	52
L- Ile	57	150	84	0
L-Leu	94	245	138	0
L-Lys	98	0	0	159
DL- Met	27	0	40	44
L-Phe	94	0	138	152
L-Thr	42	0	0	68
L-Trp	14	0	21	23
L-Val	64	167	94	0
Total	562	562	562	562

[1]0.9% saline infusion was the negative control treatment; EAAC, Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; BCAA, Ile, Leu, Val; GR1+ILV, His, Ile, Leu, Met, Phe, Trp, Val; GR1+ALT, Arg, His, Lys, Met, Phe, Thr, Trp. All AA treatments supplied 562 g/d metabolizable protein where AA were infused in amounts relative to their content in casein.

Milk and Blood Collection and Analysis

Cows were milked twice daily at 0530 and 1530 h. Milk was collected, weighed, and sampled at each milking. Samples were stored at 4°C and analyzed within 4 d for protein, fat, lactose, and urea by mid-infrared spectroscopy (ISO 9622; ISO, 2013; VVB, Doetinchem, the Netherlands). At 0730, 0930, 1130, 1330, and 1530 h on d 4 of infusion, blood samples were collected by venipuncture into 10 mL sodium heparin and potassium EDTA Vacutainers (Becton Dickinson, Rutherford, NJ) from the coccygeal vessels and from the subcutaneous abdominal vein of each cow. Arteriovenous (AV) differences across the tail are assumed to be negligible and thus samples from the coccygeal vessels are representative of mammary arterial supply (Emery et al., 1965). Samples were collected from the left and right subcutaneous abdominal veins, alternating at each time point, to account for differences between sides. Collection tubes were immediately placed in ice and centrifuged at 3,000 × *g* for 15 min at room temperature. Plasma from each time point was collected and stored at -80°C pending analysis of AA, peptides, and AA metabolites. Plasma for analysis of other metabolites was pooled over sampling time points by cow and period and stored at -20°C until analysis. Plasma AA, peptide, and AA metabolite concentrations were determined using an ultra-performance liquid chromatography-mass spectrometry system (Waters Acquity Ultra Performance LC system, Waters Corp.) as described by Haque et al. (2012). Plasma

concentrations of glucose, BHB, non-esterified fatty acids (NEFA), triacylglycerol (TAG), and urea were analysed by the Veterinary Diagnostic Laboratory (Utrecht University, the Netherlands) as described by van Knegsel et al. (2007).

Calculations and Statistical Analysis

Plasma concentrations of AA, peptides, and AA metabolites were averaged over the 5 sampling times. Milk crude protein was assumed to consist of 94.5% true protein (DePeters and Ferguson, 1992). All following calculations were based on this estimate of true protein yield. Long-chain fatty acid (LCFA) concentrations were calculated on a molar basis as 3 × TAG + NEFA. Mammary plasma flow (MPF) across the whole udder was estimated according to the Fick principle using Phe and Tyr as internal markers (Cant et al., 1993), where MPF (L/h) = [milk Phe + Tyr output (μmol/h)]/[AV Phe + Tyr difference (μmol/L)], with an allowance for 3.37% contribution of blood-derived proteins to milk Phe + Tyr (Lapierre et al., 2012). Milk output of Phe + Tyr was estimated from the afternoon milk protein yield of d 4 of infusion, corresponding to the blood samples taken that day, using mean Phe and Tyr contents reported by Mepham (1987) and Lapierre et al. (2012). Uptakes (mmol/h) of metabolites across the mammary glands were calculated as the product of their plasma AV differences and MPF. Positive uptakes indicate a net removal from plasma, whereas negative values indicate net release from the mammary glands. Mammary metabolite clearances were calculated from the model of Hanigan et al. (1998), where clearance (L/h) = (AV difference × MPF)/venous concentration. The average milk protein AA composition of that reported by Mepham (1987) and Lapierre et al. (2012) was used to calculate mammary gland AA uptake to milk true protein output ratios (U:O).

One cow did not receive the correct treatment in period 1 and was thus removed from the statistical analysis for this period (n = 4 for GR1+ALT; n = 5 for all other treatments). Variances in milk and milk component production, plasma constituent concentrations and AV differences, MPF, mammary metabolite uptakes and clearances, and mammary metabolite uptake to output ratios were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The model contained treatment and period as fixed effects and cow as a random effect. We observed no carryover effects between periods, assessed by testing for an effect of the previous treatment in the ANOVA. Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. Multiple comparisons between treatment means were made using the Tukey-Kramer method.

RESULTS

Milk Production

Daily lactation performance and DMI have been presented by Nichols et al. (2019a). The present paper reports milk production expressed on an hourly basis from the afternoon milking on d 4 of infusion (**Table 8.2**). Total milk yield was not different between SAL, EAAC, BCAA, or GR1+ALT, but tended to increase over SAL with GR1+ILV (P = 0.10). Milk protein yield increased over SAL with EAAC and GR1+ILV (P = 0.03). Milk protein yield was not different between BCAA and SAL, but BCAA infusion decreased protein yield compared with EAAC and GR1+ILV (P = 0.03), and tended to decrease protein content compared with EAAC (P = 0.09). Milk fat and lactose yield, and milk fat content, were not affected by treatment (P > 0.20). Compared with SAL, milk lactose content decreased with GR1+ILV infusion (P = 0.01) and tended to decrease with EAAC infusion (P = 0.09). Infusion of BCAA increased lactose content compared with EAAC and GR1+ILV ($P \leq 0.02$). Lactose content was higher on GR1+ALT compared with GR1+ILV (P = 0.02). Milk urea content tended to increase with GR1+ALT compared with EAAC (P = 0.10).

Table 8.2. Milk and component production of lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d[1]

	Treatment[2]						
Item	SAL	EAAC	BCAA	GR1+ILV	GR1+ALT	SEM	*P*-value
Milk, kg/h	1.30	1.46	1.35	1.50	1.43	0.088	0.09
Protein, g/h	39.2^{a}	47.4^{b}	39.4^{a}	47.4^{b}	45.3^{ab}	2.16	0.01
Protein, g/kg	30.1	32.6	29.4	31.9	31.8	1.08	0.07
Fat, g/h	58.5	57.5	62.3	66.6	60.1	4.04	0.23
Fat, g/kg	44.9	40.0	46.9	45.1	43.2	3.62	0.22
Lactose, g/h	61.5	67.1	63.8	68.1	67.1	4.02	0.25
Lactose, g/kg	47.1^{ab}	46.1^{bc}	47.4^{a}	45.5^{c}	46.9^{ab}	0.42	<0.01
Urea, mg/dL	11.2	8.8	13.4	13.6	14.3	2.16	0.07

$^{a-c}$Means within a row with no common superscripts differ (P < 0.05).

[1]Data are least squares means from the afternoon milking on d 4 of infusion.

[2]SAL, 0.9% saline; EAAC, Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; BCAA, Ile, Leu, Val; GR1+ILV, His, Ile, Leu, Met, Phe, Trp, Val; GR1+ALT, Arg, His, Lys, Met, Phe, Thr, Trp. All AA treatments supplied 562 g/d metabolizable protein where AA were infused in amounts relative to their content in casein.

Arterial Metabolite Concentrations and AV Differences

Arterial plasma concentrations of glucose, BHB, NEFA, TAG, and LCFA were not affected by AA infusions ($P \geq 0.49$; **Table 8.3**). Plasma urea concentration increased with GR1+ALT infusion over SAL and EAAC ($P \leq 0.05$), and tended to increase over SAL with BCAA (P = 0.08) and GR1+ILV (P = 0.06). Arterial plasma concentration of EAA, group 1 AA, group 2 AA, and non-branched (NB)-group 2 AA increased with EAAC infusion over SAL ($P \leq 0.05$). Individual concentrations of all EAA increased ($P \leq 0.02$) or tended to increase (Thr and Val; $P \leq 0.08$)

over SAL with EAAC, except Ile and Leu which were unaffected. Infusion of BCAA increased arterial concentration of total AA (TAA) over SAL, EAAC, and GR1+ALT ($P \leq 0.03$), and increased arterial concentration of EAA, group 2 AA, and branched-chain (BC)-group 2 AA (as a group and individually) over SAL and the other AA infusions ($P \leq 0.01$). Group 1 AA concentrations (as a group and individually) decreased with BCAA infusion compared with the other AA infusions ($P \leq 0.01$). Concentration of NB-group 2 AA (as a group and individually) decreased with BCAA compared with EAAC and GR1+ALT ($P \leq 0.01$). Infusion of GR1+ILV increased arterial concentration of TAA, EAA, group 1 AA, group 2 AA, and BC-group 2 AA over SAL ($P \leq 0.02$), and increased group 1 AA concentration and decreased NB-group 2 AA concentrations (as a group and individually) compared with EAAC ($P < 0.01$). Infusion of GR1+ALT increased group 1 AA and NB-group 2 AA over SAL and EAAC ($P < 0.01$), and tended to decrease BC-group 2 AA compared with EAAC ($P = 0.07$). In response to GR1+ILV and GR1+ALT, individual concentrations of all EAA included in the respective infusions increased over SAL ($P \leq 0.01$), with the exception of Ile during GR1+ILV infusion. Compared with EAAC, concentrations of His, Met and Phe increased ($P \leq 0.02$) and Trp tended to increase ($P = 0.09$) with GR1+ILV infusion. Infusion of GR1+ALT increased concentrations of Lys, Met, Phe, Thr, and Trp ($P \leq 0.04$), and decreased concentrations of Leu and Val ($P \leq 0.05$) compared with EAAC. Concentration of BC-group 2 AA (as a group and individually) increased over GR1+ALT with GR1+ILV infusion ($P < 0.01$; tendency for Ile, $P = 0.10$), and concentration of NB-group 2 AA (as a group and individually) increased over GR1+ILV with GR1+ALT infusion ($P < 0.01$).

Arterial concentration of NEAA tended to increase with GR1+ALT over EAAC ($P = 0.08$). Infusion of EAAC increased concentration of Orn ($P < 0.01$), decreased concentration of Ser ($P = 0.02$), and tended to decrease concentration of Ala ($P = 0.07$) compared with SAL. Compared with SAL, infusion of BCAA increased concentration of Cit ($P = 0.04$), decreased concentrations of Ala and Tyr ($P \leq 0.04$), and tended to decrease concentration of Asp ($P = 0.08$). Concentration of Cys and Tyr decreased with BCAA infusion compared with all other AA infusions ($P \leq 0.03$), Ser concentration increased over EAAC ($P = 0.01$), and Ala and Asp concentrations decreased or tended to decrease compared with GR1+ILV ($P \leq 0.03$) and GR1+ALT ($P \leq 0.07$). Concentration of Gln increased with BCAA over EAAC ($P = 0.01$) and tended to increase over GR1+ALT ($P = 0.07$) and concentration of Orn decreased with BCAA compared with EAAC and GR1+ALT ($P < 0.01$). Compared with SAL, infusion of GR1+ILV increased concentration of Tyr ($P < 0.01$) and tended to increase concentration of Cit ($P = 0.09$), and GR1+ALT infusion increased concentration of Orn and Tyr ($P < 0.01$) and tended to increase concentration of Cys ($P = 0.09$). Compared with EAAC, concentrations of Ser and Tyr increased with GR1+ILV and GR1+ALT ($P \leq 0.04$), and concentration of Orn decreased with GR1+ILV and increased with GR1+ALT ($P \leq 0.01$). Concentration of Orn increased over GR1+ILV with infusion of GR1+ALT ($P < 0.01$).

Arterial plasma concentration of 1 methyl-histidine (1M-His) tended to decrease with EAAC ($P = 0.08$) and decreased with BCAA, GR1+ILV, and GR1+ALT ($P \leq 0.03$) compared with SAL. Concentration of α-amino-n-butyric acid increased over SAL with EAAC, GR1+ILV and GR1+ALT infusions ($P \leq 0.05$), and increased over BCAA ($P < 0.01$) and tended to increase over EAAC and GR1+ILV ($P = 0.08$) with GR1+ALT infusion. Concentration of α-amino-adipic acid increased over SAL, BCAA, and GR1+ILV with infusion of EAAC and GR1+ALT ($P \leq 0.01$), and increased over EAAC with GR1+ALT infusion ($P = 0.01$). Carnosine concentration tended to be higher on EAAC, GR1+ILV, and GR1+ALT compared with SAL and BCAA ($P = 0.10$). Cystathionine concentration decreased or tended to decrease with BCAA infusion compared with SAL and all AA infusions ($P \leq 0.07$), increased with GR1+ILV and GR1+ALT infusions over SAL and EAAC ($P \leq 0.02$), and increased with GR1+ALT infusion compared with GR1+ILV ($P = 0.03$). Infusion of BCAA tended to decrease hydroxyproline concentration compared with SAL ($P = 0.07$) and decreased it compared with GR1+ILV ($P = 0.02$). Infusion of GR1+ILV and GR1+ALT increased concentration of taurine over SAL, EAAC, and BCAA ($P \leq 0.02$).

Table 8.3. Arterial plasma metabolite concentrations in lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d

	Treatment[1]						
Item	SAL	EAAC	BCAA	GR1+ILV	GR1+ALT	SEM	*P*-value
Glucose, m*M*	3.64	3.64	3.58	3.36	3.71	0.140	0.49
BHB, m*M*	0.76	0.90	0.84	0.95	0.78	0.122	0.74
NEFA, µ*M*	64	74	86	68	79	16.1	0.78
TAG, µ*M*	66	66	58	66	65	4.1	0.51
LCFA, µ*M*	262	272	260	266	277	24.6	0.98
Urea, m*M*	2.26^{a}	2.50^{a}	2.78^{ab}	2.80^{ab}	3.12^{b}	0.239	0.01
Amino acids, µ*M*							
EAA[2]	670^{a}	1351^{b}	2174^{c}	1545^{b}	1117^{ab}	147.8	<0.01
Group 1[3]	147^{a}	274^{b}	116^{a}	430^{c}	409^{c}	12.2	<0.01
Group 2[4]	556^{a}	1111^{b}	2080^{c}	1182^{b}	772^{ab}	146.1	<0.01
BC-Group 2[5]	354^{a}	753^{ab}	1933^{c}	1021^{b}	234^{a}	141.2	<0.01
NB-Group 2[6]	202^{a}	358^{b}	148^{a}	161^{a}	539^{c}	24.6	<0.01
NEAA[7]	1160	948	1054	1133	1199	58.1	0.07
TAA[8]	1831^{a}	2299^{ab}	3228^{c}	2678^{bc}	2352^{ab}	169.4	<0.01
Arg	55^{a}	90^{b}	50^{a}	55^{a}	118^{b}	6.8	<0.01
His	23^{a}	60^{b}	20^{a}	83^{c}	75^{bc}	5.0	<0.01
Ile	106^{a}	169^{a}	363^{b}	207^{a}	93^{a}	30.4	<0.01
Leu	89^{a}	206^{ab}	588^{c}	297^{b}	60^{a}	44.2	<0.01
Lys	54^{a}	116^{b}	37^{a}	48^{a}	175^{c}	9.1	<0.01
Met	17^{a}	48^{b}	11^{a}	91^{c}	77^{c}	3.1	<0.01
Phe	46^{a}	94^{b}	36^{a}	145^{c}	145^{c}	8.6	<0.01
Thr	93^{ab}	151^{b}	61^{a}	58^{a}	245^{c}	13.8	<0.01
Trp	29^{a}	38^{b}	27^{a}	45^{bc}	47^{c}	2.4	<0.01
Val	160^{ab}	378^{ac}	982^{d}	517^{c}	105^{b}	68.2	<0.01
Ala	220^{a}	162^{ab}	128^{b}	196^{a}	205^{a}	16.3	<0.01

Table 8.3 (continued). Arterial plasma metabolite concentrations in lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d

	Treatment[1]						
Item	SAL	EAAC	BCAA	GR1+ILV	GR1+ALT	SEM	*P*-value
Asn	42	36	40	43	42	2.9	0.51
Asp	15^{ab}	13^{ab}	11^{b}	16^{a}	15^{ab}	1.0	0.02
Cit	57^{a}	75^{ab}	88^{b}	84^{ab}	68^{ab}	9.3	0.05
Cys	4^{ab}	6^{a}	3^{b}	6^{a}	7^{a}	0.6	0.01
Gln	251^{ab}	183^{b}	289^{a}	222^{ab}	208^{ab}	18.0	0.01
Glu	38	33	33	32	37	3.1	0.32
Gly	311	241	270	278	313	22.8	0.11
Orn	25^{a}	41^{b}	22^{a}	26^{a}	62^{c}	2.5	<0.01
Pro	69	60	55	74	75	4.9	0.05
Ser	94^{a}	65^{b}	94^{a}	90^{a}	101^{a}	6.3	0.01
Tyr	33^{a}	34^{a}	22^{b}	67^{c}	66^{c}	2.6	<0.01
Other AA, peptides, and AA metabolites,[9] μ*M*							
1 Methyl-histidine	3.8^{a}	3.0^{ab}	2.4^{b}	2.9^{b}	2.7^{b}	0.34	<0.01
3 Methyl-histidine	3.9	3.0	3.0	3.8	2.7	0.35	0.19
α-Amino-*n*-butyric acid	11^{a}	22^{bc}	13^{ab}	22^{bc}	32^{c}	2.5	<0.01
α-Amino-adipic acid	3^{a}	6^{b}	2^{a}	1^{a}	9^{c}	0.5	<0.01
β-Alanine	3.7	3.8	3.8	3.9	2.9	0.29	0.20
Carnosine	9	11	9	12	12	1.1	0.05
Cystathionine	1.7^{ab}	2.3^{a}	0.6^{b}	3.8^{c}	5.3^{d}	0.31	<0.01
Hydroxylysine	0.5	0.4	0.3	0.4	0.4	0.09	0.37
Hydroxyproline	11^{ab}	10^{ab}	8^{a}	12^{b}	10^{ab}	0.9	0.03
Phosphoserine	0.5	0.6	0.8	0.8	0.6	0.08	0.07
Taurine	29^{a}	29^{a}	26^{a}	45^{b}	50^{b}	3.2	<0.01

[a-d]Means within a row with no common superscripts differ ($P < 0.05$).

[1]SAL, 0.9% saline; EAAC, Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; BCAA, Ile, Leu, Val; GR1+ILV, His, Ile, Leu, Met, Phe, Trp, Val; GR1+ALT, Arg, His, Lys, Met, Phe, Thr, Trp. All AA treatments supplied 562 g/d metabolizable protein where AA were infused in amounts relative to their content in casein.

[2]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

[3]Group 1 = His, Met, Phe + Tyr, Trp.

[4]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.

[5]BC-Group 2 = Ile, Leu, Val.

[6]NB-Group 2 = Arg, Lys, Thr.

[7]NEAA = Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Orn, Pro, Ser, Tyr.

[8]TAA = EAA + NEAA.

[9]Other N derivatives measured with ultra-performance liquid chromatography-mass spectrometry showing a plasma concentration higher than the limit of quantification.

Arteriovenous differences of glucose, BHB, NEFA, TAG, and LCFA were not affected by treatment ($P \geq 0.17$; **Table 8.4**). Compared with SAL, infusion of EAAC increased AV differences of EAA, group 2 AA, BC-group 2 AA, and NB-group 2 AA ($P \leq 0.02$), and tended to increase that of group 1 AA ($P = 0.10$). Individual AV differences of His, Ile, Leu, and Lys increased ($P \leq 0.04$), and of Arg and Met tended to increase ($P = 0.07$) with EAAC over SAL. Infusion of BCAA increased AV differences of group 2 AA ($P = 0.05$) and BC-group 2 AA (as a group and individually; $P < 0.05$), and decreased AV difference of Lys ($P = 0.01$) compared with SAL. Arteriovenous difference of NB-group 2 AA decreased with BCAA compared with SAL and all AA infusions ($P \leq 0.03$). Compared with all AA infusions, BCAA decreased or tended to decrease the AV difference of group 1 AA, and individual AV differences of Arg, His, Lys, Met, Phe, and Thr ($P \leq 0.07$). Infusion of BCAA tended to decrease AV difference of Trp compared with GR1+ILV ($P = 0.06$), increased AV difference of BC-group 2 AA (as a group and individually) compared with GR1+ALT ($P \leq 0.02$), and increased AV difference of Val compared with EAAC ($P = 0.02$). Infusion of GR1+ILV increased AV differences of EAA, group 2 AA, and BC-group 2 AA ($P < 0.01$), and tended to increase AV difference of group 1 AA ($P = 0.06$) compared with SAL. Individual AV differences of EAA in this infusion profile increased ($P \leq 0.03$) compared with SAL, except Met which tended to increase ($P = 0.08$) and Trp which was unaffected. Compared with EAAC, infusion of GR1+ILV increased AV differences of BC-group 2 AA (as a group and individually; $P \leq 0.04$) and decreased AV difference of NB-group 2 AA and Lys ($P \leq 0.05$). Compared with SAL, infusion of GR1+ALT tended to increase AV difference of EAA ($P = 0.09$) and group 2 AA ($P = 0.07$), and increased AV difference of NB-group 2 AA and Lys ($P < 0.01$). Arteriovenous difference of Leu tended to decrease with GR1+ALT compared with EAAC ($P = 0.07$). Arteriovenous difference of BC-group 2 AA (as a group and individually) increased over GR1+ALT with GR1+ILV infusion ($P \leq 0.01$), and AV difference of NB-group 2 AA and Lys increased over GR1+ILV with GR1+ALT infusion ($P < 0.01$).

Arteriovenous difference of NEAA tended to decrease with BCAA compared with SAL ($P = 0.07$). Infusion of EAAC tended to decrease AV difference of Ala ($P = 0.06$) and Asp ($P = 0.09$) compared with SAL. Infusion of BCAA decreased or tended to decrease AV differences of Asn and Pro ($P \leq 0.07$) compared with SAL, and decreased or tended to decrease AV differences of Asn and Gln compared with all other AA infusions ($P \leq 0.08$). Further, infusion of BCAA tended to decrease AV difference of Ser compared with EAAC ($P = 0.06$), decreased AV difference of Tyr compared with EAAC and GR1+ILV ($P \leq 0.02$), and decreased AV difference of Cys compared with GR1+ILV ($P = 0.04$). Infusion of GR1+ILV decreased AV difference of Ala ($P = 0.03$) and increased AV difference of Cys ($P = 0.03$) compared with SAL. Infusion of GR1+ALT increased or tended to increase AV difference of Orn over SAL and all AA infusions ($P \leq 0.07$). Further, infusion of GR1+ALT tended to decrease AV difference of Ala

(*P* = 0.09) compared with SAL, and tended to increase AV difference of Asp over EAAC (*P* = 0.07).

Table 8.4. Mammary gland arteriovenous differences of metabolites in lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d

	Treatment[1]						
Item	SAL	EAAC	BCAA	GR1+ILV	GR1+ALT	SEM	*P*-value
Glucose, m*M*	0.72	0.78	0.56	0.74	0.79	0.068	0.17
BHB, m*M*	0.23	0.28	0.20	0.26	0.30	0.030	0.24
NEFA, µ*M*	-2	-2	6	-8	-6	10.1	0.64
TAG, µ*M*	40	40	32	40	44	4.0	0.25
LCFA, µ*M*	118	118	102	112	140	18.7	0.52
Amino acids, µ*M*							
EAA[2]	223^{a}	299^{bc}	261^{ab}	323^{b}	277^{abc}	16.6	<0.01
Group 1[3]	52^{ab}	67^{a}	41^{b}	68^{a}	59^{a}	3.8	<0.01
Group 2[4]	185^{a}	250^{b}	231^{b}	270^{b}	233^{ab}	15.1	<0.01
BC-Group 2[5]	104^{a}	144^{bc}	170^{bd}	183^{d}	119^{ac}	12.1	<0.01
NB-Group 2[6]	81^{a}	106^{b}	61^{c}	87^{a}	113^{b}	4.4	<0.01
NEAA[7]	197	176	119	176	173	18.8	0.10
TAA[8]	420	475	380	498	443	30.3	0.08
Arg	25^{ab}	31^{a}	21^{b}	29^{a}	31^{a}	1.1	<0.01
His	10^{ab}	12^{c}	8^{b}	12^{c}	11^{ac}	0.5	<0.01
Ile	27^{a}	39^{bc}	42^{bd}	49^{d}	32^{ac}	3.3	<0.01
Leu	40^{a}	59^{bc}	64^{bd}	72^{d}	46^{ac}	4.2	<0.01
Lys	35^{a}	48^{b}	23^{c}	34^{a}	56^{b}	2.6	<0.01
Met	9^{ab}	12^{a}	7^{b}	12^{a}	10^{a}	0.7	<0.01
Phe	16^{ab}	22^{ac}	13^{b}	23^{c}	19^{abc}	1.5	<0.01
Thr	22^{ab}	27^{a}	16^{b}	25^{a}	27^{a}	1.4	<0.01
Trp	4	4	3	5	4	0.7	0.07
Val	36^{a}	46^{a}	64^{b}	62^{b}	41^{a}	4.9	<0.01
Ala	34^{a}	8^{ab}	14^{ab}	6^{b}	10^{ab}	5.8	0.03
Asn	9^{ab}	13^{a}	5^{b}	11^{a}	11^{a}	1.0	<0.01
Asp	1.6	0.1	0.8	1.2	1.8	0.38	0.06
Cit	1.8	0.3	2.7	2.5	1.5	0.69	0.14
Cys	0.2^{a}	0.5^{ab}	0.2^{a}	0.8^{b}	0.3^{ab}	0.13	0.03
Gln	54^{ab}	59^{a}	31^{b}	65^{a}	58^{ab}	6.4	0.02
Glu	28	23	21	22	28	2.5	0.11
Gly	19	13	12	13	12	4.4	0.72
Orn	13^{a}	14^{ab}	11^{a}	13^{a}	19^{b}	1.4	<0.01
Pro	11^{a}	8^{ab}	6^{b}	8^{ab}	8^{ab}	0.9	0.05
Ser	11	20	5	16	10	4.4	0.06
Tyr	14^{ab}	17^{b}	11^{a}	16^{b}	15^{ab}	1.1	0.01

$^{a-d}$Means within a row with no common superscripts differ (*P* < 0.05).

[1]SAL, 0.9% saline; EAAC, Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; BCAA, Ile, Leu, Val; GR1+ILV, His, Ile, Leu, Met, Phe, Trp, Val; GR1+ALT, Arg, His, Lys, Met, Phe, Thr, Trp. All AA treatments supplied 562 g/d metabolizable protein where AA were infused in amounts relative to their content in casein.

[2]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

Table 8.4 (continued). Mammary gland arteriovenous differences of metabolites in lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d

[3]Group 1 = His, Met, Phe + Tyr, Trp.
[4]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.
[5]BC-Group 2 = Ile, Leu, Val.
[6]NB-Group 2 = Arg, Lys, Thr.
[7]NEAA = Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Orn, Pro, Ser, Tyr.
[8]TAA = EAA + NEAA.

Mammary Plasma Flow and Metabolite Uptake

Mammary plasma flow (MPF) was faster on BCAA infusion compared with EAAC and GR1+ILV ($P \leq 0.05$; **Table 8.5**). Mammary gland net uptake of glucose, BHB, NEFA, TAG, and LCFA were not affected by treatment ($P \geq 0.35$; Table 8.5). Compared with SAL, infusion of EAAC tended to increase mammary gland net uptake of EAA ($P = 0.10$), group 1 AA ($P = 0.06$), and Met ($P = 0.07$), and increased uptake of Leu, and Phe ($P \leq 0.04$). Infusion of BCAA increased mammary uptake of EAA, group 2 AA, and BC-group 2 AA (as a group and individually) over SAL ($P < 0.01$). Mammary uptake of group 1 AA ($P = 0.10$) and NB-group 2 AA ($P = 0.06$) tended to decrease and uptake of group 2 AA tended to increase ($P = 0.06$) with BCAA infusion compared with EAAC. Infusion of BCAA increased uptake of BC-group 2 AA, Leu, and Val over all AA infusions ($P \leq 0.03$), increased uptake of Ile and decreased uptake of Lys compared with EAAC and GR1+ALT ($P \leq 0.01$), decreased Phe uptake compared with EAAC and GR1+ILV ($P \leq 0.01$), decreased Thr uptake compared with GR1+ALT ($P = 0.03$), and tended to decrease Met uptake compared with EAAC ($P = 0.06$). Infusion of GR1+ILV increased mammary uptake of EAA and BC-group 2 AA ($P \leq 0.04$), and tended to increase uptake of group 1 and group 2 AA ($P \leq 0.07$) over SAL. Individual uptakes of Ile, Leu, Phe, and Val increased or tended to increase ($P \leq 0.08$) with GR1+ILV infusion over SAL. Uptake of NB-group 2 AA tended to decrease ($P = 0.10$) and uptake of Lys decreased ($P = 0.02$) with GR1+ILV compared with EAAC. Infusion of GR1+ALT increased uptake of NB-group 2 AA, Lys, and Thr ($P \leq 0.04$), tended to increase uptake of Arg ($P = 0.09$) over SAL, did not affect uptake of any EAA group or individual EAA compared with EAAC, but increased uptake of NB-group 2 AA over BCAA and GR1+ILV ($P < 0.01$). Uptake of BC-group 2 AA tended to increase ($P = 0.06$) and uptake of Ile and Leu increased ($P \leq 0.02$) with GR1+ILV over GR1+ALT. Uptake of Lys was higher ($P < 0.01$) and uptake of Thr tended to be higher ($P = 0.08$) on GR1+ALT compared with GR1+ILV.

Mammary gland uptake of total NEAA was not affected by treatment ($P = 0.53$). Infusion of EAAC decreased mammary uptake of Ala and Pro ($P \leq 0.05$) compared with SAL. Infusion of BCAA decreased or tended to decrease uptake of Asn compared with other AA infusions ($P \leq 0.08$), and increased uptake of Cit compared with EAAC ($P = 0.04$). Infusion of GR1+ILV decreased mammary uptake of Ala and Pro and increased uptake of Cys compared

with SAL ($P \leq 0.03$). Infusion of GR1+ALT tended to increase mammary uptake of Asp ($P = 0.06$) over EAAC.

Table 8.5. Whole-mammary gland plasma flow and net uptakes of metabolites in lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d

	Treatment[1]						
Item	SAL	EAAC	BCAA	GR1+ILV	GR1+ALT	SEM	*P*-value
Plasma flow, L/h	768^{ab}	703^{b}	965^{a}	693^{b}	767^{ab}	70.1	0.04
Net mammary uptake, mmol/h							
Glucose	553.1	549.3	528.0	510.4	605.3	80.98	0.94
BHB	178.4	197.7	205.7	174.7	228.2	29.97	0.70
NEFA	-2.8	-2.4	1.7	-5.8	4.1	7.99	0.76
TAG	30.2	28.2	30.5	27.3	33.5	2.45	0.48
LCFA	87.9	82.3	93.3	76.0	103.7	10.05	0.35
Amino acids							
EAA[2]	167.4^{a}	208.9^{ab}	248.3^{b}	219.2^{b}	210.2^{ab}	11.80	<0.01
Group 1[3]	38.7	46.4	39.3	46.2	44.7	2.30	0.02
Group 2[4]	138.8^{a}	174.2^{ab}	219.2^{b}	184.0^{ab}	176.3^{ab}	10.94	<0.02
BC-Group 2[5]	77.5^{a}	100.3^{ab}	160.9^{c}	124.4^{b}	88.9^{ab}	8.00	<0.01
NB-Group 2[6]	61.3^{a}	73.9^{ab}	58.3^{a}	59.6^{a}	86.9^{b}	4.20	<0.01
NEAA[7]	145.1	123.7	116.2	119.3	136.2	13.54	0.53
TAA[8]	312.5	332.6	364.5	338.6	346.2	22.58	0.57
Arg	18.9	21.5	20.1	19.7	23.5	1.08	0.10
His	7.2	8.5	7.4	8.4	8.6	0.57	0.13
Ile	20.4^{a}	27.2^{ab}	39.9^{c}	33.6^{bc}	23.6^{a}	2.03	<0.01
Leu	30.1^{a}	40.9^{bc}	60.6^{d}	48.8^{b}	35.0^{ac}	2.49	<0.01
Lys	26.2^{ab}	34.1^{ac}	22.3^{b}	23.2^{b}	42.8^{c}	2.81	<0.01
Met	6.9	8.3	6.8	7.9	7.8	0.45	0.03
Phe	12.0^{a}	14.9^{b}	11.9^{a}	15.8^{b}	14.2^{ab}	0.74	<0.01
Thr	16.2^{a}	18.4^{ab}	15.8^{a}	16.7^{ab}	20.5^{b}	1.06	0.02
Trp	2.5	2.9	3.0	3.2	2.9	0.25	0.54
Val	27.0^{a}	32.1^{a}	60.4^{b}	42.0^{a}	30.2^{a}	3.78	<0.01
Ala	25.1^{a}	6.4^{ab}	12.8^{ab}	4.8^{b}	7.5^{ab}	4.32	0.03
Asn	7.0^{ab}	8.7^{a}	5.3^{b}	7.7^{ab}	8.2^{a}	0.65	0.01
Asp	1.2	0.1	0.8	0.9	1.5	0.30	0.06
Cit	1.3^{ab}	0.2^{b}	2.8^{a}	1.7^{ab}	1.2^{ab}	0.55	0.07
Cys	0.1^{a}	0.3^{ab}	0.2^{ab}	0.5^{b}	0.3^{ab}	0.08	0.03
Gln	40.5	41.4	30.8	44.4	44.8	5.22	0.36
Glu	21.4	16.6	20.1	15.7	21.9	2.64	0.23
Gly	13.1	9.3	11.6	8.1	9.8	3.07	0.78
Orn	10.1	10.0	10.4	9.1	14.5	1.50	0.14
Pro	8.0^{a}	5.6^{b}	6.2^{ab}	5.2^{b}	6.1^{ab}	0.51	0.02
Ser	7.3	13.5	5.0	10.4	6.9	3.15	0.16
Tyr	10.0	11.7	10.2	10.9	11.4	0.70	0.32

$^{a-d}$Means within a row with no common superscripts differ ($P < 0.05$).

Table 8.5 (continued). Whole-mammary gland plasma flow and net uptakes of metabolites in lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d

[1]SAL, 0.9% saline; EAAC, Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; BCAA, Ile, Leu, Val; GR1+ILV, His, Ile, Leu, Met, Phe, Trp, Val; GR1+ALT, Arg, His, Lys, Met, Phe, Thr, Trp. All AA treatments supplied 562 g/d metabolizable protein where AA were infused in amounts relative to their content in casein.

[2]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

[3]Group 1 = His, Met, Phe + Tyr, Trp.

[4]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.

[5]BC-Group 2 = Ile, Leu, Val.

[6]NB-Group 2 = Arg, Lys, Thr.

[7]NEAA = Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Orn, Pro, Ser, Tyr.

[8]TAA = EAA + NEAA.

Mammary Metabolite Clearance

Mammary gland clearance of glucose, BHB, NEFA and TAG was not affected by treatment ($P > 0.25$). Mammary gland clearance of LCFA tended to be higher with GR1+ALT infusion compared with GR1+ILV ($P = 0.06$; **Table 8.6**). Mammary clearance of EAA decreased in response to all AA infusions compared with SAL ($P \leq 0.02$). Infusion of EAAC decreased clearance of group 2 AA ($P \leq 0.03$) and tended to decrease clearance of NB-group 2 AA ($P = 0.10$), and decreased individual clearances of Leu, Lys, Met, and Val ($P \leq 0.03$), and tended to decrease clearance of Phe ($P = 0.09$) compared with SAL. Infusion of BCAA decreased clearance of group 2 AA and BC-group 2 AA ($P \leq 0.02$), increased clearance of Met ($P < 0.01$), and decreased clearances of Leu and Val ($P < 0.01$) compared with SAL. Infusion of BCAA increased clearance of group 1 AA and individual clearances of Met and Phe ($P < 0.01$), and increased or tended to increase clearance of His ($P \leq 0.07$) over all other AA infusions. Clearance of NB-group 2 AA, Lys, and Thr increased over EAAC and GR1+ALT ($P \leq 0.03$), and clearance of Arg tended to increase over EAAC ($P = 0.09$) with BCAA infusion. Infusion of GR1+ILV decreased mammary clearance of group 1 AA, group 2 AA, and individual clearances of Leu, Met, Phe, and Val ($P \leq 0.02$), tended to decrease clearance of BC-group 2 AA and His ($P = 0.08$), and increased clearance of NB-group 2 AA and Thr ($P \leq 0.05$) compared with SAL. Infusion of GR1+ILV increased clearance of NB-group 2 AA (as a group and individually) over EAAC ($P \leq 0.02$). Compared with SAL, infusion of GR1+ALT decreased mammary clearance of group 1 AA, NB-group 2 AA, and individual clearances of Lys, Met, and Phe, and increased clearance of Leu ($P \leq 0.05$). Infusion of GR1+ALT increased mammary clearance of BC-group 2 AA and individual clearances of Leu and Val over all other AA infusions ($P \leq 0.01$). Clearance of NB-group 2 AA (as a group and individually) increased over GR1+ALT with GR1+ILV infusion ($P \leq 0.01$).

Mammary gland clearance of NEAA was not affected by treatment ($P = 0.32$). Infusion of EAAC increased mammary clearance of Asn and Ser ($P \leq 0.01$), tended to increase clearance of Gln ($P = 0.07$), and tended to decrease clearance of Orn ($P = 0.08$) compared

with SAL, and increased Asn clearance compared with all other AA infusions ($P \leq 0.05$). Infusion of BCAA decreased or tended to decrease mammary clearance of Gln compared with all other AA infusions ($P \leq 0.06$), increased Orn clearance over EAAC and GR1+ALT ($P \leq 0.03$), decreased clearance of Ser compared with EAAC ($P < 0.01$), and decreased or tended to decrease clearance of Asn and increased clearance of Tyr over GR1+ILV and GR1+ALT ($P \leq 0.06$). Infusion of GR1+ILV tended to increase mammary clearance of Cys compared with SAL ($P = 0.06$), tended to decrease clearance of Ser compared with EAAC ($P = 0.08$), and decreased Tyr clearance compared with EAAC ($P = 0.03$). Infusion of GR1+ALT tended to decrease mammary clearance of Orn compared with SAL ($P = 0.09$). Compared with EAAC, infusion of GR1+ALT decreased clearance of Ser ($P = 0.01$), and tended to increase clearance of Asp ($P = 0.10$) and tended to decrease clearance of Tyr ($P = 0.06$).

Mammary Gland AA U:O

Infusion of EAAC did not affect the mammary gland uptake to milk protein output ratio (U:O) of any EAA group or individual EAA compared with SAL ($P > 0.95$; **Table 8.7**). Infusion of BCAA increased U:O of EAA, group 2, and BC-group 2 AA (as a group and individually) over SAL and all other AA infusions ($P < 0.01$). Infusion of GR1+ILV tended to increase U:O of BC-group 2 AA over SAL ($P = 0.07$), and tended to decrease U:O of NB-group 2 AA compared with SAL and EAAC ($P = 0.07$). Individually, U:O of Ile and Leu increased and U:O of Thr decreased with GR1+ILV compared with SAL ($P \leq 0.05$), U:O of Arg decreased or tended to decrease compared with SAL, BCAA, and GR1+ALT ($P \leq 0.09$), and U:O of Lys decreased compared with EAAC ($P = 0.03$). Infusion of GR1+ALT increased U:O of NB-group 2 AA and individual U:O of Lys over SAL and all AA infusions ($P \leq 0.05$), and increased or tended to increase U:O of Thr over all AA infusions ($P \leq 0.06$).

Infusion of EAAC decreased U:O of NEAA and individual U:O of Ala, Asp, and Pro compared with SAL ($P \leq 0.05$). Infusion of BCAA tended to decrease U:O of NEAA compared with SAL ($P = 0.09$) and tended to decrease U:O of Asn compared with EAAC ($P = 0.06$). Compared with SAL, infusion of GR1+ILV decreased U:O of NEAA and individual U:O of Ala and Pro ($P \leq 0.03$), and increased U:O of Cys ($P = 0.02$). Infusion of GR1+ALT had no effect on U:O of NEAA compared with all other treatments, decreased individual U:O of Ala and Pro compared with SAL ($P \leq 0.05$), and increased U:O of Asp compared with EAAC ($P = 0.05$). Total AA U:O increased or tended to increase over all other AA infusions in response to BCAA infusion ($P \leq 0.06$). Total AA U:O expressed on an N basis tended to increase over SAL ($P = 0.06$) and increased over all other AA infusions ($P \leq 0.03$) in response to BCAA infusion.

Table 8.6. Mammary clearances (L/h) of metabolites in lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d

	Treatment[1]						
Item	SAL	EAAC	BCAA	GR1+ILV	GR1+ALT	SEM	*P*-value
Glucose	195	194	190	194	209	34.2	0.98
BHB	330	344	309	294	449	51.2	0.36
NEFA	-19	-64	-28	-109	63	107.6	0.56
TAG	1255	1174	1233	1077	1565	180.7	0.25
LCFA	619	531	598	492	746	65.0	0.07
Amino acids							
EAA[2]	400^{a}	215^{b}	145^{b}	190^{b}	226^{b}	30.1	<0.01
Group 1[3]	409^{ab}	237^{bc}	575^{a}	127^{c}	142^{c}	54.0	<0.01
Group 2[4]	421^{a}	225^{b}	134^{b}	222^{b}	287^{ab}	40.5	<0.01
BC-Group 2[5]	355^{ab}	190^{bc}	105^{c}	166^{bc}	475^{a}	46.3	<0.01
NB-Group 2[6]	566^{ab}	312^{bc}	689^{ad}	863^{d}	249^{c}	65.8	<0.01
NEAA[7]	155	163	124	125	134	16.5	0.32
TAA[8]	227^{a}	190^{ab}	136^{b}	157^{ab}	180^{ab}	16.8	0.02
Arg	688^{ab}	381^{b}	728^{ab}	857^{a}	320^{b}	91.9	0.01
His	1099^{ab}	195^{b}	1305^{a}	120^{b}	194^{ab}	291.6	0.01
Ile	336	243	148	244	378	74.7	0.19
Leu	707^{a}	322^{b}	134^{b}	245^{b}	2087^{c}	80.1	<0.01
Lys	1464^{a}	524^{b}	1750^{a}	1705^{a}	468^{b}	171.4	<0.01
Met	982^{a}	240^{b}	1744^{c}	100^{b}	148^{b}	99.0	<0.01
Phe	415^{ab}	226^{bc}	575^{a}	132^{c}	141^{c}	54.7	<0.01
Thr	271^{ab}	160^{b}	372^{ac}	532^{c}	117^{b}	43.5	<0.02
Trp	107	91	128	79	68	15.1	0.07
Val	243^{a}	113^{b}	75^{b}	102^{b}	295^{a}	26.0	<0.01
Ala	135	47	126	28	43	33.1	0.05
Asn	210^{ab}	374^{c}	156^{b}	249^{ab}	268^{a}	24.1	<0.01
Asp	104	11	77	57	118	25.6	0.09
Cit	24	5	34	20	19	8.3	0.23
Cys	35	69	104	120	42	21.6	0.04
Gln	217^{ab}	366^{a}	120^{b}	287^{a}	290^{ab}	37.2	<0.01
Glu	2059	1863	1756	1664	2341	244.8	0.31
Gly	52	41	43	33	34	13.0	0.82
Orn	900^{ab}	398^{b}	1025^{a}	764^{ab}	374^{b}	131.0	0.01
Pro	143	110	133	80	92	16.6	0.09
Ser	104^{a}	320^{b}	57^{a}	172^{ab}	84^{a}	51.2	<0.01
Tyr	570^{abc}	755^{ab}	951^{a}	216^{c}	236^{bc}	116.8	<0.01

$^{a-d}$Means within a row with no common superscripts differ ($P < 0.05$).

[1]SAL, 0.9% saline; EAAC, Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; BCAA, Ile, Leu, Val; GR1+ILV, His, Ile, Leu, Met, Phe, Trp, Val; GR1+ALT, Arg, His, Lys, Met, Phe, Thr, Trp. All AA treatments supplied 562 g/d metabolizable protein where AA were infused in amounts relative to their content in casein.

[2]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

[3]Group 1 = His, Met, Phe + Tyr, Trp.

[4]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.

[5]BC-Group 2 = Ile, Leu, Val.

[6]NB-Group 2 = Arg, Lys, Thr.

Table 8.6 (continued). Mammary clearances (L/h) of metabolites in lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d

[7]NEAA = Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Orn, Pro, Ser, Tyr.

[8]TAA = EAA + NEAA.

Table 8.7. Mammary gland AA uptake to milk output ratios in lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d

	Treatment[1]						
Item	SAL	EAAC	BCAA	GR1+ILV	GR1+ALT	SEM	*P*-value
EAA[2]	1.23^{a}	1.28^{a}	1.84^{b}	1.35^{a}	1.35^{a}	0.078	<0.01
Group 1[3]	1.01	1.00	1.01	1.00	1.01	0.013	0.83
Group 2[4]	1.28^{a}	1.34^{a}	2.04^{b}	1.42^{a}	1.43^{a}	0.097	<0.01
BC-Group 2[5]	1.20^{a}	1.29^{a}	2.52^{b}	1.61^{a}	1.23^{a}	0.138	<0.01
NB-Group 2[6]	1.40^{a}	1.41^{a}	1.32^{a}	1.13^{a}	1.72^{b}	0.066	<0.01
NEAA[7]	0.80^{a}	0.55^{b}	0.58^{ab}	0.53^{b}	0.62^{ab}	0.055	0.03
TAA[8]	0.98^{ab}	0.86^{b}	1.11^{a}	0.88^{b}	0.91^{ab}	0.044	0.01
TAA-N[9]	1.01^{ab}	0.91^{b}	1.20^{a}	0.93^{b}	0.96^{b}	0.045	<0.01
Arg	2.55^{ab}	2.40^{ab}	2.69^{a}	2.20^{b}	2.68^{a}	0.094	0.01
His	1.10	1.07	1.10	1.07	1.14	0.043	0.74
Ile	1.25^{a}	1.40^{ab}	2.48^{c}	1.72^{b}	1.29^{ab}	0.147	<0.01
Leu	1.09^{a}	1.24^{ab}	2.24^{c}	1.48^{b}	1.12^{ab}	0.112	<0.01
Lys	1.22^{ab}	1.33^{ac}	1.04^{ab}	0.90^{b}	1.75^{c}	0.111	<0.01
Met	1.00	1.00	0.98	0.96	0.98	0.029	0.67
Phe	1.10	1.13	1.08	1.19	1.15	0.034	0.16
Thr	1.09^{ab}	1.02^{bc}	1.04^{abc}	0.93^{c}	1.19^{a}	0.033	<0.01
Trp	0.89	0.84	1.00	0.91	0.84	0.064	0.42
Val	1.29^{a}	1.29^{a}	2.91^{b}	1.68^{a}	1.32^{a}	0.182	<0.01
Ala	1.87^{a}	0.35^{b}	0.97^{ab}	0.26^{b}	0.41^{b}	0.303	0.01
Asn	0.59	0.62	0.44	0.55	0.61	0.043	0.06
Asp	0.07^{a}	0.01^{b}	0.05^{ab}	0.05^{ab}	0.08^{ab}	0.016	0.04
Cys	0.05^{a}	0.11^{ab}	0.06^{ab}	0.16^{b}	0.08^{ab}	0.023	0.03
Gln	1.70	1.42	1.25	1.53	1.58	0.182	0.48
Glu	0.52	0.33	0.48	0.31	0.47	0.061	0.10
Gly	1.49	0.82	1.21	0.71	0.90	0.299	0.37
Pro	0.25^{a}	0.14^{b}	0.19^{ab}	0.13^{b}	0.15^{b}	0.017	<0.01
Ser	0.36	0.51	0.19	0.40	0.26	0.126	0.23
Tyr	0.90	0.87	0.92	0.81	0.89	0.033	0.16

[a-c]Means within a row with no common superscripts differ ($P < 0.05$).

[1]SAL, 0.9% saline; EAAC, Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; BCAA, Ile, Leu, Val; GR1+ILV, His, Ile, Leu, Met, Phe, Trp, Val; GR1+ALT, Arg, His, Lys, Met, Phe, Thr, Trp. All AA treatments supplied 562 g/d metabolizable protein where AA were infused in amounts relative to their content in casein.

[2]EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val.

[3]Group 1 = His, Met, Phe + Tyr, Trp.

[4]Group 2 = Arg, Ile, Leu, Lys, Thr, Val.

[5]BC-Group 2 = Ile, Leu, Val.

[6]NB-Group 2 = Arg, Lys, Thr.

[7]NEAA = Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Orn, Pro, Ser, Tyr.

Table 8.7 (continued). Mammary gland AA uptake to milk output ratios in lactating dairy cows receiving abomasal infusions of saline (SAL) or 562 g/d of AA in different profiles for 5 d

[8]TAA = EAA + NEAA.

[9]TAA on a N basis.

DISCUSSION

The current results complement previously reported daily lactation performance and energy and N balance from the same study (Nichols et al., 2019a). Notably, infusion of the complete EAA profile increased energy retained as body protein, and resulted in the same milk N efficiency when compared with SAL. Infusion of GR1+ILV and GR1+ALT resulted in the same energy and N retention compared with EAAC. Milk N efficiency was not different between EAAC and GR1+ILV, but was lower on GR1+ALT compared with EAAC, and tended to be lower with GR1+ALT compared with GR1+ILV. Infusion of BCAA reduced feed intake 9% compared with the other AA infusions, resulted in negative energy balance, and resulted in lower milk N efficiency compared with EAAC. Few studies have examined the effects of imbalanced EAA profiles on mammary gland metabolism, and usually this is evaluated under conditions where single AA or groups of AA are subtracted from postruminal infusions of complete AA profiles where the lost MP supply from the subtracted AA is not compensated in the supplement (Bequette et al., 2000; Doepel and Lapierre, 2011; Doepel et al., 2016). Therefore, the objective of the present work was to examine mammary gland responses to incomplete EAA profiles at a constant supplemental MP level, with the general hypothesis that upon providing incomplete EAA profiles, intramammary metabolism of the supplemented EAA will increase in support of milk protein synthesis.

Arterial Metabolite Concentrations

Infusion of the complete EAA profile increased arterial plasma concentration of all EAA groups approximately 2-fold over SAL, and did not affect total NEAA concentration. This is in line with the observations of Doelman et al. (2015a,b) who abomasally infused an identical EAA profile and dose as the EAAC treatment in the current study. Individual concentrations of all EAA increased over SAL (tendency for Thr and Val), except Ile and Leu, where the response of these AA to EAAC infusion were not detected as significant but still increased 59 and 131% over SAL, respectively.

In general, arterial plasma EAA concentrations during the incomplete EAA infusions reflected the infused EAA profile. Infusion of BCAA increased delivery of Ile, Leu, and Val to the abomasum 162% over EAAC, and subsequently increased their arterial concentrations 115, 185, and 160%, respectively, resulting in the highest concentrations of BC-group 2 AA and total EAA in arterial plasma over all other AA infusions. To our knowledge, the BCAA treatment in this experiment represents the highest dose of postruminal Ile, Leu, and Val supplemented to dairy cattle. Arterial concentrations of all non-branched-chain EAA were

not different from SAL with BCAA infusion, despite a 1.8 kg/d difference in DMI between these treatments (Nichols et al., 2019a). This suggests catabolism of these EAA was reduced, their mobilization from body protein was increased, or both, during BCAA infusion. However, arterial concentration of 1-MHis decreased during BCAA infusion compared with SAL, in contrast with increased muscle protein turnover. Plasma urea tended to increase over SAL with BCAA in the current study, which could arise from catabolism of excess BC-group 2 AA while catabolism of non-branched-EAA was reduced.

Amino acids in the GR1+ILV and GR1+ALT profiles were delivered at 1.5 and 1.6-times their levels in EAAC infusion. As expected, arterial concentrations of group 1 AA, as a group and individually, increased or tended to increase with infusion of GR1+ILV and GR1+ALT over SAL and EAAC. Within the group 2 AA, concentrations of Arg, Lys, and Thr were not different from SAL with GR1+ILV infusion, but Ile, Leu, and Val concentrations increased (significantly or numerically) over SAL and EAAC. Following the same pattern with GR1+ALT infusion, concentrations of Ile, Leu, and Val were not different from SAL, but Arg, Lys, and Thr concentrations increased (significantly or numerically) over SAL and EAAC.

Arterial concentrations of NEAA, peptides, and AA metabolites reflect metabolism of infused EAA in the different profiles. Increased concentration of Orn with EAAC and GR1+ALT compared with the other infusions reflects metabolism of infused Arg through the urea cycle, and highest Orn concentration on GR1+ALT suggests extra infused Arg stimulated the urea cycle further than on EAAC. Arginine can be synthesised de novo in the gut and kidney (Morris, 2007), although in insufficient quantities relative to requirements for dairy cows, hence its classification as an EAA (Doepel et al., 2004). In the AA infusions where Arg was absent (BCAA and GR1+ILV), Cit concentration increased (tendency only for GR1+ILV) over SAL, which might reflect up-regulation of de novo Arg synthesis on these treatments in response to stimulation of protein anabolism by the other infused EAA. This is consistent with Doepel and Lapierre (2011) who observed a tendency for increased arterial Cit concentration when Arg was removed from a complete AA infusion. Lower Ala concentration with BCAA infusion relative to SAL, GR1+ILV, and GR1+ALT can be attributed to its higher contribution to gluconeogenesis when DMI decreased (Nichols et al., 2019a). Tyrosine is synthesised during hydroxylation of Phe (Shiman and Gray, 1998), so absence of Phe in the BCAA infusion probably accounts for the reduced Tyr concentration relative to all other AA infusions. Conversely, the highest levels of Phe in the GR1+ILV and GR1+ALT infusions likely account for the increase in arterial Tyr concentration with these treatments over the other infusions. Arterial concentrations of cystathionine, Cys, and taurine across treatments reflects the relative doses of Met infused. Catabolism of Met through its transsulferation pathway produces the intermediate cystathionine, which is metabolised to Cys, a potential fate of which is its use for taurine synthesis (Brosnan and Brosnan, 2006). Similarly, the non-proteinogenic AA α-amino-*n*-butyric acid and α-amino-adipic acid arise from metabolism of

Met, Lys, and Thr (Mabjeesh et al., 2000), and their concentrations in arterial plasma reflect the relative daily infusion rate of these EAA. In particular, concentrations of both were greatest during GR1+ALT infusion where Lys and Thr were infused in the highest amount relative to the other infusions. Concentration of 1-MHis decreased compared with SAL on all AA infusions (tendency only for EAAC), which suggests reduced skeletal muscle turnover when circulating EAA concentrations increased, and agrees with the lowest N retention on SAL and more positive N retention in response to AA infusions in this experiment (Nichols et al., 2019a). This is also in line with a similar decrease in arterial 1-MHis concentration observed by Nichols et al. (2019b) during abomasal infusion of the same EAA profile infused at 1.5-times the dose used in the current experiment. Carnosine is a storage pool of His in muscle, and tended to increase in plasma with EAAC, GR1+ILV, and GR1+ALT, possibly indicating carnosine synthesis from His during these infusions (Giallongo et al., 2015).

Mammary Plasma Flow

Mammary plasma flow was fastest during BCAA infusion, and it increased 38% over that on EAAC and GR1+ILV. Mammary plasma flow can be altered during excessive or deficient supply of milk precursors in an attempt to maintain extracellular and intracellular concentrations of precursor substrates required for milk component synthesis, particularly when single EAA or 2C compounds are deficient in arterial circulation (Cant et al., 2003). In previous studies, supplying extra MP as a mixture of EAA and NEAA (Nichols et al., 2019c), or as a mixture of EAA only (Nichols et al. 2019b), did not affect MPF compared with no extra supply of MP, which is in line with the results of the present experiment. The effects of EAA deletions on MPF have been variable in previous studies. Removal of Lys from a complete AA profile infused into the abomasum of dairy cattle did not affect MPF (Lapierre et al., 2009), while MPF increased 50% when Thr was removed from a complete AA infusion (Doepel et al., 2016). Deletion of Arg from a complete EAA profile infused postruminally did not affect MPF compared with the complete profile, but decreased it 17% relative to control levels (Doepel and Lapierre, 2011). Bequette et al. (2000) reported a 33% increase in MPF in goats when His was removed from an abomasal infusion of AA in the profile of microbial protein. In the current study, if MPF was responding to the absence of any single EAA, it should have also increased during the other incomplete infusions, i.e., GR1+ILV and GR1+ALT. The magnitude of effect on MPF when single EAA are removed from complete supplements could relate to the degree of change in arterial concentration of the other EAA (Cant et al., 2001). The BCAA infusion in the current study was relatively more imbalanced than GR1+ILV and GR1+ALT with respect to the complete profile, and this may explain why MPF was only increased in response to BCAA. This difference in magnitude of imbalance can be seen in the response of circulating EAA concentrations to the incomplete infusions, where BCAA increased the circulating concentration of only the BC-group 2 AA compared with SAL, and GR1+ILV and GR1+ALT increased the concentration of all the EAA (numerical increase only

for Ile with GR1+ILV infusion) in the infusate profile compared with SAL. In agreement, Haque et al. (2015) postruminally infused an EAA profile missing only Thr and Trp and observed no effect on MPF. Changes in blood flow are often associated with concomitant changes in arterial concentration of acetate and BHB, particularly during glucogenic infusions where the arterial concentrations of these metabolites decreases and MPF increases (Raggio et al., 2006; Curtis et al., 2018; Nichols et al., 2019b). A link between AA supply and MPF has been hypothesized to lie with the associated effects on arterial energy metabolites when EAA supply is increased (Cant et al., 2003; Cant et al., 2018). However, arterial BHB concentration was not affected by the infused AA profiles in the current study. This adds credence to the suggestion that the effect of BCAA infusion on MPF is related to the relative AA imbalance, and may indicate that the mechanisms regulating MPF under conditions of AA imbalance can override those responsible for maintaining ATP status of the gland.

Mammary AA Metabolism

Milk protein synthesis increased 21% over SAL in response to EAAC infusion. In support, mammary net uptake of total EAA and group 1 AA tended to increase 25 and 20%, respectively, and uptake of group 2 AA numerically increased 26%. The U:O of group 1 and group 2 AA followed canonical patterns (1.00 and 1.34, respectively) and were not affected by EAAC infusion, and U:O of NEAA decreased in response to EAAC compared with SAL. Together, these observations indicate that NEAA required in support of the increase in milk protein yield were likely synthesised from group 2 AA taken up in excess of their output in milk protein, and are consistent with previous reports where absorptive EAA supply is increased (Raggio et al., 2006; Haque et al., 2015; Nichols et al., 2019b). Mammary U:O of group 1 AA was not different from 1 on any treatment in this experiment, indicating these AA on the whole were used exclusively for milk protein synthesis on a net basis, irrespective of the infused EAA profile. The increase in total EAA uptake during EAAC infusion was due to the elevated arterial EAA concentration and not a change in MPF. Therefore, the decrease in mammary clearance during EAAC infusion is consistent with surplus EAA supply for mammary protein synthesis (Bequette et al., 2000). Essential AA not sequestered by the mammary gland would be available for metabolism in extra-mammary tissues, which is in line with increased body N retention and tendency for higher urine N excretion observed in this experiment during EAAC infusion compared with SAL (Nichols et al., 2019a).

The 50% increase in mammary net uptake of total EAA over SAL with BCAA infusion can be entirely attributed to the uptake of BC-group 2 AA (as a group and individually) which increased over SAL and all AA infusions. Arterial concentration of BC-group 2 AA during BCAA infusion was increased over EAAC and GR1+ILV (treatments where Ile, Leu, and Val were also infused), but AV difference of this AA group during EAAC and GR1+ILV infusion did not differ from that on BCAA infusion. The higher uptake of BC-group 2 AA can hence be attributed to the faster MPF during BCAA infusion.

The U:O of BC-group 2 AA increased with BCAA over all infusions, to approximately double the level observed on SAL, EAAC, and GR1+ALT, and 1.6-times that on GR1+ILV, in agreement with our hypothesis that their intramammary catabolism would be elevated when they were infused in the absence of other EAA. This observation suggests a high level of intramammary catabolism of Ile, Leu, and Val is possible by the lactating mammary gland, and agrees with observations by others where increased MP or BCAA caused faster intramammary catabolism of BC-group 2 AA, particularly Leu (Bequette et al., 1996; Raggio et al., 2006; Nichols et al., 2019c). The mammary gland obtains substantial N and carbon for de novo NEAA synthesis from excess uptake of AA-N from BC-group 2 AA relative to their output in milk (Lapierre et al., 2012). It might therefore be expected that with greater uptake and intramammary catabolism of BC-group 2 AA on BCAA infusion, uptake of NEAA would decrease further than with the other AA infusions, but this was not observed. Furthermore, the U:O of total AA-N increased with BCAA over the other AA infusions, driven by the increased U:O of BC-group 2 AA. Taken together, the substantial difference in mammary uptake of BC-group 2 AA, and subsequently total AA-N, relative to their output in milk, while NEAA U:O was not affected indicates that Ile, Leu, and Val likely contributed to other mammary pathways (lactose and fat synthesis, and oxidation) above what was used for intramammary NEAA synthesis. This increased intramammary AA catabolism supports the lowest milk N efficiency observed on this treatment (Nichols et al., 2019a) and the observation that despite the lower DMI, milk protein production did not differ between BCAA and SAL, and fat and lactose yield did not differ with BCAA from any infusion.

Milk protein yield with infusion of the incomplete profiles of GR1+ILV or GR1+ALT did not differ from that stimulated by the complete EAA profile. In agreement, mammary net uptake of group 1 AA did not differ between EAAC, GR1+ILV, and GR1+ALT. Differences in uptake of the group 2 AA were found between the incomplete infusions, where BC-group 2 AA uptake tended to be higher on GR1+ILV compared with GR1+ALT, and NB-group 2 AA uptake was higher on GR1+ALT compared with GR1+ILV. Infusion of GR1+ILV increased clearance of NB-group 2 AA (as a group and individually) over EAAC. Infusion of GR1+ALT increased mammary clearance of BC-group 2 AA and individual clearances of Leu and Val over all other AA infusions. Because their uptakes were not different from EAAC, higher clearance of these EAA groups is due to their relatively lower arterial concentrations on the respective incomplete EAA infusions, and reflects mammary affinity for their sequestration when supplementation of the other EAA stimulated milk protein synthesis.

The level of intramammary EAA catabolism was the same between GR1+ILV and GR1+ALT, as evidenced by the equal net uptake and equal U:O for total EAA. We hypothesised that the same level of milk protein synthesis between EAAC, GR1+ILV, and GR1+ALT in the absence of Arg, Lys, and Thr (GR1+ILV) or in the absence of Ile, Leu and Val (GR1+ALT) was maintained through intramammary compensation between the group 2 AA.

Intramammary catabolism of NB-group 2 AA tended to decrease with GR1+ILV compared with EAAC. The U:O of BC-group 2 AA numerically increased 25% over EAAC with GR1+ILV infusion. Therefore, in agreement with our hypothesis, on GR1+ILV intramammary catabolism of BC-group 2 AA could have compensated for lower levels of NB-group 2 AA. During GR1+ALT infusion, NB-group 2 AA intramammary catabolism increased over EAAC, particularly Lys and Thr, but the gland maintained the same level of excess BC-group 2 AA uptake. This difference in intramammary catabolism of the absent groups in the respective infusions, where (compared with EAAC) NB-group 2 AA catabolism tended to decrease on GR1+ILV whereas BC-group 2 AA catabolism was not different on GR1+ALT, arose primarily from the decrease in the U:O of Lys on GR1+ILV compared with EAAC, whereas on GR1+ALT the U:O of the individual BC-group 2 AA were not different from EAAC. Similarly, Lapierre et al. (2009) observed a decrease in U:O of Lys when it was removed from a complete AA infusion, although it did not drop below unity. These authors suggested that a certain level of Lys catabolism by the mammary gland is obligate. Although the U:O of Lys on GR1+ILV did not differ from that on SAL and BCAA where the ratios were > 1, the low ratios observed on GR1+ILV and BCAA (0.90 and 1.04, respectively) suggest that when intramammary BC-group 2 AA levels are high, Lys catabolism by the gland is minor or not obligate. Abundant intramammary levels of BC-group 2 AA could have compensated for the contributions of Lys to intramammary N exchanges in support of milk protein synthesis on GR1+ILV and BCAA infusion. Furthermore, the observed U:O of Lys, but also Thr on GR1+ILV, and Trp on SAL, EAAC, GR1+ILV, and GR1+ALT of < 1 suggests a portion of these EAA required for milk protein synthesis arose from sources not captured in our arteriovenous measurements, such as plasma peptides or breakdown of mammary constituent proteins (Bequette et al., 1999).

Since milk protein yield did not differ between EAAC and GR1+ILV, lower mammary U:O of Lys with GR1+ILV infusion compared with EAAC arose from the lower mammary uptake of Lys. Arterial concentrations of Arg, Lys, and Thr were all lower on GR1+ILV compared with EAAC, but only Lys uptake was affected. This suggests that Lys uptake may have been inhibited through a mechanism that did not affect the other NB-group 2 AA. Arterial Lys concentration was not different between SAL, BCAA, and GR1+ILV. Considering the similarly lower uptake of Lys observed on BCAA infusion, compared with EAAC, it is compelling to suggest that the presence of high levels of arterial BC-group 2 AA (i.e. > 753 μ*M* as observed on EAAC) inhibited Lys transport. Cationic Na^+-independent AA transport systems facilitate transfer of both Arg and Lys into bovine mammary cells (Baumrucker, 1984; Baumrucker, 1985), and react with neutral amino acids, including Leu, in lactating rat and sow mammary tissue (Calvert and Shennan, 1996; Shennan et al., 1997; Hurley et al., 2000). Lysine may also be transported into mammary cells using Na^+-dependent systems $ATB^{o,+}$ and y^+LAT1, both identified in porcine mammary tissue (Pérez Laspiur et al., 2009; Manjarin et al., 2011), and Na^+-independent LAT1 systems that have been identified in bovine mammary tissue (Bionaz

and Loor, 2011), all of which are shared by Ile and Leu. Therefore, it is possible that intracellular and extracellular concentrations of Ile and Leu when their arterial supply to mammary cells was high may have influenced the transport of Lys and Arg into mammary cells in the current experiment. If interaction on these transporters was indeed affected, the fact that Lys uptake was more susceptible than Arg might be due to its apparent flexibility for use in intramammary metabolism compared with Arg. When Lapierre et al. (2009) removed Lys from a complete AA mixture, the reduced U:O of Lys was accompanied by reduced Lys-N transfer into NEAA, while milk protein yield was not affected. In the current study, the U:O of Arg was decreased on GR1+ILV compared with GR1+ALT, but unlike Lys, its uptake was still maintained well above its milk protein output (2.20). Intracellular requirements for Arg appear to hold a constant level of priority for intramammary catabolism, evidenced by an U:O that is maintained > 2 across a range of digestive supplies (33 to 175 g/d; Lapierre et al., 2012). This ratio appears to hold whether increased digestive Arg supply arises from a mixture of EAA + NEAA fed as rumen-protected protein (Nichols et al., 2019c), or from EAA infused into the abomasum (Nichols et al., 2016; Nichols et al., 2019b). This is probably to overcome intramammary deficits of Glu and Pro, the NEAA present in the highest concentrations in milk protein, the latter of which is majorly synthesised from Arg and metabolites of its catabolism in mammary cells (Clark et al., 1975; Roets et al., 1979b).

CONCLUSIONS

When Arg, Lys, and Thr, or Ile, Leu, and Val were absent from postruminal EAA infusions and the other 7 EAA were present to compensate the MP supply, intramammary catabolism of the present group 2 AA compensated for the lower mammary uptake of the absent EAA. Mammary uptake of Lys may have been inhibited by high levels of arterial branched-chain AA during BCAA and GR1+ILV infusion, and in both treatments the intramammary catabolism of branched-chain AA could have compensated for the lower intramammary Lys level. When Ile, Leu, and Val were supplemented alone, the mammary gland maintained a high capacity for their uptake and increased their intramammary catabolism to stimulate synthesis of milk protein, and likely synthesis of fat and lactose as well. Overall, these findings illustrate flexibility in mammary uptake and intramammary catabolism of the AA within the group 2 category to support milk protein synthesis when supplemented MP level is maintained but the EAA profile of MP is incomplete with respect to casein.

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Chapter 9

General Discussion

Introduction

Gains made in the productive efficiency of dairy cattle to date have reduced resource inputs and the environmental footprint of dairy farming per unit of milk produced (Capper et al., 2009; Baumgard et al., 2017). Growing intensity in the global balance between resources to produce food for humans and feed for animals drives continued emphasis on maximizing nutrient use efficiency. Furthermore, it has become increasingly important to consider the environmental impact of food production per unit of human nutritional value, rather than simply per unit of mass (Tilman and Clark, 2014). Milk protein produced from ruminants can be particularly impactful with regards to the synthesis of healthy human-edible protein from non-human edible biomass (de Vries and De Boer et al., 2010; Pereira, 2014; Thorning et al., 2016). Maximal and efficient conversion of nitrogen (N) from dairy diets into milk protein is important to ensure a sustainable source of human-edible protein and for the social and environmental acceptability of the industry. The modern dairy cow must be fed and maintained in such a way that she can effectively consume, digest, absorb, and partition energy and protein towards high-quality milk, and it is critical that this is achieved within the bounds of her own health and welfare. Current research focus in the field of ruminant nutrition is aimed at improving the transfer efficiency of energy and N from a variety of common feedstuffs into milk, minimizing N emissions, and realizing the utility of alternative, human inedible feedstuffs for incorporation into cattle rations.

The glucogenic potential of amino acids (AA) has spurred years of research into the supply of non-AA glucogenic energy as a means to improve the transfer of dietary AA into milk protein (Clark et al., 1977; Raggio et al., 2006; Rius et al., 2010a; Nichols et al., 2016). Feeding lipogenic diets through provision of high-fiber forages, rumen-inert long-chain fatty acid (FA) supplements, or both, can increase the transfer of dietary energy into milk energy (van Knegsel et al., 2007; Boerman et al., 2015), but quantification of N utilization with high fat diets is scarce. Specifically, little attention has been paid to how lipogenic substrates affect AA utilization by mammary glands. Variable efficiencies of individual AA use by dairy cattle has been recognized (Lapierre et al., 2018), but there is still a remarkably wide knowledge gap around understanding the dynamics of these efficiencies with respect to other dietary nutrients and dietary AA profile, and how they can be most effectively managed in a feeding scheme for dairy cows. Furthermore, the mechanistic pathways through which glucogenic, lipogenic, and aminogenic substrates affect cellular regulation of milk component synthesis remain incompletely understood (Cant et al., 2018).

With this thesis, contributions have been made to the gaps in knowledge around effects of postruminal energy type, particularly lipogenic energy compared with glucogenic energy, at low and high metabolizable protein (MP) levels, and the effect of AA profile of supplemented MP. A particularly impactful aspect of this work is the characterisation of these effects at the whole-body and mammary gland level in 3 experiments with lactating

dairy cattle. This chapter aims to discuss the application of lipogenic, glucogenic, and aminogenic energy, and the AA profile of aminogenic supplements, to improve milk N efficiency (conversion of feed N into milk N), according to observations from the work described in this thesis. This discussion concludes with recommendations for feeding dairy cattle to optimize transfer of dietary energy and N into milk macronutrients, and suggestions for future research efforts.

Hypotheses and Overview

General hypotheses of this thesis were as follows:

- Postruminal fat supplementation will improve milk N efficiency at low and high MP levels, but through different whole-body metabolic effects than glucogenic energy.
- Mammary gland AA metabolism will be affected more by postruminally absorbed glucose than fat.
- Lipogenic and glucogenic energy (from protein or glucose) will stimulate milk lactose yield, but through different metabolic adaptations.
- Postruminal AA supplementation will increase milk protein yield, and the AA profile of this supplement will affect milk N efficiency and mammary gland AA metabolism.
- Signaling pathways regulating mammary secretory cell differentiation respond to glucogenic, lipogenic, and aminogenic substrate and are involved in the milk synthetic response to nutritional intervention.

The approach of this general discussion is to reflect on the validity of these hypotheses by comparing and contrasting results of the 3 experiments presented in this thesis. To test these hypotheses, 2 experiments with mid-lactation dairy cows were performed where lipogenic, glucogenic, and aminogenic substrates were supplemented. In Experiment 1 (Chapters 2, 3, and 4), isoenergetic levels of rumen-protected (RP) protein and hydrogenated palm FA were tested in a factorial arrangement. In Experiment 2 (Chapters 5 and 6), abomasal infusions of glucose and palm olein were tested at low and high MP levels, where the high MP level arose from abomasal essential AA (EAA) infusion. A third experiment (Experiment 3; Chapters 7 and 8) was conducted to test the effect of EAA profile within a constant level of supplemental MP using abomasal infusions. **Table 9.1** gives an overview of the experimental treatments tested in the chapters of this thesis. In the following discussion, most emphasis is placed on the effect of lipogenic energy at low and high MP levels, which can be compared between the treatments LP/HF and LMP-LG, and HP/HF and HMP-LG, in Experiment 1 and 2. This discussion also makes comparisons between the treatments HP/LF, HMP-C, and EAAC in Experiments 1, 2, and 3, respectively, to assess the effect of energy from protein in terms of level and the presence or absence of non-EAA (NEAA) in the supplement. Comparing HMP-C and EAAC from Experiment 2 and 3, respectively, allows discussion on the

Table 9.1. Overview of the experimental treatments in this thesis

Experiment	Treatment	Energy Supplement	Metabolizable Protein Level	Source[1]	Supply	Control Treatment Gross Energy Intake[2]
Experiment 1 (Chapters 2, 3, 4)	LP/LF*	-	Low	-	0 g/d	361 MJ/d SEM = 5.2 MJ/d
	LP/HF	Lipogenic	Low	saturated FA (C16 + C18; Hidropalm)	680 g/d	
	HP/LF	Aminogenic	High	soybean meal and rapeseed meal (SoyPass + RaPass)	2000 g/d	
	HP/HF	Aminogenic + Lipogenic	High	soybean meal and rapeseed meal + saturated FA	2000 g/d + 680 g/d	
Experiment 2 (Chapters 5, 6)	LMP-C*	-	Low	-	0 g/d	317 MJ/d SEM = 13.9 MJ/d
	LMP-GG	Glucogenic	Low	glucose	1319 g/d	
	LMP-LG	Lipogenic	Low	palm olein (monounsaturated, C16 + C18:1)	676 g/d	
	HMP-C	Aminogenic	High	essential AA (all 10, casein profile)	844 g/d	
	HMP-GG	Aminogenic + Glucogenic	High	essential AA + glucose	844 g/d + 1319 g/d	
	HMP-LG	Aminogenic + Lipogenic	High	essential AA + palm olein	844 g/d + 676 g/d	
Experiment 3 (Chapter 7, 8)	SAL*	-	Low	-	0 g/d	350 MJ/d SEM = 13.1 MJ/d
	EAAC	Aminogenic	High	essential AA (all 10, casein profile)	562 g/d	
	BCAA	Aminogenic	High	Ile, Leu, Val	562 g/d	
	GR1+ILV	Aminogenic	High	His, Met, Phe, Trp, Ile, Leu, Val	562 g/d	
	GR1+ALT	Aminogenic	High	His, Met, Phe, Trp, Arg, Lys, Thr	562 g/d	

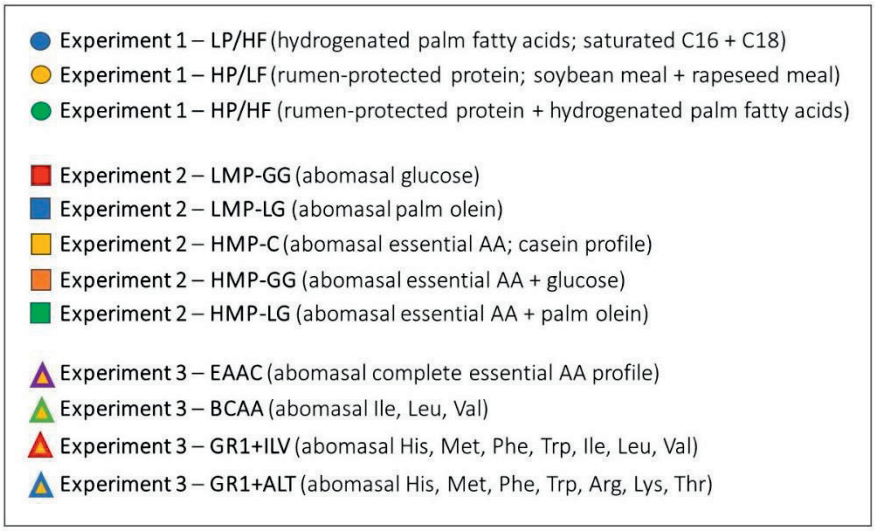
*Denotes the control treatment in each experiment.

[1]FA = fatty acid; AA = amino acid

[2]SEM = standard error of the mean according to statistical analysis of each experiment.

impact of level of MP supplementation when it is comprised of exclusively EAA. Marginal gross energy (GE) intake is the basis by which all experimental treatments can be compared across this thesis, regardless of energy type. Unless otherwise indicated, the figures in the following discussion present the marginal response in several variables against the change in GE intake (basal diet plus the respective supplements) between each treatment and its respective control within each experiment. Table 9.1 lists the daily GE intake on the control treatment in each experiment.

The following legend can be applied to the figures in this chapter, unless otherwise indicated:



Whole-Body Metabolism

Milk Yield

In Experiment 1, milk yield increased independently in response to isoenergetic fat and protein supplementation, and the effect was additive when fat and protein were supplemented together (Chapter 2). In Experiment 2, milk yield was not affected by fat supplementation, whereas isoenergetic protein supplementation increased milk yield over the control level, and this was independent of fat level (Chapter 5). This difference in milk yield in response to lipogenic supplementation at low and high MP levels in Experiment 1 and 2 (**Figure 9.1**) can be attributed, at least in part, to the different responses in dry matter intake (DMI) observed with fat supplementation in these experiments. **Textbox 9.1** discusses possible reasons for the difference in DMI response during fat supplementation between Experiment 1 and 2. If DMI had been the same in response to supplementation of isoenergetic fat and protein in Experiment 2 (between LMP-LG and HMP-C), as intended, marginal GE intake would have been 22 MJ/d on LMP-LG and milk yield would have increased 2.8 kg/d over the control (instead of decreasing 0.6 kg/d), assuming fat supplementation in Experiment 2 would stimulate proportionately the same response in milk yield relative to marginal GE intake as in Experiment 1.

Feeding HP/LF provided similar marginal GE intake compared with HMP-C and EAAC (29, 22, and 24 MJ/d, respectively), but did not stimulate the same magnitude of increase in total milk yield (2.1 kg/d on HP/LF versus 3.5 and 5.0 kg/d with HMP-C and EAAC, respectively; Figure 9.1). Protein supplementation in Experiment 1 delivered approximately 909 g/d of digestible AA, of which 449 g/d were EAA, whereas protein supplementation in Experiments 2 and 3 consisted entirely of EAA (844 and 562 g/d of digestible AA, respectively). The fact that milk yield was not stimulated to the same level on Experiment 1 despite similar GE intake between the 3 experiments is probably linked to the relationship between total milk yield and milk protein synthesis, of which the latter is more stimulated by EAA than NEAA (Metcalf et al., 1996; Doepel and Lapierre, 2010).

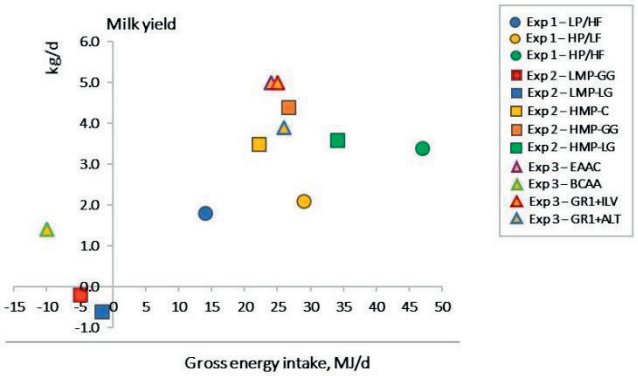


Figure 9.1. Marginal milk yield responses against marginal gross energy intake between each experimental treatment and control.

Textbox 9.1. Fat supplementation and feed intake

The level of fat supplementation in g/d between Experiment 1 and 2 was comparable (Table 9.1), along with the crude fat content of the basal diets (37 and 32 g/kg DM in Experiment 1 and 2, respectively). Fat supplementation in Experiment 1 resulted in a small negative response in DMI at the low MP level only (0.2 kg/d; no change at high MP level; Chapter 2). In Experiment 2, lipogenic supplementation decreased DMI 1.5 kg/d at the low MP level and 0.5 kg/d at the high MP level (Chapter 5). The difference in magnitude of effect on DMI between these fat supplements could have been due to the difference in their saturation level. Hypophagic effects on DMI become more severe as the degree of saturation of fat supplements decreases (Pantoja et al., 1996; Bremmer et al., 1998), and the response of gut peptides sensitive to FA supply in the gastrointestinal tract could be affected by FA saturation (Choi and Palmquist, 1996; Benson and Reynolds, 2001; Relling and Reynolds, 2007). The dynamics of gut peptide signaling to affect feed intake could also have differed when FA were delivered to the lower gut via continuous infusion in Experiment 2 rather during digestion of a total mixed ration in Experiment 1. Indeed, feeding canola oil (1 kg/d) increased plasma concentration of the gut peptide cholecystokinin but did not reduce DMI, whereas abomasal infusion of the same amount of canola oil increased plasma cholecystokinin concentration and reduced DMI (Chelikani et al., 2004).

Protein Yield and Milk Nitrogen Efficiency

Non-AA energy precursors can allow the use of available EAA for milk protein synthesis by sparing them from gluconeogenesis or energy-yielding oxidative processes. The relationship between MP level and AA partitioning towards milk protein synthesis in response to absorptive supply of glucogenic substrates had been examined in several studies prior to this thesis work (Raggio et al., 2006; Rius et al., 2010a; Nichols et al., 2016), but had not been extensively characterised with regards to lipogenic supplementation. This gap in knowledge prompted many of the hypotheses tested in this thesis. Independent of MP level, fat supplementation in Experiment 1 resulted in a tendency for an increase in milk protein yield (Chapter 2). In Experiment 2, fat supplementation did not increase milk protein yield (Chapter 5), but it also did not significantly decrease it when compared with control levels at the low MP level (LMP-C) and high MP level (HMP-C; **Figure 9.2A**). Taken together with the decrease in DMI, and thus the lower N intake, at both MP levels in response to fat supplementation, it can be stated that lipogenic supplementation in Experiment 2 did stimulate milk protein synthesis.

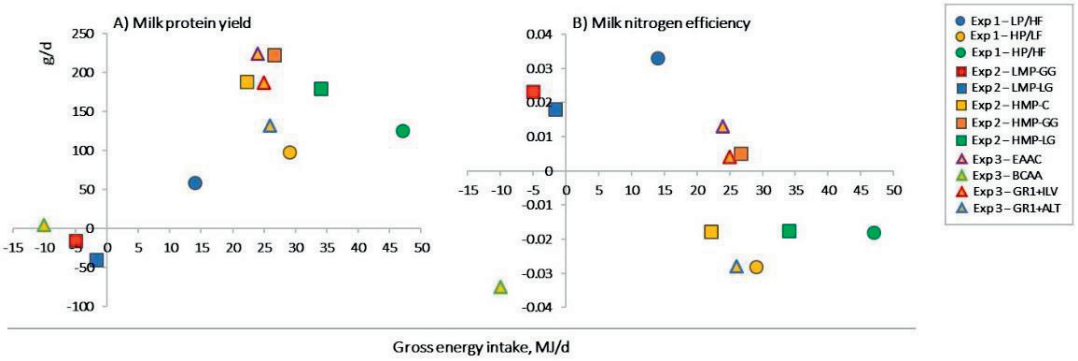


Figure 9.2. Marginal responses in milk protein yield (A) and milk nitrogen efficiency (B) against marginal gross energy intake between each experimental treatment and control.

In Experiment 1, we did not measure N balance as was done in Experiment 2. Parameters that supported AA partitioning into protein synthesis instead of catabolism with lipogenic supplementation in Experiment 1 included the tendency for milk urea to decrease at the HP level with the addition of HF (Chapter 2; see **Textbox 9.2** for comments on the suitability of milk urea content as an indicator of milk N efficiency), the highest milk N efficiency with LP/HF, the 28 g/d marginal increase in milk protein yield between HP/LF and HP/HF, and the numerically higher milk N efficiency on HP/HF compared with HP/LF (Chapter 2; **Figure 9.2B**). In Experiment 2, at the low MP level, fat supplementation numerically increased milk N efficiency over the control, whereas at the high MP level, fat supplementation did not offer any improvement in milk N efficiency (i.e., when compared

with HMP-C; Chapter 5; Figure 9.2B). The higher supply of EAA might 'override' a positive effect of extra GE intake from fat supplementation in Experiment 2 compared with that observed in Experiment 1, suggesting positive benefits of fat supplementation on milk N efficiency might be more realized at lower EAA doses. In contrast to fat supplementation, glucose supplementation at the high MP level still resulted in a positive response in marginal milk N efficiency relative to the control (Chapter 5; Figure 9.2B), and a 29% increase in marginal MP efficiency over that achieved by HMP-C and HMP-LG in Experiment 2 (**Figure 9.3**). This response is in agreement with others who have observed positive effects of glucogenic energy on milk N efficiency at high MP levels (Clark et al., 1977; Rius et al., 2010b; Cantalapiedra-Hijar et al., 2014), and is discussed further in Chapter 5.

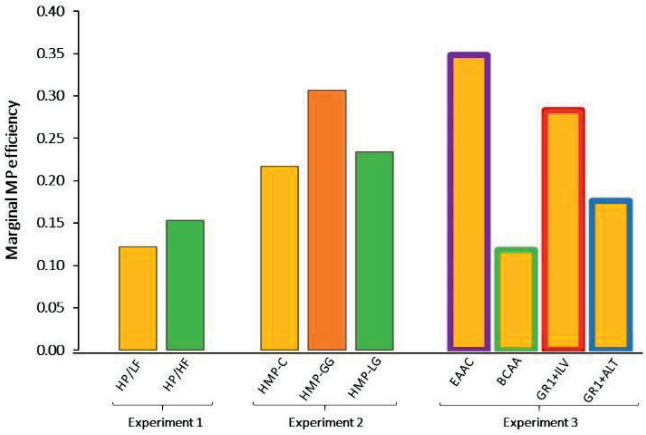


Figure 9.3. Marginal metabolizable protein (MP) efficiency calculated as the marginal milk protein yield arising from the supplemented protein relative to the marginal MP intake between each protein-supplemented experimental treatment and experimental control.

Milk protein yield was most affected by aminogenic substrates in this thesis (Figure 9.2A). This was expected, in line with generally positive milk protein responses when postruminal AA replace a portion of the MP supply in the form of RP protein sources (Chan et al., 1997; Wright et al., 1998; Schor and Gagliostro, 2001) or infused profiles of total AA or EAA alone (Doepel and Lapierre et al., 2010; Galindo et al., 2011; Nichols et al., 2016). The marginal increase in milk protein yield was similar between the complete EAA profile at both doses (189 g/d on HMP-C and 224 g/d on EAAC) and the GR1+ILV profile (187 g/d; Figure 9.2A). At the lower EAA infusion dose, EAAC and GR1+ILV in Experiment 3 resulted in a positive marginal response in milk N efficiency, whereas the higher EAA dose with HMP-C in Experiment 2 resulted in a negative marginal response (Figure 9.2B). This shows that N from postruminal protein supplemented as EAA in the profile of casein is transferred more efficiently into milk N at a lower dose than at a higher dose, and that supplying an AA profile

consisting of His, Met, Phe, Trp, and the branched-chain AA can result in a similar transfer efficiency. It is notable that energy from protein in Experiment 1 on HP/LF resulted in a marginal milk protein yield response of approximately 100 g/d less than abomasal EAA treatments, and a negative response in marginal milk N efficiency (Figure 9.2). This can be attributed to the fact that, of the absorbed AA from the RP supplement, approximately 50% were NEAA, which are not as efficiently incorporated into milk protein as EAA (Metcalf et al., 1996; Lemosquet et al., 2009; Doepel and Lapierre, 2010).

Supplementation of the complete EAA profile infused at 562 g/d achieved the greatest marginal MP efficiency of all treatments in this thesis (35%; Experiment 3; Figure 9.3). This is high relative to marginal MP efficiencies generally achieved with casein infusion (21%, Hanigan et al., 1998; 29%, Huhtanen and Hristov, 2010). Considering the quadratic prediction equation of Huhtanen and Hristov (2010) relating casein infusion level to expected milk protein yield, 844 g/d of infused casein would be used with 19% efficiency. This is slightly lower than the 22% marginal MP efficiency that was observed with this infusion dose in including only EAA in Experiment 2. In response to 562 g/d of infused casein, this equation predicts a marginal MP efficiency of 25%, where observed marginal MP efficiency was 35% in response to 562 g/d of EAA in Experiment 3. Comparison of these predicted and observed responses nicely contrasts the effect of AA supplementation as EAA (in a casein profile) compared with AA supplementation as casein (delivering both EAA and NEAA). In Experiment 2, the higher EAA infusion dose resulted in an expectedly lower marginal efficiency relative to the lower EAA infusion dose in Experiment 3. However, supplementing EAA even at a high level can still be used slightly more efficiently than when the same level of supply comes from casein. Further, considering the 909 g/d of total AA supplemented in Experiment 1, the predicted marginal MP efficiency with this dose of casein is 18%, while the observed response was 12%. In this case, casein would be expected to do better than the RP supplement where the AA profile of soybean meal and rapeseed meal varies more from the profile of milk protein. Overall, this comparison emphasises the importance of AA profile and dose when expecting supplemental MP to offer gains in efficiency of milk protein synthesis.

Textbox 9.2. Milk urea N as an indicator for milk N efficiency

It has been suggested throughout this thesis that milk urea N is not a sound indicator of changes in milk N efficiency (Chapters 2, 5, and 7). This was particularly noticeable in Experiments 2 and 3 when dietary CP contents were low (<14%) and the resulting milk urea contents were relatively low. Limits of detection in the analyses of milk urea when done by infrared may affect the reliability of this measurement (Barbano and Lynch, 1992; Weeks and Hristov, 2017). **Table 9.2** summarizes milk urea and milk N efficiency responses described in this thesis. Based on this summary, 50% of the observations in

this thesis would have given a false impression of the change in efficiency of transfer of dietary N into milk N if only milk urea was considered (shaded lines). Discrepancies in milk urea analysis when milk urea contents are at the low end of detection ranges may become more important as dairy farmers are increasingly encouraged to reduce dietary CP contents in practice. In order to accurately monitor N efficiency on-farm, alternative measurement proxies may need to be considered.

Table 9.2. Summary of responses in milk urea and milk N efficiency in this thesis[1]

Chapter	Treatment	Milk urea (response over control)	Milk N efficiency (response over control)
Chapter 2	HP/LF	↑	↓
	LP/HF	**nc**[2]	**↑**
	HP/HF	↑	nc
Chapter 5	LMP-GG	**nc**	**↑**
	LMP-LG	nc	nc
	HMP-C	↑	↓
	HMP-GG	**↑**	**↑**
	HMP-LG	↑	↓
Chapter 7	EAAC	**nc**	**↑**
	BCAA	**nc**	**↓**
	GR1+ILV	**nc**	**↓**
	GR1+ALT	nc	nc

[1]Shaded lines indicate responses where observed milk urea does not agree with observed milk N efficiency.
[2]No change.

Lactose Yield

In Experiment 1, the same level of lactose yield was produced with energy from protein and energy from fat, and the response was additive when protein and fat were supplemented together (**Figure 9.4A**). In Experiment 2, fat supplementation did not increase milk lactose yield over control levels, as observed in Experiment 1, but it did produce the same level of lactose as the control at both the low MP level (LMP-C) and at the high MP level (HMP-C), despite lower DMI. Reduced intramammary de novo milk FA synthesis arising from increased mammary uptake of long-chain FA with fat supplementation in both experiments spared some intramammary glucose for lactose synthesis (Chapters 3 and 6). Mammary gland glucose balance with fat supplementation is discussed in a following section.

Positive effects of postruminal AA supplementation on milk lactose yield were consistently observed in this thesis (Chapters 2, 5, and 7), in line with observations of others (Doepel and Lapierre et al., 2010; Galindo et al., 2011; Nichols et al., 2016). In all experiments, when the marginal increase in GE intake came from protein supplementation, positive marginal responses in lactose yield were observed (Figure 9.4A). Amino acids can contribute

to lactose yield by increasing whole-body glucose flux (Galindo et al., 2011), by contributing to galactose synthesis in the mammary gland (Bequette et al., 2006; Lapierre et al., 2013; Maxin et al., 2013), and by affecting cellular signaling pathways stimulating synthesis of milk protein (Cant et al., 2018), which may affect subsequent synthesis of lactose (Davies et al., 1983; Shennan and Peaker, 2000). No significant increases in arterial glucose concentration in response to increased absorptive protein supply were observed in this thesis (Chapters 3, 6, 8), and whole-body glucose flux in response to protein supplementation is not reported here. It is suggested in Chapter 6 that, particularly when their supply to the mammary gland is high, EAA could be contributing to galactose synthesis. These observations support previous suggestions of an effect of AA as such on milk lactose yield, that is independent of GE intake or mammary glucose supply (Lemosquet et al., 2009; Doepel and Lapierre, 2010; Lapierre et al., 2010). A relationship between mammary AA supply and lactose yield that is independent of total GE intake is highlighted in particular by the BCAA treatment in Experiment 3, where total GE intake relative to the control decreased 10 MJ/d, but resulted in a marginal lactose yield increase of 78 g/d (Chapter 7; Figure 9.4A).

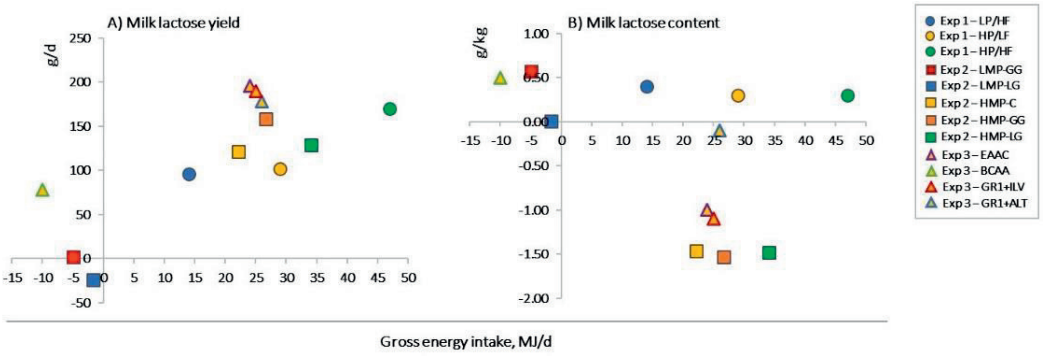


Figure 9.4. Marginal responses in milk lactose yield (A) and milk lactose content (B) against marginal gross energy intake between each experimental treatment and control.

Lactose Content

Increasing MP supply usually increases milk and lactose yield (Wright et al., 1998; Doepel and Lapierre et al., 2010; Galindo et al., 2011; Nichols et al., 2016), but either decreases (Lemosquet et al., 2007) or has no effect (Daniel et al., 2016) on lactose content. **Figure 9.4B** illustrates a clear negative marginal response in milk lactose content during postruminal EAA infusions in this thesis (except BCAA and GR1+ALT), which differed from that where absorptive AA supply was increased through RP protein. When postruminal MP supply was increased by EAA infusions (in AA profiles that are complete or almost complete with respect to casein), milk protein yield responses were greatest (≥132 g/d; shaded lines, **Table 9.3**) and were driven by increases in milk protein content, not solely by increases in

total milk yield. This pattern, where protein content and yield increases, and lactose content decreases but yield increases, is substantiated by observations of others in response to postruminal EAA infusions (Doepel and Lapierre, 2010; Nichols et al., 2016).

Table 9.3. Marginal changes in milk yield, and yield and content of protein and lactose in response to supplemented metabolizable protein relative to the control treatment in each experiment[1]

Experiment	Treatment	Milk, kg/d	Protein, g/d	Protein, g/kg	Lactose, g/d	Lactose, g/kg
Experiment 1	HP/LF	+2.1	+98	+1.2	+102	+0.3
	HP/HF	+3.4	+126	+0.3	+170	+0.3
Experiment 2	HMP-C	+3.5	+189	+2.5	+122	-1.5
	HMP-GG	+4.4	+223	+2.6	+158	-1.5
	HMP-LG	+3.6	+179	+2.0	+129	-1.5
Experiment 3	EAAC	+5.0	+224	+2.0	+196	-1.0
	BCAA	+1.4	+5	-1.3	+78	+0.5
	GR1+ILV	+5.0	+187	+1.0	+190	-1.1
	GR1+ALT	+3.9	+132	+0.6	+178	-0.1

[1]Shaded lines indicate treatment responses where the marginal milk protein yield response was positive and the marginal response in lactose content was negative.

These observations suggest that at high levels of mammary EAA supply in profiles that are relatively complete with respect to casein, milk protein synthesis may play a relatively larger role in regulation of total milk volume than that of lactose synthesis. Under conditions of highly stimulated protein synthesis, lactose yield appears to be driven more by increased total milk yield, because lactose content decreases. In line with this proposed hypothesis, it is interesting to consider the HP/HF treatment in Experiment 1, where the change in total milk yield is very similar to that on HMP-C and HMP-LG in Experiment 2, but the marginal increase in lactose yield is greater and the marginal increase in protein yield is lower. It is also interesting to consider the GR1+ALT treatment in Experiment 3, where the milk protein yield increase was the lowest of those associated with a negative lactose content (numerically closest to the next lowest (HP/HF) than the next highest (HMP-LG)), and lactose content in this treatment only decreased 0.1 g/kg where all others decreased by at least 1.0 g/kg. Together this suggests that when protein synthesis is not as highly stimulated, lactose synthesis is more influential in regulating milk volume than protein synthesis.

Lactose is the principle regulator of cellular osmotic pressure in mammary secretory cells. Therefore, changes in relative secretion rate of protein must influence lactose synthesis in order to also affect changes in milk yield. This could be mechanistically linked with the interactions between protein and lactose in milk secretory vesicles (Davies et al., 1983), and the effects on the osmotic gradient of the vesicles when protein content increases (Shennan and Peaker, 2000). For example, under conditions of increased AA flux into mammary cells via Na^+-dependent transporters, Na^+/K^+ ATPase activity may be up-regulated to balance intracellular Na^+ concentration and facilitate AA influx (McGivan and Pastor-Anglada, 1994).

Increased intracellular K^+ concentration may thereby result in reduced lactose content to maintain constant osmotic pressure of secretory cells. By this hypothesis, both protein and lactose secretion could increase, but maintenance of cellular osmotic balance via lactose would result in lactose secretion being relatively lower than that of protein, resulting in lower milk lactose content.

Mammary Gland Metabolism

Characterising mammary gland metabolite utilization, particularly in response to lipogenic substrates, was a major focus of this work. Nutrient utilization by bovine mammary glands is suited to study by arteriovenous (AV) difference methodology because of tissue drainage through an easily accessible vein, and secretion by the glands of biosynthetic products through a separate network of epithelial ducts (Linzell, 1974; Cant et al., 2016). For in vivo flux measured by AV difference, sampling of representative metabolite supply in blood at pre- and post-organ sites and a reliable measure of tissue blood flow is essential to the validity of the resulting data (Mepham, 1982). In the work described in Chapters 3, 6, and 8, mammary plasma flow (MPF) was calculated using the Fick principle. A description of this principle for calculation of MPF, along with assumptions and limitations, is given in **Textbox 9.3**.

Textbox 9.3. Measurement of mammary gland metabolite flux

Blood flow calculation

To estimate mammary plasma flow (MPF) with the Fick principle, the following parameters must be known: the amount of a given marker taken up by the tissue over a given time interval, the concentration of the marker in the arterial supply to the tissue, and the concentration of the marker in the venous outflow from the tissue. The studies in this thesis used Phe and Tyr as markers, according to the assumption that Phe is not oxidized within the mammary gland, is only converted to Tyr via hydroxylation, and that both AA are transferred quantitatively from blood to milk (Mepham, 1982). Therefore, the output of Phe + Tyr in milk true protein represents that which was taken up by the mammary gland, and the concomitant measurement of arterial and venous concentrations of these AA over the milking interval allows the calculation of their arteriovenous (AV) difference. Subsequently, MPF can be calculated according to the following equation:

MPF (L/h) = [milk Phe + Tyr output (μmol/h)] / [AV Phe + Tyr difference (μmol/L)]

In this thesis, milk output of Phe + Tyr was estimated using mean Phe and Tyr contents in milk true protein reported by Mepham (1987) and Lapierre et al. (2012). Milk crude protein was assumed to consist of 94.5% true protein (DePeters and Ferguson, 1992), with a 3.37% contribution from blood-derived proteins (Lapierre et al., 2012). Free amino acids in plasma were measured for calculation of MPF and mammary net flux.

Underestimation or overestimation of flow

The Fick principle requires the assumption that there is no oxidative loss of Phe or Tyr in the udder. For Phe, this assumption is apparently valid, even at high arterial supplies (Lemosquet et al., 2010). However, if Phe and Tyr from the arterial supply were to be oxidized in the mammary gland, MPF may be underestimated. In addition, a change in the turnover rate of constitutive proteins or the uptake of Phe- or Tyr-containing plasma peptides could affect the amount of Phe and Tyr available in the gland for protein synthesis which, if not accounted for, would affect the accuracy of the estimate of MPF (Crompton et al., 2014).

Accurate measurement of AV difference is also imperative to accurate estimation of MPF and metabolite uptake. To obtain estimates of AV difference that represent mammary biosynthetic processes over a given interval, blood samples must be taken frequently to minimize effects of variation in feed intake pattern (Bequette et al., 1998), to account for dynamics of nutrient absorption and transit times of metabolites through the gland (Cant et al., 1993), and other diurnal patterns of cows that may affect mammary blood flow that are unrelated to the dietary intervention under examination (i.e. standing and lying times, activity level). Some non-mammary venous backflow may appear in the abdominal vein due to valvular incompetence of the pudic vein after repeated dilation to accommodate the hemodynamic alterations associated with several lactations (Linzell, 1960; Thivierge et al., 2001). Using younger cows (≤ third lactation) is a suggested technique to mitigate the influence of this effect on metabolite AV difference estimates (Bequette et al., 1998; Thivierge et al., 2001).

Mammary Plasma Flow

Local vasodilator release by mammary cells alters blood flow to the gland to maintain intramammary ATP balance (Cant et al., 2003; Madsen et al., 2015). This energy-sensitive vasoactive response may allow mammary blood flow to increase or decrease according to arterial concentrations of energy-yielding metabolites (Cieslar et al., 2014). Thus, effects of energy-type and AA supply on arterial concentrations of glucose, acetate, and β-hydroxybutyrate (BHB), would be expected to affect mammary blood flow. In line with this

expectation, **Figure 9.5** suggests that in the experiments of this thesis, energy-type or the AA profile of aminogenic energy had a greater influence on MPF than changes in total GE intake.

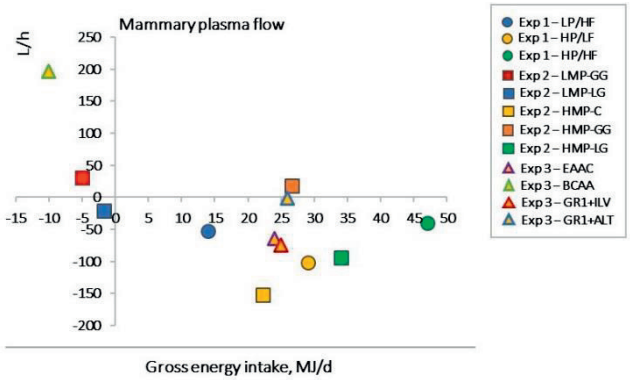


Figure 9.5. Marginal response in mammary plasma flow against marginal gross energy intake between each experimental treatment and control.

Declines in MPF were observed when GE intake was increased by postruminal EAA supplementation, particularly in Experiment 2 in response to 844 g/d of EAA infusion (HMP-C; Figure 9.5). Exceptions to this pattern can be observed with glucose infusion, where the positive effect of glucose on MPF was independent of MP level in Experiment 2 (discussed in Chapter 6), and with BCAA in Experiment 3 where the increase in MPF can be attributed to the great imbalance in mammary gland AA supply with this infusion (discussed in Chapter 8). Decreases in mammary blood flow when MP supply is increased may be linked to concomitant increases in arterial acetate or BHB concentration during EAA infusions (Cant et al., 2003). Increases in MPF in response to reduced circulating acetate and BHB during glucogenic infusions are well characterised (Rigout et al., 2002; Lemosquet et al., 2009; Curtis et al., 2018; Chapter 6), but a well-defined relationship between arterial concentrations of EAA and BHB, and MPF is missing at present. Nevertheless, responses in arterial EAA and BHB concentrations in the current work may explain the observations in MPF. **Figure 9.6** illustrates the marginal change in arterial BHB concentration relative to the marginal change in arterial EAA concentration. In all treatments where postruminal EAA supply was increased, the marginal change in arterial EAA concentration was positive. Marginal change in arterial BHB concentration was also positive (significantly or numerically increased within experiments), except in response to HP/HF in Experiment 1 and HMP-GG in Experiment 2. Mechanisms to explain the increase in BHB concentration with increased EAA supply are not well defined, but may be related to increase in ketogenic AA (Leu and Lys) supply, decreased efficiency of BHB utilization by peripheral tissues, or increased mobilization of endogenous energy stores to support protein synthesis (Ørskov et al., 1999).

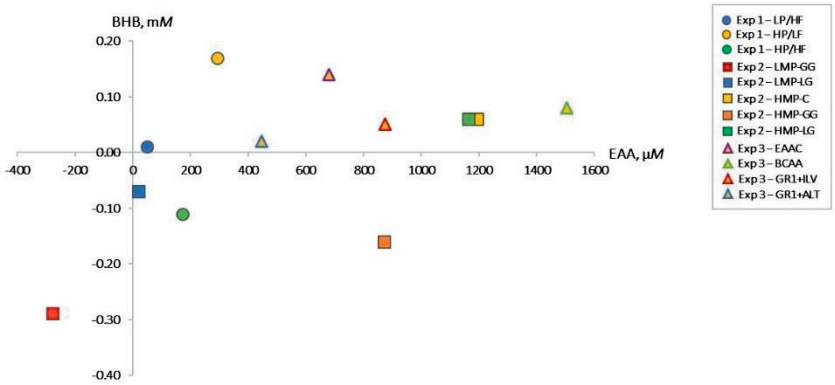
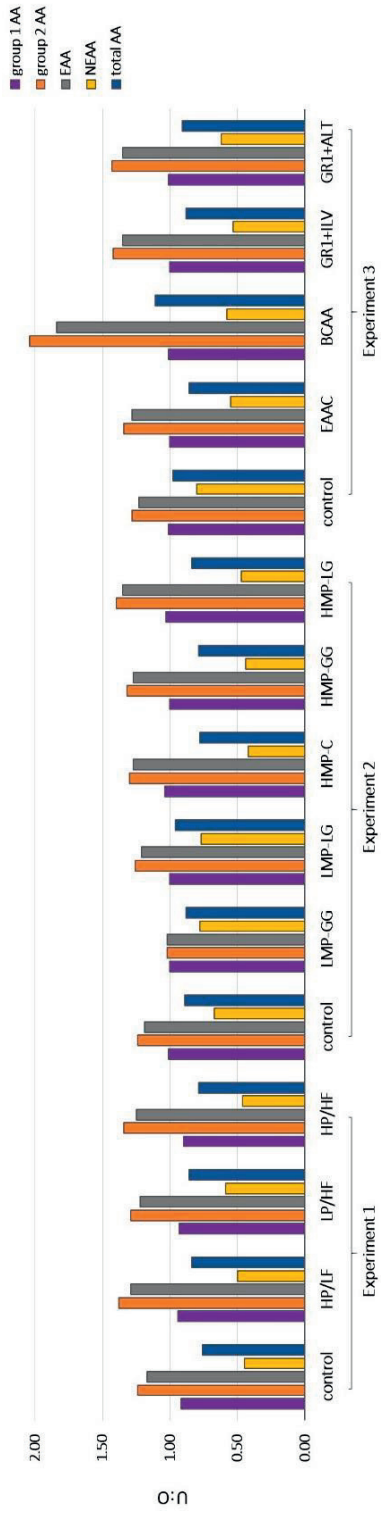


Figure 9.6. Marginal change in arterial β-hydroxybutyrate (BHB) concentration against marginal change in arterial essential amino acid (EAA) concentration between each experimental treatment and control.

Intramammary Amino Acid Metabolism

Mammary gland uptake to milk output (U:O) of AA offers an indication of intramammary AA use. Amino acids that are sequestered by the gland and not secreted in milk are used for mammary tissue protein synthesis or catabolism. Mammary AA net balance is generally comparable across experiments (**Figure 9.7**). According to canonical patterns, group 1 AA (His, Met, Phe+Tyr, Trp) are taken up in a 1:1 ratio with their output in milk protein, group 2 AA (Arg, Ile, Leu, Lys, Thr, Val) are taken up in excess of their output in milk protein, and NEAA uptake is deficient relative to their appearance in milk protein. Assumptions around these groups with regard to mammary gland utilization have been discussed in further detail in Chapter 1. In Experiment 1, mammary U:O of group 1 AA was farther from unity (average of 0.92 across treatments) compared with that in Experiment 2 and 3, where it averages 1.0 across treatments and experiments (Figure 9.7). This has been attributed to the practical setting under which this net balance was measured (discussed in Chapter 3). Because of the theoretical 1:1 transfer of group 1 AA into milk protein, their U:O should not be affected by dietary treatments. In agreement, the marginal changes in group 1 AA U:O were not different from zero with all treatments in this thesis (**Figure 9.8A**). Postruminal AA supplementation resulted in positive marginal responses in EAA U:O and group 2 AA U:O, and negative responses in NEAA U:O, with the exception of protein supplementation (with and without fat supplementation) in Experiment 1 where the marginal change in NEAA U:O was positive (**Figure 9.8B, C, D**). This can be attributed to the relatively lower EAA supply in the protein supplement in Experiment 1. During protein supplementation, the mammary gland will typically sequester greater proportions of group 2 AA and less NEAA, and group 2 AA will be used for de novo NEAA synthesis (Roets et al., 1983; Raggio et al., 2006; Lapierre et al., 2009). A striking example of mammary gland capacity for group 2 AA extraction can be seen with BCAA infusion in Experiment 3 (discussed



in Chapter 8), where the U:O ratio of group 2 AA increased approximately 1.5-times that of the other EAA infusion treatments in Experiment 2 and 3.

A main objective of this work was to characterise the effect of postruminal fat supplementation on mammary gland AA metabolism. Overall, results in this thesis show that fat supplementation has little effect on mammary gland AA utilization. Fat supplementation at the low MP level in Experiment 1 and 2 resulted in marginal increases in U:O of NEAA, where this increase was numerically highest of all treatments with LP/HF (Figure 9.8D). In both experiments, intramammary utilization of NEAA would have supported milk protein synthesis at the low dietary MP levels. More net NEAA uptake by the mammary gland means less NEAA would be re-circulated from peripheral circulation to the splanchnic bed and catabolized in the liver. Therefore, fat supplementation, particularly at low MP levels, has the potential to reduce AA catabolism by increasing utilization of NEAA for protein synthesis. Notably, glucose infusion at the low MP level reduced U:O of group 2 AA and increased U:O of NEAA relative to the control level (discussed in Chapter 6), whereas energy from fat did not produce this response in Experiment 1 or 2.

Figure 9.7 (left). Mammary gland amino acid (AA) net balance across experiments, as represented by the mammary gland uptake to milk output (U:O) for group 1 AA, group 2 AA, essential AA (EAA), non-EAA (NEAA), and total AA.

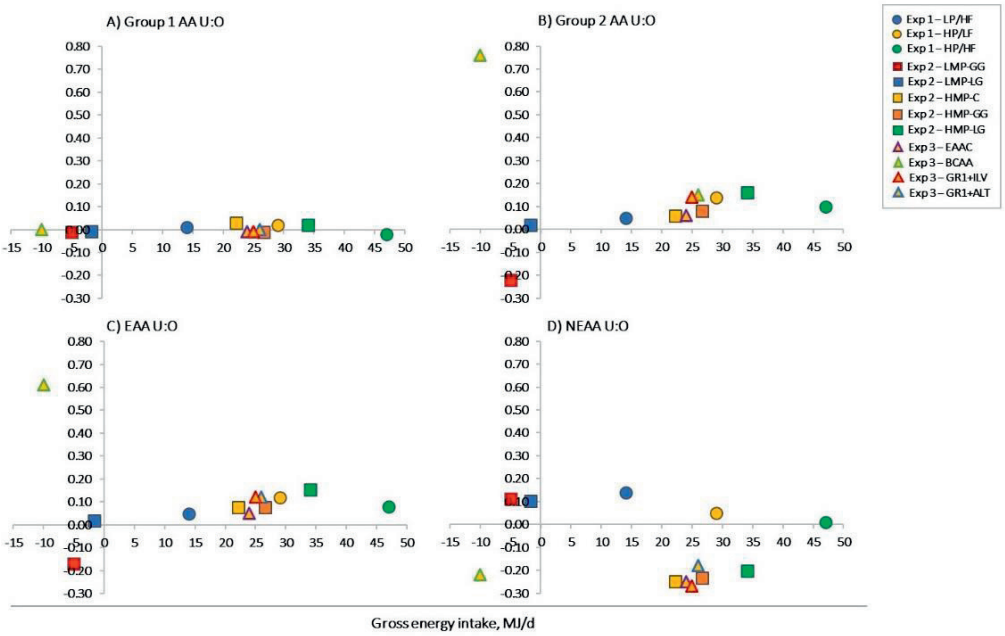


Figure 9.8. Responses in marginal mammary uptake to milk protein output (U:O) of group 1 amino acids (AA; A), group 2 AA (B), essential AA (EAA; C), and non-EAA (NEAA; D) against marginal gross energy intake between each experimental treatment and control.

Mammary Glucose Balance

In general, glucose uptake by the mammary gland does not correspond to milk lactose output (Cant et al., 2002; Rigout et al., 2002; Qiao et al., 2005). This is illustrated in all experiments, where mammary glucose uptake was either in considerable excess of estimated requirements for milk lactose output on most treatments (assuming 1.05 g glucose is required to synthesize 1 g of lactose; Dijkstra et al., 1996), or deficient (**Figure 9.9**). Glucose uptake by the gland is also required for fat synthesis (estimated 0.31 g glucose required per 1 g fat; Dijsktra et al., 1996). Excess intramammary glucose over requirements for lactose and fat will be oxidized to provide carbon for NEAA synthesis and to yield ATP for fat, protein, and lactose synthesis (Smith et al., 1983; Qiao et al., 2005; Lemosquet et al., 2009).

Infusions at the high MP level in Experiment 2, with the exception of HMP-GG, resulted in mammary glucose uptake that was deficient for lactose synthesis. Interestingly, when EAA were infused at a lower dose in Experiment 3, the proportion of glucose uptake relative to requirements for lactose synthesis was in excess, albeit to a lesser degree as in Experiment 1. Arterial glucose concentrations or glucose AV differences were not affected by EAA infusions in either experiment (Chapters 6 and 8), but a greater marginal decrease in MPF with the high EAA infusion dose in Experiment 2 compared with Experiment 3 resulted in numerically lower glucose uptake. This level of EAA infusion stimulated lactose output, even

with apparently insufficient glucose. Amino acids can contribute to the carbon substrate required for galactose synthesis in support of lactose yield (Lapierre et al., 2013; Maxin et al., 2013), which could occur when high levels of EAA depress MPF such that glucose uptake is deficient for mammary synthetic processes (Chapter 6).

A hypothesis of this thesis was that postabsorptive lipogenic energy would stimulate lactose yield to the same extent as glucogenic energy (from glucose or protein), and that this would be due to changes in intramammary glucose partitioning. Lipogenic energy did stimulate lactose yield, but calculated glucose spared from the reduction in do novo FA synthesis with fat supplementation would not have covered the required glucose for lactose synthesis (Chapters 3 and 6). It is often suggested that extra lactose observed when long-chain FA are supplemented would arise from glucose spared from de novo FA synthesis (Chilliard, 1993; Hammon et al., 2008). This contributes some glucose, but it apparently does not account for the entirety. Intramammary glucose metabolism must still respond in other ways in these circumstances, perhaps by reducing oxidation or by using other carbon sources for galactose, such as AA, when extra energy comes from fat.

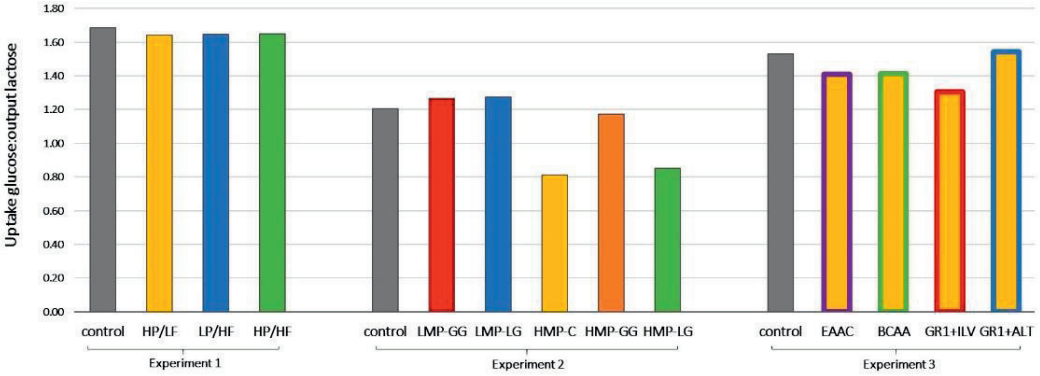


Figure 9.9. Ratio of mammary gland glucose uptake to glucose required for lactose output (estimated using assumptions of Dijkstra et al., 1996) across experiments.

Effects At The Cellular Level

Activation of milk protein synthesis in mammary secretory cells is responsible for the majority of the control over net AA uptake (Cant et al., 2018). The mammalian target of rapamycin complex 1 and the integrated stress response network are cellular pathways that regulate protein synthesis in mammary cells in vivo up to 36 h after exposure to EAA (Rius et al., 2010a; Toerien et al., 2010). However, activation of these pathways does not appear to be maintained over several days in response to such stimuli (Doelman et al., 2015a,b; Nichols et al., 2017). It has been hypothesised that endoplasmic reticulum (ER) biogenesis and secretory cell differentiation, modulated through the unfolded-protein response (UPR) may be activated in response to long-term nutritional intervention (Nichols et al., 2017). This hypothesis was tested in Chapter 4, where an important finding was that protein supplementation activated *XBP1* (X-box binding protein 1) mRNA, in particular at the low fat level, indicated by the increased proportion of the spliced (active) form relative to the unspliced (inactive) form. The product of spliced *XBP1* translation is a transcription factor that stimulates expression of proteins involved in cellular differentiation to the secretory phenotype, including rough ER formation and secretory vesicle maturation (Huh et al., 2010). This observation suggests that mammary glands might modulate their secretory capacity in response to increased AA supply through mechanisms of adaptive ER homeostasis and secretory cell differentiation. Expression of genes related to this pathway were also measured in Experiment 2. A key finding from preliminary analysis of that dataset is that expression of spliced *XBP1* mRNA increased at the high MP level (P = 0.01; **Table 9.4**), in agreement with Chapter 4 and the findings of Nichols et al. (2017). In contrast with results presented in Chapter 4, the activation of *XBP1* mRNA did not depend on fat level, as indicated by the absence of significant LG × AA interactions (Table 9.4). It can be speculated that the presence or absence of interaction between supplemented protein and fat may be related to the type of fat supplemented (saturated versus monounsaturated), or to differences in DMI during fat supplementation such as those observed between Experiment 1 and 2. Taken together, these results further support the hypothesis that the UPR may play a role in the maintenance of milk production responses to protein supplementation.

Table 9.4. Expression (arbitrary units) of X-box binding protein 1 (XBP1) in mammary cells of lactating dairy cows receiving abomasal infusions of saline (control; C), glucose (GG), and palm olein (LG) at low (LMP) and high (HMP) metabolizable protein levels for 5 d[1]

	Treatment[2]							*P*-value[3]				
Item	LMP-C	LMP-GG	LMP-LG	HMP-C	HMP-GG	HMP-LG	SEM	GG	LG	AA	GG × AA	LG × AA
XBP1												
spliced	1.03	0.80	0.91	1.45	1.47	1.23	0.162	0.50	0.26	<0.01	0.41	0.74
unspliced	1.02	0.80	0.78	1.25	0.96	0.79	0.118	0.08	0.02	0.57	0.81	0.45
*XBP1*s/*XBP1*u	1.03	1.10	1.25	1.22	1.56	1.77	0.168	0.26	0.04	0.01	0.45	0.36

[1]Values are least-squares means (n = 6) from milk fat collected during the morning milking on d 5 of each period. Briefly, milk was collected by hand from individual cows within 10 min post-milking. Samples were immediately centrifuged at 2000 × *g* for 10 min. Approximately 1 g of the supernatant cream layer was transferred into 6 mL TRIzol Reagent (Invitrogen, Life Technologies Inc., Carlsbad, CA), mixed vigorously, snap frozen in liquid N_2, and stored at -80°C. Total RNA was isolated according to TRIzol manufacturer's instructions for handling samples with a high fat content. Total RNA concentrations and purity were determined by optical density measurement using a Nano-Drop ND-1000 (ThermoFisher Scientific). Synthesis of cDNA and real-time quantitative PCR was carried out according to the methods described by Dieho et al. (2017). Primers were designed as described in Chapter 4. NormFinder (Andersen et al., 2004) identified ribosomal protein S9 (*RPS9*) as the most stable housekeeping gene across period and treatment. Fold changes in gene expression relative to LMP-C were calculated by the $2^{-\Delta\Delta Ct}$ method after normalizing to *RPS9*.

[2]Low protein (LMP) or high protein (HMP) abomasal infusion treatments: LMP-C, 0.9% saline; LMP-GG, 1319 g/d glucose; LMP-LG, 676 g/d palm olein; HMP-C, 844 g/d of a complete EAA mixture in the same profile and amount as found in 1.5 kg casein; HMP-GG, 1319 g/d glucose + 844 g/d EAA; HMP-LG, 676 g/d palm olein + 844 g/d EAA. Infusions of LMP-GG, LMP-LG, and HMP-C supplied 20.5 MJ/d, and HMP-GG and HMP-LG supplied 41.0 MJ/d.

[3]GG = effect of energy from glucose; LG = effect of energy from fat; AA = effect of protein level. Statistical analysis was performed as described in Chapter 5.

What Does This Mean For The Dairy Industry?

On-Farm

Dairy farmers and ruminant nutritionists have several objectives when formulating a ration for a particular herd of cattle. Feeding protein in such a way as to minimize feed costs, increase milk production, and keep N excretion at or below legislative limits will be among these objectives, particularly in intensively farmed areas such as the Netherlands. This thesis focused on 2 dietary strategies to reduce N losses by improving transfer of dietary N into milk N (milk N efficiency), those being dietary energy source and absorbed AA profile.

Energy from rumen-bypass fat increases yield of fat- and protein-corrected milk (Chapter 2 and 5), and if adequately saturated it does not affect DMI or diet digestibility (Chapter 2). In dairy diets, if N intake is reduced but sufficient energy is provided, milk protein yield can be increased if AA catabolism is reduced and endogenous AA are mobilized. Based on the results presented in Chapters 2 and 5, lipogenic and glucogenic energy sources in the diet both have potential to achieve improved transfer of AA into milk protein, particularly at low MP levels. Hidropalm (hydrogenated long-chain palm FA) supplementation improved milk N efficiency at the low MP level and high MP level when extra MP came from rumen-bypass soybean meal and rapeseed meal in Chapter 2. Palm olein supplementation increased milk N efficiency at the low MP level, but did not affect it when extra MP came from EAA in a casein profile in Chapter 5. An important element in the aim to improve milk N efficiency is the potential reduction in N excretion in manure that might accompany it. The results of Chapter 5 suggest that at high MP levels, glucogenic energy may have a greater capacity to reduce manure N output, particularly through urinary N excretion, compared with lipogenic energy. On the other hand, fat supplementation does not interfere with mammary gland AA use (Chapters 3 and 6), whereas increased arterial glucose concentrations arising from glucogenic energy does elicit changes in mammary gland AA metabolism, related to effects on whole-body N and energy partitioning (Chapter 6). As precision feeding of ruminants becomes more important, energy supplements that do not interact with direct mammary gland use of postruminal AA supplements could be impactful with respect to minimizing their transformation in the postabsorptive system.

A key concept that should be emphasised in practice is that milk protein synthesis in ruminants does not function according to the single limiting AA concept (**Textbox 9.4**). Results presented in Chapters 7 and 8 exemplify the metabolic flexibility of dairy cattle to deal with different EAA profiles at the whole-body and mammary gland level. The AA profile of protein supplements impacts milk N efficiency, and may be more impactful than energy type, although conclusions on interactions between AA profile and energy type cannot be explicitly drawn from the experiments in this thesis. Supplementing low (12-14%) CP diets with approximately half a kilogram of rumen-bypass EAA in the profile of casein can stimulate milk

protein yield to levels that are the same or higher as when 16% CP diets are fed where no attention is paid to the EAA profile of MP. The advantage to this approach is that there is no loss in milk protein production and milk N efficiency can be increased to levels around 35%, whereas feeding a 16% CP diet may result in similar milk protein production but milk N efficiencies > 30% would not be expected. If milk N efficiencies of 35% would be achieved in practice while milk protein production is maintained, this would offer appreciable benefits in terms of reducing N excretion to environment with no negative impact on milk protein production levels. Currently no RP product or individual rumen-bypass EAA exist that allow supplementation of the EAA profile of casein, which is the main limiting factor in the ability to implement this feeding scheme in practice. In the absence of this technology, efforts should be made to formulate rations with protein sources that contribute favourably to MP supply as a proportion of CP.

Textbox 9.4. The designation of 'limiting' amino acids for milk protein synthesis

In any diet fed to dairy cattle, certain EAA will be absorbed in limited quantities relative to other EAA, or relative to their requirement for synthesis of a certain level of milk protein. In this way, the limit on the number of milk protein molecules that can be produced, on a net basis, is indeed determined according to von Liebig's Law of the Minimum (von Liebig, 1863). However, the problem lies when this theory is translated into the assumption that the addition of a single EAA will stimulate protein synthesis. Evidence against the single limiting AA response in dairy cattle includes stimulation of milk protein yield by mutually exclusive sets of EAA (Schwab et al., 1976), equal losses in milk protein yield when Met, Lys, His, Phe, or Leu are subtracted from the duodenal EAA supply (Weekes et al., 2006; Doelman et al., 2015a,b), and equal stimulation of milk protein yield in the absence of group 2 AA profiles (Chapter 7). Under a range of nutritional and physiological states, mammary glands can regulate sequestration of those EAA that may be in limited quantities relative to requirements. Further, intramammary metabolism allows certain AA to compensate for low circulating levels of others. Splanchnic AA metabolism is also not static – the mammary gland 'pull' of EAA may reduce hepatic catabolism of certain EAA in coordination with mammary set points for protein synthesis (Raggio et al., 2004; Lapierre et al., 2005).

Perhaps the more accurate statement is that any EAA can be limiting when the bounds of the mammary gland's capacity to adapt to varied EAA supplies has been reached. In practice, it is important to recognize that supplementing single rumen-protected AA such as Met and Lys may give positive responses in some cases, but not in all, and the return on that response with respect to milk N efficiency will vary depending on the adaptation

of the gland to derive other EAA from the diet. Single EAA should only be supplemented in rations after careful consideration of the AA composition of the other diet ingredients in order to be efficient from both an N use and financial perspective.

Sustainability

With the rising premium on arable land and the use of human-edible crops for animal feed, there is heightened importance for farmers to meet their on-farm objectives in tandem with global pressure for efficient resource use management. In making recommendations regarding feeding lipogenic versus glucogenic rations, level and form of dietary protein, and management of the supply of these nutrients through forages and concentrates, considering the impacts and trade-offs of such ration ingredients from a full production chain analysis will be important for farmers and nutritionists in the future (Mottet et al., 2017).

On dairy farms, matching resource-use efficiency with milk N efficiency can be achieved by utilizing energy or N sources in a ration that could not be used more efficiently in another production stream, but that also provide a positive return on that energy or N source by increasing milk protein yield. In common dairy rations, glucogenic energy mainly comes from grain crops (in particular corn) that require arable land for production, and on a global scale may be used with higher efficiency in other production chains (Banerjee, 2011). However, feeding glucogenic by-products such as distillers grains may circumvent the need to increase land use to increase milk production (Schingoethe et al., 2009; Mottet et al., 2017). Ingredients such as rumen-inert fats and RP protein or AA sources, such as those employed in this thesis, can be incorporated into these feeding schemes. These ingredients do offer improvements in energy and protein efficiency at the animal level, but this is only impactful at the resource-use level if their production for and use in the dairy industry results in net positive outcomes on relevant sustainability indices. For example, saturated fat supplements arising from palm oil processing could represent an efficient use of co-products in the dairy production sector, if the production of palm oil does not come at a greater cost to environmental sustainability (Hospes et al., 2017) than that of feeding an alternative product to cattle. Several EAA including Lys and Trp are produced large-scale by fermentation processes using overproducing strains of bacteria with ammonia as the main source of N (Leuchtenberger et al., 2005). Indeed, if AA destined for rumen protection would be synthesized in large batches from non-protein N sources and fed to dairy cattle, this would also represent use of a non-human-edible resource stream. Many current research efforts are placed on determining the nutritive value and optimal processing methods for biomass and crop-residues with less potential for other production chains (Nayan et al., 2018), and the potential value of co-products or waste from other processing streams (Schingoethe et al., 2009; Ban et al., 2017; Reynolds et al., 2019).

Challenges Ahead – The Next Steps

To date, advances in dairy cattle production have resulted from technology developed from the understanding of basic dairy cow biology. Despite the scope of current knowledge around dairy cattle nutrition, challenges lie ahead and must be faced with innovative research efforts. Areas that require continued focus are the development of a wider profile of rumen-bypass EAA products, obtaining further data on the requirements of AA in various stages of lactation and under various physiological states, and tools to incorporate these data into feeding schemes for dairy cattle.

Abundant research efforts using postruminally infused AA have contributed greatly to our understanding of protein nutrition in dairy cows. While this knowledge contributes to our fundamental understanding of AA metabolism, the absence of a wider range of rumen-bypass EAA represents a major hurdle in the current ability to translate AA nutrition and metabolism research into practice. Therefore, production of RP protein and AA products is a critically important avenue for future innovation.

Investigation into the fundamental aspects of AA metabolism in dairy cattle should continue, with focus placed on optimizing ideal AA profiles and their use through the postabsorptive system. Examples of functional aspects of AA nutrition that require greater attention include elements of nutrient synchrony with respect to whole-body and mammary gland AA metabolism, and how postabsorptive efficiencies of particular AA may change with physiological state of the cow (e.g. lactation stage, health status, production potential). Furthermore, measurement techniques to determine the efficacy of AA supplementation and to provide indications of milk N efficiency would benefit farmers and nutritionists and aid in monitoring of overall on-farm N efficiency.

Concluding Remarks

The objective of this thesis was to investigate effects of postruminal absorption of different energy substrates (lipogenic, glucogenic, aminogenic) and AA profiles, at the whole-body and mammary gland level with respect to their application for improving milk N efficiency in dairy cattle. The studies presented in these chapter have contributed to the gaps in knowledge surrounding the effects of postruminal energy type, particularly lipogenic energy compared with glucogenic energy, at low and high MP levels, and the effect of AA profile of supplemented MP by investigating lactation performance, energy and N balance, net metabolite flux across the mammary gland, and mammary cell regulation of biosynthetic processes. Based on the work presented in this thesis, the following conclusions can be made and recommendations can be given:

- Postruminal fat increases the transfer of AA into milk protein at low MP levels, and may improve this transfer at high MP levels. Lipogenic energy from saturated FA improved

milk N efficiency when extra MP came from rumen-bypass soybean meal and rapeseed meal supplying approximately 900 g/d of EAA + NEAA. Lipogenic energy from monounsaturated FA did not affect milk N efficiency when extra MP came from 844 g/d of EAA in a casein profile.

- Increased circulating FA largely do not affect mammary gland AA metabolism, in contrast to increased circulating glucose.
- Postruminal fat supplementation stimulates the same level of lactose yield as postruminal protein (when arising from rumen-bypass soybean meal and rapeseed meal) or glucose. Regardless of supplemental energy source, mammary glucose uptake was not the sole regulator of milk lactose output.
- Supplementation of rumen-bypass EAA into low (12-14%) CP diets has potentially great impact for improving the transfer of dietary N into milk N by dairy cattle. With this approach, supplemented EAA can be used with marginal efficiencies up to 35%.
- With respect to the efficiency of incorporation of supplemented MP into milk protein, the following order applies: EAA in casein profile (low level) > EAA in casein profile (high level) > soybean + rapeseed meal.
- With regard to EAA profile of MP, the most similar response in milk production and milk N efficiency to a complete EAA profile can be achieved by supplying group 1 AA plus Ile, Leu, and Val.
- Gene expression at the mRNA level in mammary cells suggests mammary glands modulate their secretory capacity in response to increased EAA supply through mechanisms of adaptive endoplasmic reticulum homeostasis and secretory cell differentiation. The particular signaling pathway may change depending on the presence of extra energy from fat supplementation.
- The absence of a wider profile of rumen-bypass EAA reflecting casein represents a significant hurdle in the current ability to translate AA nutrition and metabolism research into practice. Therefore, production of RP protein and AA products is a critically important avenue for future innovation.
- Dairy cattle display impressive flexibility to produce milk protein, lactose, and fat from lipogenic, glucogenic, and aminogenic dietary ingredients. Striking a balance between high milk production maintained from healthy cows, low N emissions, and overall resource-use efficiency is both a great necessity and a great challenge. With this in mind, farmers and nutritionists should consider the impact of ration ingredients from a full production chain analysis when making decisions on ration formulation.

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SUMMARY

Ruminants play a key role in converting human-inedible feedstuffs into high-quality human edible food. In this way, they can contribute positively to the global feed-food competition. In dairy cattle, the type of energy and protein delivered through dietary ingredients are important factors influencing nutrient transfer from feed into milk components. In particular, the capture of dietary nitrogen (N) into milk N (milk N efficiency) is important to the profitability of dairy farms and impacts the level of N emissions to the environment.

At low and high dietary protein levels, increased supply of glucogenic nutrients can improve the postabsorptive transfer of amino acids (AA) into milk protein. Lipogenic nutrients can increase milk energy output through the direct transfer of dietary fatty acids (FA) into milk, but the impact of lipogenic supplements and their interaction with protein supply on milk N efficiency and N partitioning at the whole-body and mammary gland level represented a significant knowledge gap. Furthermore, knowledge was lacking on whole-body energy and N metabolism of cows receiving similar metabolizable protein (MP) levels that differ in AA profile. Therefore, a key objective of this thesis was to investigate effects of postruminal absorption of lipogenic, glucogenic, and aminogenic energy sources at the whole-body and mammary gland level with respect to their application for improving milk N efficiency in dairy cattle.

In the first experiment, isoenergetic levels of rumen-protected protein (xylose-treated soybean meal and rapeseed meal) and rumen-inert hydrogenated palm FA (C16:0 and C18:0) were tested in a 2 × 2 factorial arrangement using 56 Holstein-Friesian cows in a randomized complete block design (**Chapters 2, 3, and 4**). This study demonstrated independent and additive stimulation of milk yield when protein and fat were supplemented at isoenergetic levels (**Chapter 2**). Energy from protein and fat increased milk yield 1.9 and 1.6 kg/d, respectively. Notably, energy from fat produced the same level of lactose yield as energy from protein. Milk N efficiency was highest when fat was supplemented at the low protein level (32%), and was lowest when protein was supplemented at the low fat level (26%). Importantly, results of Chapter 2 support the addition of rumen inert hydrogenated palm FA as an efficient energy source to improve N utilization by lactating cows at high and low MP levels, although the effect on milk N efficiency was more pronounced at the low MP level.

Results of **Chapter 3** illustrate the metabolic flexibility of the mammary gland in its use of aminogenic versus lipogenic substrates for milk synthesis. Energy from protein increased arterial essential AA (EAA) concentration, but to a greater extent when fat was not supplemented, suggesting that supplemental energy from fat alters EAA absorption across the gut or utilization of EAA by extra-mammary tissues. Mammary plasma flow was not affected by energy from protein or fat. Energy from fat had no effect on mammary net uptake

of any AA group, but increased uptake of triacylglycerol (TAG) and long-chain fatty acids (LCFA). Mammary net uptake of total EAA and group 2 AA was increased in response to energy from protein, regardless of dietary fat level. Greater milk protein synthesis in response to energy from protein was supported by increased intramammary metabolism of group 2 AA, evidenced by an increase in their mammary uptake to milk protein output ratio (U:O). Notably, neither energy from protein nor energy from fat affected mammary glucose balance, and mammary glucose uptake was in excess of estimated requirements for the observed lactose and fat synthesis. Results of Chapter 3 show that postruminal fat supplementation has little effect on mammary gland AA utilization, and suggests that factors other than mammary glucose supply regulate lactose yield when extra energy is supplemented from protein and fat.

Considering the responses in milk production and mammary gland AA utilization observed in Chapters 2 and 3, the effects of energy from protein and fat on expression of genes associated with mammary gland cellular pathways contributing to energy generation and secretory capacity were studied using RNA isolated from milk fat (**Chapter 4**). mRNA expression of enzymes regulating branched-chain AA catabolism and of mitochondrial malic enzyme suggested that energy from protein affected cellular energy-yielding pathways differently in the presence or absence of energy from fat, and may suggest a link between regulation of branched-chain AA catabolism and anaplerotic flux through the tricarboxylic acid cycle. Energy from protein increased de novo FA yield (Chapter 2) but did not affect expression of genes related to FA synthesis. Energy from protein may increase mammary secretory capacity through endoplasmic reticulum biogenesis and secretory cell differentiation, suggested by increased expression of the active form of X-box binding protein 1. Stimulation of protein synthetic activity when energy from protein and fat are supplemented together may have been supported through increased expression of protein phosphatase 1 regulatory subunit 15A. Results of Chapter 4 show that mammary cells use aminogenic and lipogenic precursors differently in support of milk component production when AA and FA supply is altered by dietary intervention. They also suggest that mammary cells respond to increased AA supply through mechanisms of increased secretory capacity and secretory cell differentiation, dependent on the presence of extra energy from fat supplementation.

A second experiment (**Chapters 5 and 6**) was conducted to test the effects of energy from abomasally infused glucogenic (glucose) or lipogenic (palm olein; mainly C16:0 and C18:1) substrates at low and high MP levels (infused EAA mixture in the profile of casein at a constant 844 g/d). Six Holstein-Friesian dairy cows were housed in climate respiration chambers and were used in a 6 × 6 Latin square design where each experimental period consisted of 5 d of continuous abomasal infusion followed by 2 d of no infusion. Postruminal glucose and palm olein did not affect total milk, protein, or lactose yields, and did not affect

milk production differently at the high MP level than at the low MP level (**Chapter 5**). Similarly, alterations in whole-body energy and N partitioning observed in response to glucose or palm olein infusion were largely independent of MP level. Glucose infusion promoted energy retention in body tissue, increased arterial plasma glucose and insulin concentrations, decreased plasma concentrations of β-hydroxybutyrate, non-esterified FA, and LCFA, reduced milk fat yield and milk energy output, and improved milk N efficiency. Palm olein infusion increased milk fat yield and milk energy output, increased arterial plasma TAG and LCFA concentrations, and had no effect on milk N efficiency. Infusing EAA (the high MP level) increased milk protein, fat, and lactose production without negatively affecting energy balance, but decreased milk N efficiency. Important differences found between glucogenic and lipogenic energy were that regardless of MP level, glucose supplementation promoted energy retention and improved milk N efficiency, whereas palm olein supplementation partitioned extra energy intake into milk and had no effect on milk N efficiency.

Increased absorptive supply of glucose and palm olein differently affected mammary gland metabolite utilization, irrespective of MP level (**Chapter 6**). In response to glucose infusion, arterial plasma concentration of group 2 AA decreased and mammary plasma flow increased, both regardless of MP level. The observed reduction of intramammary catabolism of group 2 AA (lower mammary U:O) at the low MP level was attributed to the anabolic effects of insulin on extra-mammary peripheral tissues. Regardless of MP level, palm olein did not affect arterial AA concentrations or mammary AA utilization. Infusion of EAA (the high MP level) increased arterial EAA concentrations to 2.5-times that of the low MP level, and mammary net uptake of EAA increased. Mammary clearance of EAA decreased with EAA infusion, suggesting EAA were in excess of their requirement for milk protein synthesis. This is consistent with lower milk N efficiency and higher N retention observed in Chapter 5 at the high MP level. Intramammary catabolism of group 2 AA increased at the high MP level, and that of non-EAA decreased, suggesting group 2 AA supported the increase in milk protein yield during EAA infusion. Mammary net glucose uptake did not equilibrate with estimated requirements for milk lactose or fat synthesis, except during glucose infusion at the low MP level. This deficit may have been mitigated through contribution of EAA carbon to galactose, and decreased de novo synthesis of 16-carbon FA. Similar to the findings of Chapter 3, results of Chapter 6 suggest that lactose secretion is not solely dependent on mammary glucose supply, and illustrate flexibility of mammary metabolite utilization when absorptive supply of glucogenic, lipogenic, and aminogenic substrates are increased.

When extra N intake comes from MP with a desirable EAA profile for milk protein synthesis, milk N efficiency can be improved. Therefore, a second key objective of this thesis was to investigate the impact of altering EAA profile of MP and determine the effect on milk N efficiency, whole-body N partitioning, and mammary gland metabolism. The third

experiment (**Chapters 7 and 8**) examined the effects of EAA profile within MP supply by removing different groups of EAA from a complete EAA profile, but compensating with the supply of the other EAA such that the total supplemented MP level remained constant. Abomasal infusion was used to deliver the following EAA profiles: 1) a complete EAA mixture, 2) Ile, Leu, and Val (BCAA), 3) His, Ile, Leu, Met, Phe, Trp, Val (GR1+ILV), and 4) Arg, His, Lys, Met, Phe, Thr, Trp (GR1+ALT). Within each infusion, EAA were infused in amounts relative to their content in casein, and the total infused EAA level was constant at 562 g/d. Five Holstein-Friesian dairy cows were housed in climate respiration chambers and treatments were applied according to a 5 × 5 Latin square design. Each experimental period consisted of 5 d of continuous abomasal infusion followed by 2 d of no infusion.

An important result of this study was that compared with a complete EAA profile, the same level of total milk, protein, fat, and lactose yield can be achieved whether Arg, Lys, and Thr, or Ile, Leu, and Val are absent, if the other 7 EAA are present to compensate the MP supply (**Chapter 7**). Supplementing only Ile, Leu, and Val reduced feed intake, was inhibitory to efficient milk protein synthesis, increased the proportion of N intake excreted in urine, and resulted in negative energy balance. Most notably, extra MP from a complete EAA profile resulted in 25% greater milk protein yield with the same milk N efficiency as the saline control, extra MP from the GR1+ILV profile resulted in the same milk N efficiency and N excretion in manure as the complete EAA profile, and extra MP from the GR1+ALT profile reduced milk N efficiency to below control levels and resulted in higher manure N excretion.

When Arg, Lys, and Thr, or Ile, Leu, and Val were absent from the infusions, intramammary catabolism of the present group 2 AA compensated for the lower mammary uptake of the absent group 2 AA, evidenced by greater U:O of the absent group (**Chapter 8**). Notably, mammary uptake of Lys may have been inhibited by high levels of arterial branched-chain AA during BCAA and GR1+ILV infusion, and in both treatments the intramammary catabolism of branched-chain AA could have compensated for the lower intramammary Lys level. When Ile, Leu, and Val were supplemented alone, the U:O of these EAA increased to approximately double the level as on the other infusions, suggesting the mammary gland maintained a high capacity for their uptake at this infusion level, and increased their catabolism to stimulate milk protein synthesis and likely milk fat and lactose synthesis as well. Results in Chapter 8 illustrate flexibility in mammary uptake and intramammary catabolism of AA within the group 2 category to support milk protein synthesis when the supplemented MP level is maintained but the EAA profile is incomplete with respect to casein.

Altogether, the experiments described in this thesis contribute to the gaps in knowledge around effects of postruminal energy type, particularly lipogenic energy compared with glucogenic energy, at low and high MP levels, and around effects of AA profile of MP. Results show that postruminal fat increases the transfer of AA into milk protein at low MP levels, and may improve this transfer at high MP levels. Further, postruminal fat has the

potential to reduce AA catabolism by increasing utilization of non-EAA for milk protein synthesis, particularly at low MP levels. Aside from this, in contrast to increased levels of circulating glucose, increased levels of circulating FA largely do not affect mammary gland AA metabolism. Furthermore, postruminal fat supplementation stimulates the same level of lactose yield as postruminal protein or glucose. With regard to AA profile of MP, similar responses in milk production and milk N efficiency to a complete EAA profile can be achieved by supplying group 1 AA plus Ile, Leu, and Val. Postruminal supplementation of EAA can result in increased milk protein yields at milk N efficiencies (approximately 36%) that are the same or greater than that achieved at a low dietary MP level. In conclusion, dairy cattle display impressive flexibility to produce milk protein, fat, and lactose from lipogenic, glucogenic, and aminogenic dietary ingredients. Capitalizing on these flexibilities is necessary to promote efficient nutrient transfer from feed into milk components.

About the Author

Curriculum Vitae | Scientific Output | Training & Supervision Plan

Acknowledgements

ABOUT THE AUTHOR

Curriculum Vitae

Kelly Elizabeth Nichols was born on July 9, 1991 and grew up in Halifax, Nova Scotia, Canada. She obtained her high school diploma from Sacred Heart School of Halifax in 2009, after which she studied at the University of Guelph (Guelph, Ontario, Canada) and obtained a BSc in Animal Biology in 2013. During her BSc, Kelly gained research experience in the Department of Pathobiology of the Ontario Veterinary College, where she worked on a project concerned with early embryonic loss during equine pregnancies. She also performed an undergraduate research project focusing on dairy cattle nutrition and worked as a research assistant in the Lactation Systems Biology Lab in the Department of Animal Biosciences. She began an MSc with this research group in 2013 which focused on amino acid and energy metabolism in dairy cattle. Her MSc thesis, *Regulation of translation by essential amino acids and glucose in mammary glands and skeletal muscle of lactating dairy cows*, was completed in 2015 and resulted in 2 peer-reviewed scientific journal publications. Kelly began her PhD in 2015 at the Animal Nutrition Group of Wageningen University (Wageningen, the Netherlands) in conjunction with Wageningen Livestock Research under the VDN Feed4Foodure research theme 'reducing nitrogen losses in dairy cattle', resulting in this thesis. In 2017, Kelly was a member of the board of the Wageningen Institute of Animal Science (WIAS) graduate school, and was the chair of its associated PhD Council. Beginning in May 2019, Kelly will continue her research in the field of dairy cattle nutrition and metabolism as a postdoctoral researcher.

Peer-reviewed scientific publications

Nichols, K., J. J. M. Kim, M. Carson, J. A. Metcalf, J. P. Cant, and J. Doelman. 2016. Glucose supplementation stimulates peripheral branched-chain amino acid catabolism in lactating dairy cows during essential amino acid infusions. Journal of Dairy Science. 99:1145-1160.

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Doelman, J., L. L. McKnight, M. Carson, K. Nichols, D. F. Waterman, and J. A. Metcalf. 2019. Postruminal infusion of calcium gluconate increases milk fat production and alters fecal volatile fatty acid profile in lactating dairy cows. Journal of Dairy Science. 102:1274-1280.

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Nichols, K., H. van Laar, A. Bannink, and J. Dijkstra. 2019. Mammary gland utilization of amino acids and energy metabolites differs when dairy cow rations are isoenergetically supplemented with protein and fat. Journal of Dairy Science. 102:1160-1175.

ABOUT THE AUTHOR

Contributions to conferences, symposia, and other scientific output

Nichols, K., M. Carson, J. J. M. Kim, J. A. Metcalf, J. P. Cant, and J. Doelman. 2014. Glucose does not stimulate milk protein yield of dairy cows when essential amino acids are in excess supply. In: Journal of Dairy Science, Vol. 97 (Suppl. 1): 208. 2014 Joint Annual Meeting of ADSA-ASAS, Kansas City, Missouri, USA.

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Nichols, K., A. Bannink, and J. Dijkstra. 2018. Responses to incomplete essential amino acid profiles at the same metabolizable protein supply in lactating dairy cows. In: Proceedings of the 10th International Symposium on the Nutrition of Herbivores. Advances in Animal Biosciences. 9:313.

Training and Supervision Plan

Completed in fulfilment of the requirements for the education certificate of the Wageningen Institute of Animal Sciences (WIAS)

The Basic Package (2 ECTS[1])	Year
WIAS Introduction Course, Wageningen, the Netherlands	2015
Philosophy and Ethics of Science Course, Wageningen, the Netherlands	2015
Disciplinary Competences (13 ECTS)	
Writing PhD Research Proposal	2015
WIAS Advanced Statistics of Experimental Design, Wageningen, the Netherlands	2015
Laboratory Animal Science, obtained DEC article 9 status, Utrecht, the Netherlands	2015
Orientation on Mathematical Modelling in Biology, Wageningen, the Netherlands	2016
Indirect Calorimetry and Selected Applications, Krakow, Poland	2016
Professional Competences (7 ECTS)	
Scientific Networking Course, Wageningen, the Netherlands	2015
Project and Time Management Course, Wageningen, the Netherlands	2016
WIAS Science Day 2016 Organizing Committee	2016
WIAS-Associated PhD Student (WAPS) Council Secretary	2016-2017
WAPS Council Chair	2017-2018
Wageningen PhD Council – WAPS Representative	2017-2018
WIAS Board Member	2017-2018
Presentation Skills (3 ECTS)	
American Dairy Science Association – American Animal Science Association Joint Annual Meeting, Orlando, Florida, USA (oral presentation)	2015
International Symposium on Energy and Protein Metabolism and Nutrition, Krakow, Poland (oral presentation)	2016
International Symposium on the Nutrition of Herbivores, Clermont-Ferrand, France (poster presentation)	2018
Teaching Competences (6 ECTS)	
Supervising MSc theses (5x)	2014-2018
Supervising Internships (2x)	2016-2017
Practical Supervision	
Principles of Animal Nutrition	2016-2017
Laboratory Animal Science	2017

Total 31 ECTS

[1]One ECTS credit equals a study load of 28 hours.

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