Universidade Federal de Minas Gerais

Rumen-protected lysine for dairy cows: stability, bioavailability and lactation performance

Victor Marco Rocha Malacco

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Versão final

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ATA DE DEFESA DE TESE DE VICTOR MARCO ROCHA MALACCO

Às 09:00min horas do dia 13 de dezembro de 2019, reuniu-se, na Escola de Veterinária da UFMG a Comissão Examinadora de Tese, indicada pelo Colegiado em reunião no dia 10/10/2019, para julgar, em exame final, a defesa da tese intitulada: Rumen-protected lysine for dairy cattle: stability, bioavailability and lactation performance, como requisito final para a obtenção do Grau de **Doutor em Zootecnia área de concentração Produção de Ruminantes.**

Abrindo a sessão, o Presidente da Comissão, Prof. Ronaldo Braga Reis, após dar a conhecer aos presentes o teor das Normas Regulamentares da Defesa de Tese, passou a palavra ao (a) candidato (a), para apresentação de seu trabalho. Seguiu-se a argüição pelos examinadores, com a respectiva defesa do candidato (a). Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento da tese, tendo sido atribuídas as seguintes indicações:

	Aprovada	Reprovada
Prof.(a)/Dr.(a) Ronaldo Braga Reis	X	
Prof.(a)/Dr.(a) Hélton Mattana Saturnino	X	
Prof.(a)/Dr.(a) MARINA DE ARRUDA CAMARGO DANES	X	
Prof.(a)/Dr.(a) Maria L. MARIUS NEVES PEREIRA	X	
Prof.(a)/Dr.(a) SANDRA GESTEIRA COELHO	X	

Pelas indicações, o (a) candidato (a) foi considerado (a): [X] Aprovado (a)

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Para concluir o Doutorado, o(a) candidato(a) deverá entregar 10 volumes encadernados da versão final da tese acatando, se houver, as modificações sugeridas pela banca, e a comprovação de submissão de pelo menos um artigo científico em periódico recomendado pelo Colegiado dos Cursos. Para tanto terá o prazo máximo de 60 dias a contar da data defesa.

O resultado final, foi comunicado publicamente ao (a) candidato (a) pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ata, que será assinada por todos os membros participantes da Comissão Examinadora e encaminhada juntamente com um exemplar da tese apresentada para defesa.

Belo Horizonte, 13 de Dezembro de 2019.

Assinatura dos membros da banca:

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“A colheita é comum, mas o capinar é sozinho…”

João Guimarães Rosa, 1956.

“You gain strength, courage, and confidence by every experience in which you really stop to look fear in the face. You are able to say to yourself, 'I lived through this horror. I can take the next thing that comes along.”

Eleanor Roosevelt

DEDICATÓRIA a minha família.

SUMMARY

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LIST OF ABBREVIATIONS

AA – Amino acid
AASS - Aminoadipic semialdehyde synthase
ADF – Acid detergent fiber
ATCC - American Type Culture Collection
AUC - Area under curve
BCS – Body condition score
BW- Body weight
$BW^{0.75}$ – Metabolic body weight
CON – Control
CP - Crude protein
CR – Clearance rate
CV – Coefficient of variation
DIM – Days in milk
DM – Dry matter
DMI – Dry matter intake
EAA – Essential amino acids
ECM – Energy correct milk
FA - Fatty acids
FCM – Fat correct milk
IVGTT – Intra venous glucose tolerance test
LKR - Lysine ketoglutarate reductase
Lys – Lysine
MCP – Microbial crude protein
MDBK - Madin-Darby bovine kidney epithelial
Met – Methionine
MP – Metabolizable protein
MUN - Milk urea nitrogen
N – Nitrogen
NUE – Nitrogen use efficiency
RDP – Rumen degradable protein
RP – Rumen protected
RP Lys - Rumen protected Lys
RUP – Rumen undegradable protein
SCC – Somatic cell count
SD – Standard deviation
SDH - saccharopine dehydrogenase
SI – Small intestine
TAA – Total amino acids
TMR – Total mixed ratio

RESUMO

A principal hipótese explorada nesta tese é que o valor da lisina protegida da degradação ruminal (**RP-Lis**) é função de sua estabilidade quando pré-misturada em alimentos, inércia no rúmen, biodisponibilidade pós-ruminal e dos potenciais impactos nos parâmetros de produção quando fornecida na dieta de vacas em lactação. A segunda hipótese testada foi que a lisina e o suprimento de energia atuam na regulação de genes importantes para o catabolismo da lisina em bovinos. Para testar essas hipóteses, foram realizados quatro estudos. Os estudos avaliaram a estabilidade, a biodisponibilidade do produto e o desempenho de vacas leiteiras em lactação alimentadas com dieta a base de milho, deficiente em Lis e suplementadas com RP-Lis e determinaram os efeitos dos níveis extracelulares de Lis e do suprimento de energia na expressão do mRNA da enzima aminoadípico semialdeído sintase (**AASS**) como um indicador específico para o catabolismo de Lis. No geral, a associação das práticas de armazenamento antes da suplementação com RP-Lis e a baixa biodisponibilidade observada no estudo de biodisponibilidade podem resultar em menor quantidade de Lis digestível no intestino delgado do que o previsto, o que levaria a ausência de resposta positiva a suplementação nas vacas em lactação. Além disso, vários aminoácidos podem ser limitantes quando a maior parte da proteína da dieta é derivada do milho e seus subprodutos, o que também poderia atenuar o aumento esperado da Lis plasmática e a resposta em produção e componentes do leite. O aumento da Lis no meio de cultura e também o fornecimento de uma fonte de energia adicional não resultou em alterações na expressão da AASS e confirma dados anteriores de estudos *in vivo* com aumento da oferta de lisina pós-ruminal. Juntas, as informações apresentadas nesta tese destacam a importância de se manter a integridade do sistema de proteção da lisina nos produtos em todas as etapas, armazenamento, alimentação e no rumen, o que resultará na correta quantidade de lisina livre pós-ruminal.

Palavras-chave: aminoácidos, proteína, vacas em lactação.

ABSTRACT

The guiding hypothesis explored in this dissertation is that the value of rumen-protected lysine (**RP-Lys**) is a function of its stability when premixed in feeds, inertness in the rumen, postruminal bioavailability, and impact on production parameters when fed to lactating dairy cows. A second hypothesis tested was that lysine and energy supply act to regulate genes that are critical for lysine catabolism in bovine. To test these hypotheses, four related studies were performed. The studies evaluated the rumen-protected Lys product stability, bioavailability and lactating dairy cow's performance due to its use as a supplement in Lys deficient corn-based diets and determined the effects of extracellular Lys levels and energy supply on aminoadipate-semialdehyde synthase (**AASS**) mRNA expression as an indicator of a gene specific to Lys catabolism. Overall, summarizing all those studies related to the RP-Lys we conclude that association of the storage practices before feeding RP-Lys and the low bioavailability observed in the bioavailability trial might result in a lower delivery than predicted for digestible Lys in the small intestine resulting in a lack of response in the lactation trial. In addition, multiple AA may have been limiting when most of the dietary protein was derived from corn products which could also dampen the expected increase in plasma Lys and increase of milk yield and milk components. The increase in the Lys in the media and providing an additional energy source did not translate in alterations in the AASS expression and confirms data from *in vivo* studies with increased postruminal lysine supply. Taken together, the information presented in this thesis highlights the importance of maintaining the integrity of the protection technology for RP-Lys through all sequential segments leading to postruminal presence of free lysine.

Keywords: amino acids, protein, dairy cows.

CHAPTER I– INTRODUCTION

Ration formulation that targets the maximal N efficiency diets for dairy cattle is a desirable objective for achieving optimal nitrogen utilization and improved dairy system profitability given that protein typically represents about 40% of total cost of the diet for lactating dairy cows (St-Pierre and Sylvester, 2005). Furthermore, reductions in nitrogen (**N**) intake and N excretion have environmental benefits as livestock systems impact air and water quality and excess N can contribute negatively to eutrophication and soil acidity (Castillo et al., 2000).

Conversely, several nutrition models indicate that low crude protein (**CP**) diets, especially corn-based diets, commonly result inadequate postruminal flow of lysine (**Lys**), and contribution to metabolizable protein, to the small intestine. Because lactating dairy cows have a high need for protein as a source of amino acids, and supplementation with the optimal amino acid profile in the diet may reduce nitrogen excretion while also not affecting production, it is critical to develop feeding strategies that accomplish both reduced CP intake and optimal postruminal AA supply. Based on these goals the recommended level for Lys and Met in metabolizable protein range from 2.2% to 2.4% for Met and 6.6 to 7.2% for Lys resulting in Lys to Met ratio in the metabolizable protein (MP) of approximately 3 (NRC, 2001a; Arriola Apelo et al., 2014a). Thus, diets balanced to the established AA requirements offer the opportunity to provide similar or greater amounts of limiting AA in the small intestine.

Lysine is an essential amino acid for dairy cattle. Because the rumen microbes are able to effectively hydrolyze, deaminate and decarboxylate amino acids from the diet the success of postruminal Lys supplementation depends on a protective coating to avoid Lys utilization by rumen microorganisms but permits absorption by the cow (Berthiaume et al., 2000). Increasing the supply of Lys to dairy cows, using rumen protection technologies, is a strategy to increase Lys availability for absorption and potential for use by the lactating mammary gland for protein synthesis. Although the potential value of feeding rumen protected Lys has been recognized for some time (Donkin et al., 1989) there are still only a few studies reported using protected Lys (**RP-Lys**) sources (Robinson, 2010). One of the hallmarks of the studies that have been published for RP-Lys is a small nonsignificant response in milk production and milk protein composition and yield (Mullins et al., 2013; Giallongo et al., 2016; Weiss, 2019). The lack of strong and positive results due to Lys supplementation may have been due to Lys is first limiting

but not sufficiently supplied. This may also be related to the requirement calculation, feeding method and strategy, or Lys supplementation alone resulting in an imbalance/deficiency in another essential AA. Therefore, a better understanding of the observed failures in RP-Lys supplementation should be further investigated.

In addition to the resilience against the rumen environment, RP-Lys technologies must also resist breakdown during the mechanical mixing process with common feedstuffs, the impact of exposure to moist feeds in the diet, and the effects of acidic and basic feeds. A lack of protection from these factors may decrease the Lys delivered from a RP-Lys source. In many cases the characterization of the stability of RP-Lys in feeds and the mechanical mixing process are overlooked while rumen stability is documented. It is important to recognize this potential effect of feed mixing in assessing rumen protection strategies and postruminal delivery of AA to dairy cattle.

The duodenum, commonly referred to as the small intestine, is the primary site for AA absorption in dairy cows, therefore, ensuring that dietary AA are able to reach the small intestine for digestion and absorption is crucial to improving AA nutrition and potentiating a production response. Determining the bioavailability of individual essential amino acids (**EAA**) in the small intestine is difficult. The dairy NRC (2001) uses an empirical equation to predict EAA flows, but such an approach likely misses some of the subtler changes in supply and cannot be used outside of the range of the data used to derive the equations. Plasma amino acid concentrations reflect AA supply and rumen-protected aminoacid supplementation and have been used as an index of dietary AA supply but are subject to fluctuations in intake, supply and physiological status (Weekes et al., 2006). Changes in plasma AA with acute changes in postruminal AA are possible (Weekes et al., 2006) and provide a relative assessment of level of rumen-protection combined with digestibility and absorption across the small intestine. All these steps are crucial to increase the postruminal supply of AA and as a strategy to increase N use efficiency and dairy cattle performance.

The work described in this thesis is an evaluation of a rumen-protected Lys product including test of stability in feeds and in the rumen, relative estimates of post ruminal bioavailability, and impact on production parameters when fed to lactating dairy cows receiving a Lys deficient corn-based diet. In supporting a greater understanding of postabsortive lysine metabolism, the effects of lysine and energy supply were explored in an in vitro system of bovine cellular metabolism. Taken together the information uncovers important criteria necessary for the full evaluation of RP Lys for lactating dairy cows.

CHAPTER II - LITERATURE REVIEW

2.1. Current protein recommendation for dairy cattle

Dietary crude protein **(CP)** for ruminants and nonruminants is expressed as the nitrogen content of feeds multiplied by a factor of 6.25. However, in balancing diets for ruminants the partitioning of CP to rumen degradable (**RDP**) and rumen undegradable (**RUP**) creates a level of complexity necessary in defining and meeting the CP needs of the animal (NRC 2001). Regardless, many practicing nutritionists in the field are still considering CP in balancing diets (Schwab and Broderick, 2017) despite recognition since the 1990s that requirements for dairy cattle are met through a combination of RUP, RDP, and the dynamics between RDP and ruminal energy available for microbial protein synthesis (NRC, 2001). Optimal feeding strategies combine balances for the amount of N and amino acids that maximize the microbial protein synthesis linked to the right amount of rumen undegradable protein and AA profile that meets the requirements for metabolizable protein by the cow (NRC, 2001a; Schwab and Broderick, 2017).

Meeting the amino acid requirements for dairy cows hinges on optimizing the rumen environment to maximize the synthesis of microbial protein and flow of amino acids from microbial protein to the small intestine (**SI**) and then augmenting with rumen undegradable protein and amino acids to match the post-absorptive requirements of the cow for maintenance, growth, pregnancy, if applicable and lactation. The NRC (2001) model divides dietary protein into RDP, and RUP. Both microbial CP and RUP flowing out the rumen and are digested in the abomasum and small intestine, the AA from which are then absorbed in the SI for host utilization. Thus, conceptually the total supply of MP fulfilling the AA needs of the cow is met from ruminally synthesized microbial CP, RUP and, to a much lesser extent, endogenous CP reaching the SI (Clark et al., 1992; NRC, 2001a). In practice, CP that could be easily measured in the feed, however the contribution of CP to metabolizable protein is dependent of several factors such as total N to fermentable carbohydrate, protein solubility, rumen digestibility and passage rate, and consequently because of this complexity computer models are necessary to estimate rumen parameters and relationships (Zanton, 2017) and reduction of parameter estimates to measurable chemical and physical attributes in feeds is still a challenge.

Protein requirements of dairy cows have been assessed in dose-response studies (NRC, 2001a). Similar diets are fed to the animals containing increasing amounts of CP and milk

production is tracked as the response variable. The point at which in milk volume or milk protein production reaches a plateau with increasing supply of crude protein is identified as adequate, or requirement. The NRC (2001), compared protein milk yield and milk protein yield response to changes in the dietary CP concentration using a data set of 393 means from 82 protein studies. They observed that milk yield increased quadratically as diet CP concentrations increased and the maximum milk production was obtained at 23% of CP. The milk production expected increases is 0.75 kg/d to 1 % of CP increases between 15 to 16 percent of diet CP and increasing dietary CP 1% unit from 19 to 20% would be expected to increase milk yield by 0.35 kg/d.

Increasing dietary crude protein in diets for lactating dairy cattle to support high levels of milk production could increase the N excretion and reduce the nitrogen use efficiency (**NUE**) (Olmos Colmenero and Broderick, 2006). These authors reported an increase in milk yield, with increasing dietary CP concentration up to 165 g/kg of DM, after which point, they observed a decline in milk yield.

In excess, the crude protein is extensively degraded in the rumen exceeding the capacity of rumen microorganism assimilate the amino acid and ammonia, which leads to an increase in ammonia concentration in the rumen. Ammonia can be removed from the ruminal environment, mainly via diffusion, and can subsequently be returned to the rumen or lost as urea through urine, feces and milk (Russell et al., 1991). Overfeeding crude protein often results in a decrease in profit margin due to the high costs of protein supplements and low efficiency of ruminants in using nitrogen (Broderick, 2003; Hristov and Giallongo, 2014).

Cows are inefficient in using N (Hanigan et al., 2018; Vandaele et al., 2019) but it is probably not an inherent limitation of the specie and might be related to the overestimation of the requirements. Ruminants have a nitrogen use efficiency (**NUE**) that is typically 20–35% of intake N. In a metanalytical study Spek et al. (2013) presented NUE for modern lactating dairy cow that averaged 26.1% in North America and 27.4 in Northern Europe. These low efficiency use rates are associated with high CP supplementation and results in increasing N excretion in urine and feces which may contribute to environmental pollution (Hristov et al., 2011; Powell and Rotz, 2015).

The concentration of N in urine, especially in the form of urea, varies more than N in feces. After excretion, fecal N is relatively stable, but urinary urea N can transform rapidly to ammonium (NH_4+) and can subsequently be lost as NH_3. Ammonium can also nitrify and denitrify, forming NO_3^- and N_2O (the most potent greenhouse gas emitted from agricultural

systems) (Edouard et al., 2016). As reducing the environmental footprint of milk production concern (FAO, 2006), especially with the reduction of N pollution, there is a need to increase the efficiency of nutrient utilization so as to minimize nutrient excretion.

The maximum efficiency is not necessarily aligned with the best performance related to cost, animal longevity, efficiency of nutrient use, or with environmental conservation practices. Therefore some degree of deficiency and reduced animal performance may be acceptable in justifying the marginally deficient nutrient supply (Owens et al., 2014).

Chibisa and Mutsvangwa (2013) showed that milk yield decreased by 3 kg per cow per day and milk protein yield decreased by 140 g per cow per day when dairy cows were fed diets containing 15.2% CP compared to 17.3% CP. Lee et al. (2012) observed a reduction in milk yield in dairy cows fed a diet deficient in metabolizable protein compared to a diet adequate in metabolizable protein. Colmenero and Broderick (2006) studied the crude protein content of diets fed to dairy cattle ranging from 13.5 to 19.4 % CP and observed a trend towards decreased DMI of 0.7 kg/d (P = 0.22) and milk production 2 kg (P = 0.1 in a quadratic fashion) with reduced CP feeding.

Owens et al. (2014) in order to examine the relationship between performance and dietary protein concentration, used a dataset from 13 published trials. Data were analyzed by regressing the performance data in relation to dietary CP concentration and tested for significance of linear, quadratic and cubic dietary CP responses including diet as a fixed factor. The authors observed with the equations that decreasing dietary protein from 18% to 17, 16, and 15% of diet DM would be expected to decrease expected daily fat corrected milk (**FCM**) yield by an average of 1.4, 3.0, and 5.0 kg/d below FCM yield with 18% CP. Although the changes identified by the authors were not statistically different in single studies the magnitude of the effect, if consistent would be economically important.

A primary goal for the modern dairy nutritionist is formulating diets that meet the metabolizable protein needs of dairy cows by feeding to match the least amount of protein content possible without causing detrimental effects in milk production and on this way reduce the N excretion. Some of the uncertainty in response to reducing CP in diets may be the result of inaccuracies in predicting rumen metabolism and microbial protein synthesis, in predicting RUP, estimating flow and postruminal digestion or a combination of these factors. Furthermore, because AA supply with reduced CP is close to borderline sufficiency the effects of these inaccuracies may be amplified. Although many diets, when fed at low CP levels, may be deficient in Lys and methionine (NRC, 2001a) because of that overfeeding crude protein (>

17% of the diet) has overcome potential amino acid deficiencies for high producing dairy cows. However, it may be possible to achieve high milk yield and milk protein yield in dairy cows with lower CP diets by supplementation using ruminal protected AA especially for those AA that are limiting for milk production and optimal health. Thus, supplementing rumen protected Lys and Met is a strategy to increase or maintain milk yield when dietary CP content are lower (Armentano et al., 1997; Dinn et al., 1998).

2.2. Current amino acid recommendation for dairy cattle

Amino acids are designated as dietary essential and non-essential depending on the body's ability to synthesize it in adequate quantities to meet tissue needs or not. Nine AA cannot be formed in cellular metabolism and must be absorbed from the gastrointestinal tract (His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val). Three amino acids (Arg, Cys, Tyr) are denoted as "semi-essential" because they can be synthesized but supplementation improve performance, Arg is synthesized in the urea cycle, or because they can spare other AA requirements since they are used partly to synthesize, Cys and Tyr could spare Met and Phe requirements. The remaining 8 AA are referred to as "non-essential" (Broderick, 2016)

High milk production requires high output of amino acids, which means that the CP intake needs to be increased and/or a supplemented, to improve the AA ratio of the protein that reach the small intestine.

A survey conducted by the Dairy Herd Management show that 81 % of the nutritionists and veterinarians formulate diets to balance for AA content (Dairy Herd Network, 2010), aiming to reduce CP level of the diet. The value of feeding diets with reduced CP levels is recognized as a means to reduce feed costs but also as a means to reduce N output in the manure (Arriola Apelo et al., 2014a). Thus, rumen protected amino acid supplementation could be used as a strategy to avoid the lost in milk and solids production due to the use of the low protein diets (Broderick et al., 2008).

Initial studies explored the sequence of AA limitations in dairy cows fed with corn-based diets and Lys and methionine were identified as the two main limiting amino acids (Schwab et al., 1976; NRC, 2001a). However, histidine may be the first limiting amino acid when high producing dairy cows are fed with high forage diets due to the low content of rumen undegradable in the diet, the reliance in the microbial protein to supply metabolizable protein and the low level of His in microbial protein (Vanhatalo et al., 1999; Lee et al., 2012). Despite recognizing the potential limitation in His with high forage diets current nutritional standards for dairy cows in the US (NRC, 2001) provide recommendations for methionine 2,5 % of MP

and Lys 7.2 % of MP only. Histidine has been shown to limit milk production in lactating cows in some studies, but histidine requirements in the diets have not been defined.

Metabolizable protein, the sum of the microbial protein, protein which escapes degradation in the rumen and endogenous protein secreted into the gastrointestinal tract prior to the duodenum, represents the total protein which is available to the animal for maintenance, growth, fetal growth during gestation, and milk production (NRC, 2001a). Together, RUP and microbial protein have been estimated to represents 50 to 80% of the total protein flow to the small intestine (Bach et al., 2005). The range in this estimate creates an inaccuracy in predicting protein flow from the rumen. However, Pacheco et al. (2012) comparing four rumen sub models predictions with observed values of duodenal flows of EAA concluded that recent commercial models may more accurately predict the flow of EAA to the duodenum. It would appear that additional work is needed to test, refine and implement models that permit greater precision in AA nutrition for dairy cows.

There are two different approaches that have been used to determine the AA requirements of lactating cows. The proportional approach adopted by the (NRC, 2001a) and (INRA, 2007) which includes a broken stick model, a dose-response relationship between the % of individual AA in MP supply and milk protein yield or percentage. The % observed at the breakpoint represents the proportion of this AA in MP supply required to maximize the targeted output (Lapierre et al., 2014). The second is the factorial approach which takes into account the AA needs for each individual function with a defined AA composition and a defined efficiency of transfer. This approach is used by CNCPS (Fox et al., 2004), AMTS and Amino cow, two commercially available models designed to predict MP and metabolizable (or digested) AA supply with needs using feed input data (Pacheco et al., 2012).

Without quantitative estimates and knowledge of the supply of the amino acids reaching the small intestine coupled with knowledge of the AA requirements of lactating cows the response to AA supplementation might be random and inconsistent (Robinson, 2010; Pacheco et al., 2012). Deficient post-ruminal AA supply, related to the limitation of one or more AA available to tissue, will result in limitations for protein synthesis. According with the limiting AA theory, the most limiting AA dictates the efficiency of utilization of absorbed AA and it could explain the observed performance limitations. When the first limiting AA supply is corrected the performance will be improved until other AA becomes limiting in the synthesis process (Schwab et al., 1992).

Attempts in identify the first single limiting AA for milk protein synthesis or sequentially limiting AA have failed (Cant et al., 2018) leading to the belief that there may be

limiting set of EAA that are needed simultaneousness to promote protein synthesis. The fact that milk production could be affected by the same magnitude when different amino acids are removed from duodenal EAA supply or the fact that insulin or glucose could change the milk protein synthesis even when plasma EAA concentrations are reduced tends to support the theory that AA are required as a group (Mackle et al., 2000; Weekes et al., 2006; Toerien et al., 2009; Doelman et al., 2015; Cant et al., 2018).

The optimal amounts of Lys and Met requirements indicated by the NRC (2001) model are 7.2 and 2.4 % of metabolizable protein respectively. Schwab, (2012) presented a compilation of breakpoint estimates for required concentrations of Lys and Met in MP for maximal content and yield of milk protein for the NRC (2001), CPM, and AMTS models using the NRC (2001) database re-evaluated by Schwab et al., (2009) and Whitehouse et al. (2009). The results observed by the authors are different for the updated NRC (2001) version when compared with the first version of the NRC (2001) mostly because of the difference in feed inputs for some of the studies. They also observed differences in the estimative for different models which was expected since the models differ in the approach for predicting supplies of AA.

Knowledge about Lys and methionine requirements in lactating cattle has made significant progress (Schwab et al., 1992; Rulquin et al., 1993) and, by providing recommendations and adequately supplementing the animals, an improvement in performance is observed (Leonardi et al., 2003; Noftsger and St-Pierre, 2003). The same effort must be made in an attempt to establish requirements for all essential amino acids and thus be able to replace the crude protein as the main reference in the diets formulation (LaPierre et al., 2019). The use of low crude protein diets supplemented with amino acids protected from ruminal degradation seems to be the best economically productive and environmental approach in milk production systems.

Estimations of the optimal supply of individual AA, as defined in Doepel et al (2004), using a meta-analysis approach are presented in Table 1. These authors used studies where AA or proteins were infused postruminally in order to determine the influence of AA supply on the variation of the efficiency of conversion of AA into milk and to estimate requirements for EAA. The results indicate a similar pattern of amino acid MP regardless of the method used to evaluate the data available in the literature.

Table 0-1 Ideal concentrations of Lys and Met in MP for milk protein yield in lactating dairy cows (% metabolizable protein)

Amino acid	Segmented linear model	Logistic model
Arginine	4.8	4.6
Histidine	2.4	2.4
Isoleucine	5.3	5.3
Leucine	9.4	8.9
Lys	7.2	7.2
Methionine	2.5	2.5
Phenylalanine	5.2	5.5
Threonine	5.1	5
Tryptophan	-	-
Valine	6.1	6.5

2.3. Rumen protected amino acid technologies

The great advantage of ruminants, the symbiotic association of rumen bacteria with the animal in the rumen, can be both beneficial with regard to the protein nutrition because of the ability to convert non-protein N into a high-quality protein through microbial protein synthesis but also detrimental due to the degradation of supplemented free amino acids by the rumen microorganisms (Wu and Papas, 1997). Consequently, supplementation in a non-protect form usually results in an AA utilization by the ruminal microorganism and no changes in the metabolizable protein. The initial attempts to supplement unprotected Lys for lactating cows suggested that this could be a viable alternative. Early research (described in Wu and Papas, 1997) evaluated ruminal Lys escape using pulse dose infusion, reported ruminal escape of free Lys that varied from 10 to 29% of the infused dose and suggest that free Lys might represent an option for enhancing Lys supply. However this approach was later discarded due to the confirmation of the low rumen escape rate especially when the source of Lys was mixed with TMR (Robinson et al. 2004) where ruminal escape rate is only 7.2 % of the free lysine. Consequently, the amount of Lys escaping the rumen relative to the quantity provided in the diet necessitated other approaches to supplementation.

Thus, AA supplementation success depends of the adequate coating protection to avoid the Lys utilization by rumen microorganisms (Berthiaume et al., 2000). The protecting derivate must be then stable in the rumen but release the amino acid in the abomasum/small intestine. The main industrial methods used to protect AA are: 1) the production of AA analogs, which are more stable under ruminal condition; 2) coating with fat, mixtures of fats and proteins, proteins treated with formaldehyde or calcium soaps of long chain fatty acids; 3) encapsulation

with polymer compounds resistant to rumen degradation, but which are hydrolyzed in the abomasum (Alves, 2004).

Wu and Papas (1997) defined desirable characteristics of rumen-protected amino acid pellets (Fig 1). This authors also listed the criteria that should be meet for a polymer used to formulate the rumen-stable delivery system: 1. Physiologically inert, non-absorbable, unchanged in excretions in animals such as rats, dogs, cows and steers, 2. Non-mutagenic; 3. Produces no adverse effect following chronic feeding; 4. Thermally stable or non-degradable at temperatures experienced during process and storage; 5. Insoluble in ruminal fluid, but soluble in abomasal fluid; 6. Soluble in common volatile organic solvents for coating applications. The water solubility of Lys is so high that the development of RP-Lys has been a great challenge which to date has favored ruminal protection technologies for Lys that utilize hydrogenated fat and/or minerals. These are advantageous because they are already registered as feed ingredients, regarded as safe and do not require special testing for approval as feed supplements (Watanabe et al., 2006).

A variety of rumen protected products are available in the market with different coated protection forms and amino acid cores. Other factors such as the mechanical mixing process, diet moisture, acidity and the time that RP-Lys product is exposed to feed could decrease the AA retained in the rumen protected products. Several studies have evaluated the stability of the ruminal protected amino acids in the feeding and mixing process, and the bioavailability through the performance response of the cows and also using the plasma amino acid concentrations changes due to supplementation

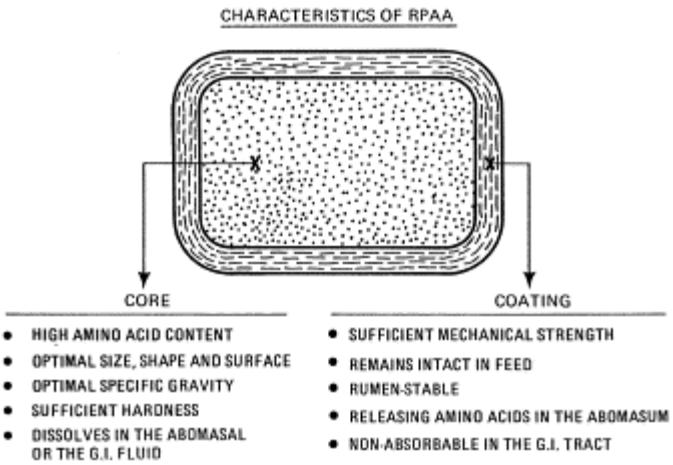

Figure 0-1 Desirable characteristics of rumen-protected amino acid pellets (adapted Wu and Papas, 1997).

As indicated above most of the Lys rumen protected amino acids available in the market are lipid-coated. Studies that evaluated the stability of the Lys coated products exposed to the mixing process concluded that methods of manufacture together with the physical and chemical composition of the diets can affect the amounts of Lys provided from the lipid coated products to the ruminants (Ji et al., 2016; Reiners et al., 2017, 2018). Those studies also found a wide variation in the stability of the products related to the capacity of retain the Lys which clearly impacts the final bioavailability of the product.

Increases in the plasma AA concentrations in response to postruminal delivery of a RP AA have been used to determine the ability of RP-Lys products to be absorbed across the duodenum. Whitehouse et al. (2017) using this principle and the dose-response technique determined the relative bioavailability of 27 products tested in 13 different experiments using the free AA dose technique. There was a wide range (5 to 85%) in the bioavailability between products delivered postruminally at different levels. This high variability observed may reflect the difficulties in creating a coating process that effectively protects Lys from ruminal degradation while allowing consistent release of the encapsulated Lys in the intestine. This inability to consistently provide products that deliver predictable payloads of Lys may help to explain the challenges faced by the industry in effectively developing an RP Lys source and documenting the effects of postruminal Lys supply (Robinson, 2010).

2.4. Postruminal amino acids supplementation

Lysine and Met have been the primary focus of the research on postruminal AA supplementation because those two amino acids are identified as the first limiting in a typical corn-based diets in US. Postruminal infusion of 6 to 12 g/d of Met (Pisulewski et al. 1996) increased milk protein yield and content in a linear fashion. A meta-analysis of 129 RP-Met studies determined that supplementation increased milk protein content and yield and slightly increased milk production (Patton, 2010). A more recent metanalysis (Zanton, 2017) indicated that infusing or supplementing with sources of Met which increased Met in MP resulted in increases in both milk protein and fat yield.

In contrast, postruminal methionine supplementation, when combined with Lys, frequently increases milk production (Lean et al., 2017). Post ruminal infusion of a complete mixture of amino acids increases in milk protein yield, although, when Lys was eliminated from

the EAA mixture milk production and milk protein production were similar to saline infusion (Weekes et al., 2006; Lapierre et al., 2009). Guo et al. (2017) investigated responses of milk protein synthesis and mammary AA metabolism to a graded decrease of post ruminal Lys supply used 4 lactating goats in a 4 × 4 Latin square experiment. Treatments included a graded decrease of Lys content in the infusate to 100 (complete), 60, 30, or 0% of Lys in casein. When Lys was removed from the infusate there was a linear decrease in milk yield and a tendency for an increase in milk fat to protein ratio. Milk protein content and yield were linearly decreased by graded Lys deficiency. For the authors these data suggest that a decrease in milk protein yield linked to graded Lys deficiency was mainly a result of the varied physiological status, indicated by the elevated circulating glucagon and glucose, rather than a result of the decreased mammary Lys uptake or depressed signals in the mTOR pathway.

In other studies, when Lys was infused postruminally there are inconsistent effects on milk production and composition (Rulquin et al., 1993; Tucker et al., 2017). Increasing post ruminal Lys supply failed to alter mRNA transcripts of key genes in Lys and protein catabolism in liver and mammary tissue despite the fact that these genes are regulated in other species by lysine supply and there was an increase in alpha amino adipic acid, a breakdown product of Lys, with increasing lysine supply (Tucker et al. 2017). The data suggest that hepatic Lys metabolism in bovine is not responsive to Lys supply at the transcription level, and that the availability of Lys to extrahepatic tissue may be determined by hepatic Lys metabolism.

The determination of Lys requirements together with other essential amino acids are the first step to optimize the nutrition and efficiency of N use by dairy cows. However, the profile of amino acids that reach the mammary gland, and are available for milk protein synthesis, is different from that one which is available to be absorbed in the small intestine. If the ultimately goal is to maximize milk protein synthesis and N use efficiency then the next step to be taken is understand the potential modifications of the AA profile supplied, post absorptive metabolism, and also the portioning of the available AA between mammary gland and other tissue (Bequette and Nelson, 2006).

Overall only a small number of studies have evaluated RP Lys (RP-Lys) supplementation alone without another RP AA. For most there is a small or nonsignificant response in milk production and milk protein composition and yield (Mullins et al., 2013; Giallongo et al., 2016; Weiss, 2019). Robinson (2010), systematically evaluated the literature to determine if duodenal Lys level and RP Lys impacts the performance of dairy cows. Results demonstrate that Lys supplementation decreased DM intake as much as 1.9% when compared with control groups and increased the milk/DM ratio. In the 13 studies that were used for the

analysis there were no other differences in milk yield, milk protein production or N efficiency due to Lys feeding (Robinson, 2010). Awawdeh (2016) reported an increase in milk production and protein content when cows were supplemented with RP-Lys and RP-Met compared with cows supplemented only with RP-Met or not supplemented suggesting a co-limitation in Lys and Met.

Several hypotheses could explain the lack of response to RP-Lys feeding especially in diets that were formulated to be lysine deficient. Among them, is the potential that a single level of Lys feeding may create an AA imbalance at the intestine to impair Lys absorption (Robinson et al., 2011). Other explanations include insufficient supply of a colimiting essential amino acid (Robinson, 2010; Mullins et al., 2013; Lee et al., 2019), or increased hepatic catabolism and loss of absorbed Lys to other tissues including the mammary gland (Doepel et al., 2004; Swanepoel et al., 2010; Nichols et al., 2019). Evaluating these hypotheses will require a more detailed understanding of metabolism of AA and the relationship to other nutrients including energy metabolism.

Although Lys and Met have been identified as potentially limiting in corn based diets, histidine is been identified as also colimiting amino acid in the corn based diets fed to dairy cattle in the US and as first limiting for cows feed with diets based on grass silage and a cereal-based supplement (Korhonen et al., 2000). Hadrová et al. (2012) evaluated the effect of duodenal infusion of histidine on milk yield, milk composition, and plasma amino acids in lactating dairy cows, failed to observe any difference for milk production or composition despite of the increase in plasma His concentration.

Most of the beneficial effects of His supplementation have been observed when low protein diets were fed to dairy cows (Kim et al., 1999; Lee et al., 2012) and have led to the working hypothesis (Lee et al. 2012) that low CP diets increase the dependency on the microbial crude protein (**MCP**) to supply to meet MP supply and that MCP may be limiting in His. Despite recognition as one of the top three limiting amino acids for high yielding dairy cows, the benefits of postruminal supplementation of Met Lys and His combined has been small. A more thorough understanding of feeding strategies, evaluation of amino acid sources, and determination of cow requirements are necessary to improve response supplementation and to achieve the goal of improving performance and efficiency of dairy cows N use.

2.5. Postabsorptive Lys metabolism

Once absorbed by the intestine AA are first transported to liver through portal blood. Is well recognized that approximately 1/3 of the EAA amino acid pool that disappears from the

lumen of the small intestine will be oxidized by the portal drained viscera, primarily enterocytes and another third by the hepatic tissue (Hanigan et al., 2004; Pacheco et al., 2006; Larsen et al., 2015). Removal of amino acids by the splanchnic tissues (intestine and liver) is not constant for all AA, and the composition of the post-splanchnic AA availability to other tissues is altered relative to absorbed AA profile (Hanigan and Cyriac, 2006). It is recognized that uptake and oxidation of amino acids is a mass action reaction and increases in of AA availability will result in higher oxidation by peripheral tissues and consequently in lower efficiency use (Hanigan et al., 1998). The AA removed from the circulation by intestine and liver can be used for several anabolic processes; for example, synthesis of export proteins (e.g., albumin, peptide hormones, and immune proteins) and constitutive proteins, as well as for catabolic processes such as gluconeogenesis and complete oxidation (Larsen et al., 2015).

Hepatic extraction of Lys is less than 10% of portal drained viscera Lys flux, therefore intestinal oxidation is mainly responsible for reducing the net flux of absorbed Lys to the mammary gland. The gut oxidation of Lys in sheep accounted for 29% of whole body Lys oxidation (Lapierre and Lobley, 2001) and this estimate did not account for oxidation of AA derived from the gut lumen (i.e. during absorption). Van Goudoever et al. (2000) observed, in pig, a negligible oxidation of systemic (arterial) Lys, although the luminal-derived Lys catabolism represents 30% of whole-body Lys oxidation. These authors also showed that the source of Lys used by these tissues is sensitive to protein status, although Lys is catabolized by the intestines and accounts for 31% of whole-body Lys oxidation in high protein fed piglets, it is strongly suppressed when protein becomes the limiting nutrient. The data would suggest that intestinal lysine supply does not translate to a linear increase in Lys supply to other tissues therefore a linear response in milk production to increased postruminal Lys supply, if possible, would not be expected to be linear.

Liver represents only 2% of the body weight but is highly active and account for 25% of the whole-body oxygen use (Huntington and Reynolds, 1987). The high blood flow through the liver, that can reach 2000 l h^{-1}, and the fact that AA could be extracted in the first or second pass through this organ, results in an clearance capacity of up to two-thirds of circulating AA (Reynolds et al., 1988; Wray-Cahen et al., 1997; Hanigan et al., 2004). The AA clearance by the liver vary widely between AA. Bequette et al. (2003) indicated that although the regulation of hepatic removal and oxidation of AA is still not understood, there must be coordination between the demand by peripheral tissues and hepatic removal of AA which suggests that AA catabolism by liver is a potential point of regulation (Figure 2).

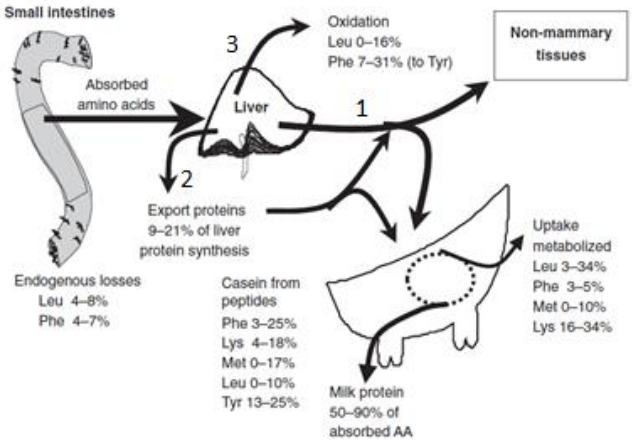

Figure 0-2 Summary of studies in lactating dairy cows and goats where the metabolic fates and partition of amino acids were monitored by tracer kinetics in vivo. There are represented the three options when AA are presented to the liver. 1- AA may pass directly through and become available to peripheral tissues. 2- AA may be extracted for synthesis of proteins, constitutive of liver matrix or exported as plasma proteins. 3- AA may be oxidized with the nitrogen potentially lost as urea. (Bequette et al. 2004).

Lys catabolism, that occurs mainly through the saccharopine-dependent pathway (Fig. 3), is coordinate by combined action of Lys ketoglutarate reductase (**LKR**; EC 1.5.1.8) and saccharopine dehydrogenase (**SDH**; EC 1.5.1.9) (Benevenga and Blemings, 2007). These enzymes reside on a bi-functional protein complex, aminoadipic semialdehyde synthase (**AASS)** (Markovitz et al., 1984), present in numerous tissues (Kiess et al., 2008; Gatrell et al., 2013).

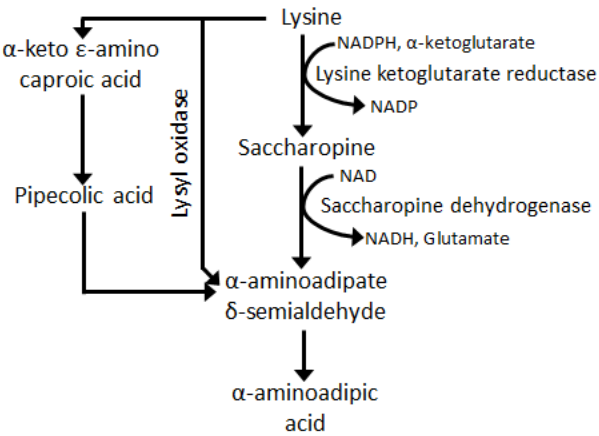

Figure 0-3 Lys catabolism in mammals. Involvement of Lys ketoglutarate reductase and saccharopine dehydrogenase in catabolism of Lys to α-aminoadipic acid constitutes the saccharopine-dependent pathway of Lys catabolism.

In liver Lys is metabolized via the saccharopine-dependent pathway to α-aminoadipic acid. Activities of both enzymes in the AASS protein have been shown to be sensitive to Lys supply (Muramatsu et al., 1984; Blemings et al., 1994) and dietary protein content (Kiess et al., 2008). Increase in the activity of those enzymes could result in a high activity of this complex and high oxidation rate of Lys when this AA is supplemented. Kiess et al., (2008) observed higher LKR activity when mice were fed with 46% casein diet compared with a 21% casein diet. However, the observed increases in AASS mRNA did not translate to an increase in AASS protein abundance (Kiess et al., 2008). Tucker et al., (2017) did not observe a change in AASS due to post abomasal Lys infusion in early lactation dairy cows, although there was an observed increase in plasma α-aminoadipic acid.

The reduction of Lys oxidation by saccharopine-dependent pathway could be an opportunity to increase Lys availability for peripherical tissues including mammary gland. Therefore, the fully understand the regulation of AASS expression and activity may provide important strategies to reducing postabsorptive Lys catabolism and increasing Lys use for protein synthesis in mammary gland and other tissues.

2.6. Summary and conclusions

Lys is the first limiting amino acid for high producing lactating dairy cows when fed a corn-based diet. There are few sources of proteins that can meet the shortfall of Lys when corn-based diets are fed and protein sources that are available are often expensive and/or are inconsistent on the amount of Lys that is biologically available to the cow.

The use of rumen protected AA is a well-established method to augment the profile of AA needed to maintain high levels of milk and milk protein production and maximizing the N use efficiency. Products that provide rumen protected Lys must be evaluated for both rumen protection against microbial degradation and also evaluated for postruminal delivery of Lys that can be absorbed and utilized by the lactating dairy cow. Milk protein production and plasma amino acid concentrations are response variables that have been used previously to assess the efficacy of rumen protected Lys products in meeting the Lys needs of lactating dairy cows. Matching Lys supply to requirements depends complete knowledge of rumen dynamics and the impact on postruminal Lys supply and the post absorptive Lys metabolism. Identifying processes that are sensitive to altered AA supply may prove beneficial in determining the source of inefficient N utilization.

The guiding hypothesis explored in this dissertation is that value of RP Lys is a function of its stability when premixed in feeds, inertness in the rumen, post ruminal bioavailability, and impact on production parameters when fed to lactating dairy cows. A second hypothesis tested was that lysine and energy supply regulate genes that are critical for lysine catabolism in bovine. Taken together this dissertation explores important criteria necessary for the full evaluation of RP Lys for lactating dairy cows and subsequent use of absorbed lysine in dairy cattle.

2.7. References

Alves, D. 2004. Nutrição aminoacídica de bovinos. Curr. Agric. Sci. Technol. 10.

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

Arriola Apelo, S.I., A.L. Bell, K. Estes, J. Ropelewski, M.J. de Veth, and M.D. Hanigan. 2014. Effects of reduced dietary protein and supplemental rumen-protected essential amino acids on the nitrogen efficiency of dairy cows. J. Dairy Sci. 97:5688–5699.

Awawdeh, M.S. 2016. Rumen-protected methionine and lysine: Effects on milk production and plasma amino acids of dairy cows with reference to metabolisable protein status. J. Dairy Res. 83:151-155.

Bach, A., S. Calsamiglia, and M.D. Stern. 2005. Nitrogen metabolism in the rumen. J. Dairy Sci. 88:E9–E21.

Benevenga, N.J., and K.P. Blemings. 2007. Unique aspects of lysine nutrition and metabolism. J. Nutr. 137:1610S-1615S.

Bequette, B.J., M.D. Hanigan, and H. Lapierre. 2003. 19 Mammary Uptake and Metabolism of Amino Acids by Lactating Ruminants. Amin. Acids Anim. Nutr. 347.

Bequette, B. J., and K. Nelson. 2006. The roles of amino acids in milk yield and components. Pages 26-39 in Proc. 17th Florida Ruminant Nutrition Symposium, Gainesville, FL. Univ. of Florida, Gainesville, FL.

Berthiaume, R., H. Lapierre, M. Stevenson, N. Coté, and B.W. McBride. 2000. Comparison of the In Situ and In Vivo Intestinal Disappearance of Ruminally Protected Methionine. J. Dairy Sci. 83:2049-2056.

Blemings, K.P., T.D. Crenshaw, R.W. Swick, and N.J. Benevenga. 1994. Lysine-α-ketoglutarate reductase and saccharopine dehydrogenase are located only in the mitochondrial matrix in rat liver. J. Nutr. 124:1215–1221.

Broderick, G.A. 2003. Manipulation of the Dietary N-Fractions to Improve Ruminal Microbial Synthesis and Yield 1 81–89.

Broderick, G.A. 2016. Precision nutrition for ruminants. Pages 1–6 in Annals The 4th Global Feed and Food Congress, Antalya, Turkey.

Broderick, G.A., M.J. Stevenson, R.A. Patton, N.E. Lobos, and J.J.O. Colmenero. 2008. Effect of supplementing rumen-protected methionine on production and nitrogen excretion in lactating dairy cows. J. Dairy Sci. 91:1092–1102.

Cant, J.P., J.J.M. Kim, S.R.L. Cieslar, and J. Doelman. 2018. Symposium review: Amino acid uptake by the mammary glands: Where does the control lie?. J. Dairy Sci. 101:5655–5666.

Castillo, A.R., E. Kebreab, D.E. Beever, and J. France. 2000. A review of efficiency of nitrogen utilisation in lactating dairy cows and its relationship with environmental pollution. J. Anim. Feed Sci. 9:1–32.

Chibisa, G.E., and T. Mutsvangwa. 2013. Effects of feeding wheat or corn-wheat dried distillers grains with solubles in low-or high-crude protein diets on ruminal function, omasal nutrient flows, urea-N recycling, and performance in cows. J. Dairy Sci. 96:6550–6563.

Clark, J.H., T.H. Klusmeyer, and M.R. Cameron. 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. J. Dairy Sci. 75:2304–2323.

Dairy Herd Network. 2010. Amino Acid Balancing Survey.

Dinn, N.E., J.A. Shelford, and L.J. Fisher. 1998. Use of the Cornell Net Carbohydrate and Protein System and rumen-protected lysine and methionine to reduce nitrogen excretion from lactating dairy cows. J. Dairy Sci. 81:229–237.

Doelman, J., R. V Curtis, M. Carson, J.J.M. Kim, J.A. Metcalf, and J.P. Cant. 2015. Essential amino acid infusions stimulate mammary expression of eukaryotic initiation factor 2Bε but milk protein yield is not increased during an imbalance. J. Dairy Sci. 98:4499–4508.

Doepel, L., D. Pacheco, J.J. Kennelly, M.D. Hanigan, I.F. López, and H. Lapierre. 2004. Milk Protein Synthesis as a Function of Amino Acid Supply. J. Dairy Sci. 87:1279–1297.

Edouard, N., M. Hassouna, P. Robin, and P. Faverdin. 2016. Low degradable protein supply to increase nitrogen efficiency in lactating dairy cows and reduce environmental impacts at barn level. animal 10:212–220.

FAO. 2006. World Reference Base for Soil Resources 2006. A Framework for International Classification, Correlation and Communication.

Fox, D.G., L.O. Tedeschi, T.P. Tylutki, J.B. Russell, M.E. Van Amburgh, L.E. Chase, A.N. Pell, and T.R. Overton. 2004. The Cornell Net Carbohydrate and Protein System model for evaluating herd nutrition and nutrient excretion. Anim. Feed Sci. Technol. 112:29-78.

Gatrell, S.K., L.E. Berg, J.T. Barnard, J.G. Grimmett, K.M. Barnes, and K.P. Blemings. 2013. Tissue distribution of indices of lysine catabolism in growing swine. J. Anim. Sci. 91:238–247.

Giallongo, F., M.T. Harper, J. Oh, J.C. Lopes, H. Lapierre, R.A. Patton, C. Parys, I. Shinzato, and A.N. Hristov. 2016. Effects of rumen-protected methionine, lysine, and histidine on lactation performance of dairy cows. J. Dairy Sci. 99:4437–4452.

Van Goudoever, J.B., B. Stoll, J.F. Henry, D.G. Burrin, and P.J. Reeds. 2000. Adaptive regulation of intestinal lysine metabolism. Proc. Natl. Acad. Sci. 97:11620–11625.

Guo, C.L., Y.T. Li, X.Y. Lin, M.D. Hanigan, Z.G. Yan, Z.Y. Hu, Q.L. Hou, F.G. Jiang, and Z.H. Wang. 2017. Effects of graded removal of lysine from an intravenously infused amino acid mixture on lactation performance and mammary amino acid metabolism in lactating goats. J. Dairy Sci. 100:4552–4564.

Hadrová, S., L. Křížová, M. Richter, J. Třináctý, and M. Dračková. 2012. The effect of duodenal infusion of histidine on milk yield, milk composition, and plasma amino acids in dairy cows. J. Anim. Feed Sci. 21.

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

Hanigan, M. D.; Cyriac, J., 2006: Current status of amino acid requirement models for lactating dairy cows. Tri-State Dairy Nutrition Conference, 25– 26April 2006, Columbus, OH, 133–145.

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

Hanigan, M.D., R.R. White, S.I.A. Apelo, M. Aguilar, K.A. Estes, and A. Myers. 2018. Predicting post-absorptive protein and amino acid metabolism. Rev. Bras. Zootec. 47.

Hristov, A.N., and F. Giallongo. 2014. Feeding protein to dairy cows-what should be our target? Pages 75–84 in Proceedings of the 23rd Tri-State Dairy Nutrition Conference, Fort Wayne, Indiana, USA, 14-16 April 2014. Ohio State University.

Hristov, A.N., M. Hanigan, A. Cole, R. Todd, T.A. McAllister, P.M. Ndegwa, and A. Rotz. 2011. Review: Ammonia emissions from dairy farms and beef feedlots. Can. J. Anim. Sci. 91:1–35

Huntington, G.B., and C.K. Reynolds. 1987. Oxygen consumption and metabolite flux of bovine portal-drained viscera and liver. J. Nutr. 117:1167–1173.

Inra. 2007. Alimentation des bovins, ovins et caprins—besoins des animaux—valeurs des aliments—tables INRA 2007.

Ji, P., H.A. Tucker, R.E. Clark, M. Miura, and C.S. Ballard. 2016. Short communication: Effect of on-farm feeding practices on rumen protected lysine products. J. Dairy Sci. 99:1242–1246.

Kiess, A.S., B.M. Cleveland, M.E. Wilson, H. Klandorf, and K.P. Blemings. 2008. Protein-induced alterations in murine hepatic α-aminoadipate δ-semialdehyde synthase activity are mediated posttranslationally. Nutr. Res. 28:859–865.

Kim, C., J. Choung, and D.G. Chamberlain. 1999. Determination of the first-limiting amino acid for milk production in dairy cows consuming a diet of grass silage and a cereal-based supplement containing feather meal. J. Sci. Food Agric. 79:1703–1708.

Korhonen, M., A. Vanhatalo, T. Varvikko, and P. Huhtanen. 2000. Responses to graded postruminal doses of histidine in dairy cows fed grass silage diets. J. Dairy Sci. 83:2596–2608.

Lapierre, H., L. Doepel, E. Milne, and G.E. Lobley. 2009. Responses in mammary and splanchnic metabolism to altered lysine supply in dairy cows. Animal 3:360–371.

Lapierre, H., L. Doepel, D. Pacheco, and D.R. Ouellet. 2014. Amino acid requirements and post-absorptive metabolism in cattle: Implications for ration formulation. Pages 166–178 in Proc. 25th Annual Florida Ruminant Nutrition Symposium. Department of Animal Sciences, University of Florida, Gainesville.

Lapierre, H., and G.E. Lobley. 2001. Nitrogen recycling in the ruminant: A review. J. Dairy Sci. 84:E223–E236.

LaPierre, P.A., D. Luchini, D.A. Ross, and M.E. Van Amburgh. 2019. Effects of Precision Essential Amino Acid Formulation on a Metabolizable Energy Basis for Lactating Dairy Cows.

Larsen, M., C. Galindo, D.R. Ouellet, G. Maxin, N.B. Kristensen, and H. Lapierre. 2015. Abomasal amino acid infusion in postpartum dairy cows: Effect on whole-body, splanchnic, and mammary amino acid metabolism. J. Dairy Sci. 98:7944–7961.

Lean, I.J., M.B. de Ondarza, C.J. Sniffen, J.E.P. Santos, and K.E. Griswold. 2017. Meta-analysis to predict the effects of metabolizable amino acids on dairy cattle performance. J. Dairy Sci. 101:340–364.

Lee, C., A.N. Hristov, T.W. Cassidy, K.S. Heyler, H. Lapierre, G.A. Varga, M.J. de Veth, R.A. Patton, and C. Parys. 2012. Rumen-protected lysine, methionine, and histidine increase milk protein yield in dairy cows fed a metabolizable protein-deficient diet. J. Dairy Sci. 95:6042–6056.

Lee, C., N.E. Lobos, and W.P. Weiss. 2019. Effects of supplementing rumen-protected lysine and methionine during prepartum and postpartum periods on performance of dairy cows. J. Dairy Sci. 12:11026-11039.

Leonardi, C., M. Stevenson, and L.E. Armentano. 2003. Effect of two levels of crude protein and methionine supplementation on performance of dairy cows.. J. Dairy Sci. 86:4033–42.

Mackle, T.R., D.A. Dwyer, K.L. Ingvartsen, P.Y. Chouinard, D.A. Ross, and D.E. Bauman. 2000. Effects of insulin and postruminal supply of protein on use of amino acids by the mammary gland for milk protein synthesis. J. Dairy Sci. 83:93–105.

Markovitz, P.J., D.T. Chuang, and R.P. Cox. 1984. Familial hyperlysinemias. Purification and characterization of the bifunctional aminoadipic semialdehyde synthase with lysine-ketoglutarate reductase and saccharopine dehydrogenase activities.. J. Biol. Chem. 259:11643–11646.

Mullins, C.R., D. Weber, E. Block, J.F. Smith, M.J. Brouk, and B.J. Bradford. 2013. Short communication: Supplementing lysine and methionine in a lactation diet containing a high concentration of wet corn gluten feed did not alter milk protein yield. J. Dairy Sci. 96:5300-5305.

Muramatsu, K., R. Takada, and K. Uwa. 1984. Adaptive responses of liver and kidney lysine-ketoglutarate reductase and lysine oxidation in rats fed graded levels of dietary lysine and casein. Agric. Biol. Chem. 48:703–711.

Nichols, K., A. Bannink, and J. Dijkstra. 2019. Energy and nitrogen balance of dairy cattle as affected by provision of different essential amino acid profiles at the same metabolizable protein supply. J. Dairy Sci. 102:8963–8976.

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

NRC. 2001. NRC. 2001.. Nutr. Requir. Dairy Cattle. 7th rev. ed. Natl. Acad. Press , Washington, DC.

Olmos Colmenero, J.J.O., and G.A. Broderick. 2006. Effect of Dietary Crude Protein Concentration on Milk Production and Nitrogen Utilization in Lactating Dairy Cows. J. Dairy Sci. 89:1704-1712.

Owens, F.N., S. Qi, and D.A. Sapienza. 2014. Invited Review: Applied protein nutrition of ruminants—Current status and future directions. Prof. Anim. Sci. 30:150–179.

Pacheco, D., R.A. Patton, C. Parys, and H. Lapierre. 2012. Ability of commercially available dairy ration programs to predict duodenal flows of protein and essential amino acids in dairy cows. J. Dairy Sci. 95:937–963.

Pacheco, D., C.G. Schwab, R. Berthiaume, G. Raggio, and H. Lapierre. 2006. Comparison of net portal absorption with predicted flow of digestible amino acids: Scope for improving current models?. J. Dairy Sci. 89:4747–4757.

Patton, R.A. 2010. Effect of rumen-protected methionine on feed intake, milk production, true milk protein concentration, and true milk protein yield, and the factors that influence these effects: A meta-analysis. J. Dairy Sci. 93:2105–2118.

Pisulewski, P.M., H. Rulquin, J.L. Peyraud, and R. Verite. 1996. Lactational and systemic responses of dairy cows to postruminal infusions of increasing amounts of methionine. J. Dairy Sci. 79:1781–1791.

Powell, J.M., and C.A. Rotz. 2015. Measures of nitrogen use efficiency and nitrogen loss from dairy production systems. J. Environ. Qual. 44:336–344.

Reiners, J.N., J.E. Held, C.L. Wright, and D.W. Brake. 2018. Lysine retained among 2 lipid-coated lysine products after exposure to alfalfa or corn silage with different amounts of acidity. J. Dairy Sci. 101:5159–5165.

Reiners, J.N., J.E. Held, C.L. Wright, Q. Qiao, G.D. Djira, B.R. Brunsvig, K.M. Reza, and D.W. Brake. 2017. Lysine bioavailability among 2 lipid-coated lysine products after exposure to silage. Transl. Anim. Sci. 1:311–319.

Reynolds, C.K., G.B. Huntington, H.F. Tyrrell, and P.J. Reynolds. 1988. Net portal-drained visceral and hepatic metabolism of glucose, L-lactate, and nitrogenous compounds in lactating Holstein cows. J. Dairy Sci. 71:1803–1812.

Robinson, P.H. 2010. Impacts of manipulating ration metabolizable lysine and methionine levels on the performance of lactating dairy cows: A systematic review of the literature. Livest. Sci. 127:115-126.

Robinson, P.H., J.M. Moorby, M. Arana, and T. Graham. 2004. Effect of feeding a high- or low-rumen escape protein supplement to dry Holstein cows and heifers within 3 weeks of calving on their productive and reproductive performance in the subsequent lactation. Anim. Feed Sci. Technol. 114:43–57.

Robinson, P.H., N. Swanepoel, I. Shinzato, and S.O. Juchem. 2011. Productive responses of lactating dairy cattle to supplementing high levels of ruminally protected lysine using a rumen protection technology. Anim. Feed Sci. Technol. 168:30–41.

Rulquin, H., P.M. Pisulewski, R. Vérité, and J. Guinard. 1993. Milk production and composition as a function of postruminal lysine and methionine supply: a nutrient-response approach. Livest. Prod. Sci. 37:69-90.

Russell, J.B., R. Onodera, and T. Hino. 1991. Ruminal protein fermentation: new perspectives on previous contradictions. In Physiological aspects of digestion and metabolism in ruminants, pp. 681-697.

Schwab, C., and L.L.C. Schwab Consulting. 2012. The principles of balancing diets for amino acids and their impact on N utilization efficiency. Pages 1–15 in Proceedings of the 23rd Ruminant Nutrition Symposium. University of Florida, Gainsville, FL.

Schwab, C.G., C.K. Bozak, N.L. Whitehouse, and M.M.A. Mesbah. 1992. Amino acid limitation and flow to duodenum at four stages of lactation. 1. Sequence of lysine and methionine limitation. J. Dairy Sci. 75:3486–3502.

Schwab, C.G., and G.A. Broderick. 2017. A 100-Year Review: Protein and amino acid nutrition in dairy cows. J. Dairy Sci. 100:10094–10112. doi:10.3168/jds.2017-13320.

Schwab, C.G., L.D. Satter, and A.B. Clay. 1976. Response of Lactating Dairy Cows to Abomasal Infusion of Amino Acids. J. Dairy Sci. 59:1254–1270.

Schwab, C.G., N.L. Whitehouse, D. Luchini, and B. Sloan. 2009. Reevaluation of the breakpoint estimates for the NRC (2001) required concentrations of lysine and methionine in metabolizable protein for maximal content and yield of milk protein. J. Dairy Sci 92:103.

Spek, J.W., J. Dijkstra, G. van Duinkerken, W.H. Hendriks, and A. Bannink. 2013. Prediction of urinary nitrogen and urinary urea nitrogen excretion by lactating dairy cattle in northwestern Europe and North America: A meta-analysis. J. Dairy Sci. 96:4310–4322.

St-Pierre, N.R., and J.T. Sylvester. 2005. Effects of 2-hydroxy-4-(methylthio) butanoic acid (HMB) and its isopropyl ester on milk production and composition by Holstein cows. J. Dairy Sci. 88:2487–2497.

Swanepoel, N., P.H. Robinson, and L.J. Erasmus. 2010. Amino acid needs of lactating dairy cows: Impact of feeding lysine in a ruminally protected form on productivity of lactating dairy cows. Anim. Feed Sci. Technol. 157:79-94.

Toerien, C.A., D.R. Trout, and J.P. Cant. 2009. Nutritional stimulation of milk protein yield of cows is associated with changes in phosphorylation of mammary eukaryotic initiation factor 2 and ribosomal s6 kinase 1. J. Nutr. 140:285–292.

Tucker, H.A., M.D. Hanigan, J. Escobar, P.H. Doane, and S.S. Donkin. 2017. Hepatic expression of aminoadipate semialdehyde synthase is unchanged by postruminal lysine supply in lactating dairy cows. J. Dairy Sci. 100:1009–1018.

Vandaele, L., K. Goossens, J. De Boever, and S. De Campeneere. 2019. Several roads lead to Rome: about improving nitrogen efficiency in cattle. Wageningen Academic Publishers.

Vanhatalo, A., P. Huhtanen, V. Toivonen, and T. Varvikko. 1999. Response of dairy cows fed grass silage diets to abomasal infusions of histidine alone or in combinations with methionine and lysine. J. Dairy Sci. 82:2674–2685.

Watanabe, K., A.H. Fredeen, P.H. Robinson, W. Chalupa, W.E. Julien, H. Sato, H. Suzuki, K. Katoh, and Y. Obara. 2006. Effects of fat coated rumen bypass lysine and methionine on performance of dairy cows fed a diet deficient in lysine and methionine. Anim. Sci. J. 77:495–502.

Weekes, T.L., P.H. Luimes, and J.P. Cant. 2006. Responses to amino acid imbalances and deficiencies in lactating dairy cows. J. Dairy Sci. 89:2177–2187.

Weiss, W.P. 2019. Effects of feeding diets composed of corn silage and a corn milling product with and without supplemental lysine and methionine to dairy cows. J. Dairy Sci. 102:2075-2084.

Whitehouse, N., C. Schwab, D. Luchini, T. Tylutki, and B. Sloan. 2009. Comparison of optimal lysine and methionine concentrations in metabolizable protein estimated by the NRC (2001), CPM-Dairy (v. 3.0. 10) and AMTS. Cattle (v. 2.1. 1) models. J. Dairy Sci 92:103.

Whitehouse, N.L., C.G. Schwab, and A.F. Brito. 2017. The plasma free amino acid dose-response technique: A proposed methodology for determining lysine relative bioavailability of rumen-protected lysine supplements. J. Dairy Sci. 100:9585–9601.

Wray-Cahen, D., J.A. Metcalf, F.R.C. Backwell, B.J. Bequette, D.S. Brown, J.D. Sutton, and G.E. Lobley. 1997. Hepatic response to increased exogenous supply of plasma amino acids by infusion into the mesenteric vein of Holstein-Friesian cows in late gestation. Br. J. Nutr. 78:913–930.

Wu, S.H.W., and A. Papas. 1997. Rumen-stable delivery systems. Adv. Drug Deliv. Rev. 28:323–334.

Zanton, G.I. 2017. Protein and amino acid nutrition. 3rd ed. D.K. Beede, ed. American Dairy Science Association, Champaign, IL.

CHAPTER IV– ARTICLE II

Short communication: Increase in Lysine concentration and additional energy did not alter AASS gene expression in MDBK cells

2.1. ABSTRACT

The objective for this study was to determine the effects of extracellular Lys levels and energy supply on AASS mRNA expression. MDBK cells at 80% confluence were cultured in DMEM and exposed for 24 h to 0.5 % bovine serum albumin DMEM with antibiotic-antimycotic solution. The basal DMEM contained 0.8 mM Lysine. Cells received one of the 12 treatments as follows: basal DMEM media no additional Lys or energy source (Control), addition of 1mM of propionate (BLPr); addition of 1mM of lactate to the control media (BLL); addition of 1mM of alanine to the control media (BLA); addition of 0.8 mM of Lys to the control media (2XLB); addition of 0.8 mM Lys and 1mM of propionate to the control media (2XLPR); addition of 0.8 mM Lys and 1mM of lactate to the control media (2XLL); addition of 0.8 mM Lys and 1mM of alanine to the control media (2XLA); addition of 1.6 mM of Lys to the control media (3XLB); addition of 1.6 mM Lys and 1mM of propionate to the control media (3XLPR); addition of 1.6 mM Lys and 1mM of lactate to the control media (3XLL); addition of 1.6 mM Lys and 1mM of alanine to the control media (3XLA). Treatments were applied in duplicate to 3 separate MDBK cell replicates. Abundance of cDNA for each sample was quantified using real-time PCR. The 22DDCT method was used to calculate relative changes in gene expression determined from real-time quantitative PCR. There was no overall effect of treatment on AASS expression ($P = 0.16$), however, increasing Lys concentration in the media reduced AASS gene expression in a linear fashion ($P < 0.01$). Furthermore, the additional of energy to the cultures, regardless of source, did not alter the relative expression of AASS (P=0.59). We conclude that although MDBK cells have utility in the study of cellular metabolism and control of gene expression and display expression of AASS that additional information is necessary.

Keywords: amino acids, aminoadipic semialdehyde synthase, protein

2.2. SHORT COMMUNICATION

The high price of protein sources (Moraes et al., 2018) associated with the inefficiency of N use (Spek et al., 2013) and the impacts of N excretion on the environment (Arriola Apelo et al., 2014a) have necessitated a move towards formulating diets for dairy cattle with low crude protein (**CP**) content (St-Pierre and Sylvester, 2005). However, diets formulated with low CP content are typically associated with reductions in milk volume and milk component yield, mostly because of the reduction in the AA profile of metabolizable protein leading to a deficiency of essential amino acids (Arriola Apelo et al., 2014b). One strategy to avoid the drop in performance includes supplementation with rumen-protected essential amino acids (Hristov and Giallongo, 2014).

Lysine (**Lys**) is considered the first limiting amino acid for growth and milk synthesis in dairy cows' fed corn-based diets (NRC, 2001b). However, Lys supplementation does not always translate into an increase in milk production or milk protein yield (Robinson, 2010). The lack of effect on production may be related to an inability to model the impact of a postruminal increase in Lys supply on Lys metabolism. Specifically an increase in supply may increase Lys catabolism, in liver and other tissues, which potentially diverts Lys through the oxidative pathway for energy production and away from protein synthesis (Papes et al., 1999).

Lysine catabolism, in mammals, occurs mainly through the saccharopine-dependent pathway, which is coordinated by the combined actions of lysine ketoglutarate reductase (LKR; EC 1.5.1.8) and saccharopine dehydrogenase (SDH; EC 1.5.1.9) (Benevenga and Blemings, 2007). These enzymes are present in several tissues and reside within aminoadipic semialdehyde synthase (AASS), a bi-functional protein complex (Markovitz et al., 1984; Kiess et al., 2008; Gatrell et al., 2013). Lysine is catabolized to acetoacetyl-CoA and is further converted to ketone body acetoacetate, by acetoacetyl-CoA hydrolase (Voet et al., 2016), that can be used as energy source by extrahepatic tissues.

The activities of both enzymes in the AASS have been shown to be sensitive to Lys supply (Muramatsu et al., 1984; Blemings et al., 1994) and dietary protein content (Kiess et al., 2008). In the liver, LKR and SDH could also participate in the control of the N balance through Lys degradation into α-aminoadipic semialdehyde.

Lysine oxidation increases when intracellular Lys and saccharopine increases through allosteric activation of LKR and SDH by phosphorylation (Foster et al., 1993; Papes et al., 1999). An increase in AASS activity due to dietary Lys supply could reflect a portion of the observed inefficiency in milk production and milk composition response to increased postruminal lysine as a result of rumen protected lysine supplementation. Conversely a reduction in AASS would be expected to increase Lys availably to extrahepatic tissue and promote mammary protein synthesis when lysine is limiting. Previous studies have shown that reductions in AASS mRNA abundance in the liver could represent 50 % of reduction in the LKR activity and reduced Lys catabolism (Cleveland et al., 2008).

The reduction of lysine oxidation by saccharopine-dependent pathway may be an opportunity to increase Lys availability to peripherical tissues where the need for AA for protein synthesis is great, particularly lactating mammary gland. Therefore, to fully understand Lys use efficiency it is critical to understand the regulation of AASS expression and activity and role in Lys catabolism in dairy cows. Previous studies have shown that postruminal lysine infusion and postruminal protein infusions fail to induce AASS mRNA in lactating dairy cows (Tucker, 2013; Tucker et al., 2017) despite an increase in oxidation of lysine with increasing supply of postruminal protein (Tucker, 2013). Therefore, the role of AASS in Lys catabolism in lactating dairy cows is presently unclear. To address this knowledge gap, we explored the effects of Lys to induce AASS in a bovine cell culture model of Lys metabolism. We hypothesized that (1) increasing Lys would increase AASS mRNA expression due to increased substrate concentration and (2) that energy supplied to cells could alter the catabolism of Lys. Therefore, the objective for this experiment was to determine the effects of extracellular Lys levels and energy supply on AASS mRNA expression as an indicator of a gene specific to Lys catabolism.

For this study Madin-Darby bovine kidney epithelial (MDBK) cells (NBL-1 ATCC CCL-22) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were propagated in sterile polystyrene 182-cm^2 canted-neck, 0.2 µm vent cap flasks (DOT Scientific, Burton, MI) in Dulbecco's Modified Eagle's Medium (DMEM) containing 0.80 mM L-Lysine Hydrochloride, 5.5 mM D-glucose, and 1mM sodium pyruvate (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Corp., St. Louis, MO), 100 units/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B (Antibiotic-Antimycotic Solution; Sigma-Aldrich Corp., St. Louis, MO) at 37°C in 5% CO_2 and 95% air. Media was refreshed every two to three days. Cells were dislodged from the plates using trypsin-EDTA (Sigma-Aldrich Corp., St. Louis, MO) and subcultured in 35 mm 6-well plates (Corning, Glendale, AZ) at a density of 5×10^5 cells well and incubated in 10 % FBS

DMEM with antibiotic-antimycotic, as indicated above, to achieve approximately 80% confluence before exposure to treatments.

The MDBK cells at 80% confluence were cultured in DMEM as previously described (White et al., 2012) and exposed for 24 h to 0.5 % bovine serum albumin (BSA) (Probumin; Millipore, Billerica, MA) DMEM with antibiotic-antimycotic solution. The basal DMEM contained 0.8 mM Lysine (Sigma-Aldrich Corp., St. Louis, MO). Cells received one of the 12 treatments as follows: basal DMEM media no additional Lys or energy source (Control), addition of 1mM of propionate (BLPr); addition of 1mM of lactate to the control media (BLL); addition of 1mM of alanine to the control media (BLA); addition of 0.8 mM of Lys to the control media (2XLB); addition of 0.8 mM Lys and 1mM of propionate to the control media (2XLPR); addition of 0.8 mM Lys and 1mM of lactate to the control media (2XLL); addition of 0.8 mM Lys and 1mM of alanine to the control media (2XLA); addition of 1.6 mM of Lys to the control media (3XLB); addition of 1.6 mM Lys and 1mM of propionate to the control media (3XLPR); addition of 1.6 mM Lys and 1mM of lactate to the control media (3XLL); addition of 1.6 mM Lys and 1mM of alanine to the control media (3XLA). Treatments were applied in duplicate to three separate MDBK cell replicates.

After exposure to treatments for 24 h, media was removed and cells were disrupted and lysed by additional of 175 µL Buffer RLT (RNeasy Mini Kit, Qiagen Inc., Germantown, MD). Cell lysates from two wells (duplicate) were combined to a 1.5 mL microcentrifuge tube and stored at -80°C pending subsequent RNA isolation.

Cell lysates were homogenized by vortexing and processed using the RNeasy Mini Kit and RNase-Free DNase Set according to manufacturer directions (Qiagen Inc., Germantown, MD). Purified RNA was quantified for each sample by absorbance at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and sample purity was determined by the ratio of absorbance at 260 nm to 280 nm. Only samples with a ratio of 260:280 greater than or equal to 1.8 were used for further analysis. A 1 µg aliquot of purified RNA from each sample was reverse transcribed to cDNA using an Omniscript RT Kit (Qiagen Inc., Germantown, MD), random decamers (Invitrogen by Thermo Fisher Scientific Corp, Carlsbad, CA) and oligo-dT (Qiagen Inc., Germantown, MD). Abundance of cDNA for each sample was quantified using real-time PCR, Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Inc., Santa Clara, CA) and primers described below. A no-reverse-transcriptase template control was formed by combining equal quantities of purified RNA from each sample. A cDNA pool was formed from equal quantities of cDNA from each sample and then diluted with DNase-free water in a 1:3 dilution series to generate a standard curve. DNase-

free water served as the no-template control. The abundance of AASS (EC 1.5.1.8 and EC 1.5.1.9) mRNA was determined using quantitative real-time PCR. Primers were as follows: bovine AASS, AACAGAGATGCGTTTCCTGCCCTT (forward), AGGAAAGTTTCACGGCCAGATGCT (reverse); and bovine GAPDH, CATGTTTGTGATGGGCGTGAACCA (forward), TGATGGCGTGGACAGTGGTCATAA (reverse). Reactions were as follows: 1 cycle at 95°C for 3 min; 40 cycles of 95°C for 10 s, 60°C for 20 s; and 1 cycle of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s.

The 22DDCT method was used to calculate relative changes in gene expression determined from real-time quantitative PCR (Livak and Schmittgen, 2001). All samples, standards, and controls were analyzed in triplicate and mean values normalized to GAPDH abundance within each sample. Data are expressed as arbitrary units of mRNA relative to GAPDH abundance within sample. The resulting data were statistically analyzed using SAS software (version 9.3, SAS Institute Inc., Cary, NC). The data are reported as least squares means ± standard error of the mean. The MIXED procedure of SAS was used to analyze the effects of treatments on the AASS gene expression. Analyses of variance was performed using the Proc Mixed procedure of SAS. The model accounted for the fixed effects of treatment. Preplanned were built using coefficients for equally spaced treatment and used to determine the linear and quadratic effects of increasing Lys concentration in the media on the AASS gene expression. The addition of an extra energy source in the AASS gene expression was determined using preplanned comparisons. Means were considered different when $P < 0.05$ and tended to differ if $P \leq 0.05$. Tukey-Kramer studentized adjustments were used for multiple comparisons to separate treatment means.

There was no overall effect of treatment on AASS expression ($P = 0.16$), however, increasing Lys concentration in the media reduced AASS gene expression in a linear fashion ($P < 0.01$). Furthermore, the additional of energy to the cultures, regardless of source, did not alter the relative expression of AASS (P=0.59).

Liver, kidney and intestine are known to have higher AASS activity and therefore greater capacity for lysine catabolism and therefore may modulate Lys availability to other tissues such as the mammary gland. (Gatrell et al., 2013). We utilized MDBK cells for these experiments because they have been shown to display metabolism that resembles bovine liver and have been used previously as a model for study of functional control of metabolism and gene expression (Bionaz et al., 2008; Elgendy et al., 2017).

There is evidence that lysine catabolism catalyzed by the AASS in the liver tissue is an obligatory or inevitable process, and suppression of this catabolism would decrease the Lys

requirement by 26%, which would indirectly increase Lys supply to extrahepatic tissues. (Klasing, 2008). We hypothesized in the present study that the increase of available Lys concentrations in the media would result in increase in AASS expression and sought to make this determination as a first step in studying the control of AASS in bovine. From the data collected we reject our initial hypothesis and conclude that AASS in not upregulated in response to increasing Lys supply.

Concentrations of lysine tested in the current experiment represent 1X, 2X and 3X the concentration found in DMEM where 1X is 0.8 mmol/L. These qualities are 30 to 50X the concentration in portal and arterial blood (Blouin et al., 2002; Chapter 3). Supraphysiological lysine, such as the concentrations used here, can lead to erroneous conclusions when assessing changes in AA metabolism (Cantor et al., 2017) including the inability of cells to utilize additional Lys from the media due to saturation of transport mechanisms or reduction in the mitochondrial lysine uptake and consequently lysine oxidation (Benevenga and Blemings, 2007). The activity of LKR in the liver is sensitive to Lys concentration and concentrations exceeding 15 mmol/L resulted in decreased enzyme activity, eventually reaching complete inhibition when media lysine concentrations reached 75 mmol/L (Pink et al., 2011). A 50% reduction in LKR activity has been observed in murine hepatic cells line correlated to a 20% decrease in AASS mRNA abundance (Cleveland et al., 2008). Information of LKR inhibition in MBDK cells with increasing lysine is not available but our data would suggest a similar response to excessive lysine that is controlled at the transcript level.

Because lysine metabolism may be influenced by the supply of energy (Papes et al., 1999) we explored the effects of increasing Lys in the presence of several cellular energy sources, however the addition of energy source in the media had not effect on AASS gene expression. In the absence of any measures that would indicate energy insufficiencies or energetic challenges to cellular metabolism it is unlikely that the MDBK cells used in the present experiment were metabolically stressed, consequently the provision of additional energy, regardless of the substrate, was without effect.

We conclude that although MDBK cells have utility in the study of cellular metabolism and control of gene expression and display expression of AASS that additional information is necessary, relative to effect of limiting lysine concentrations, before this cell model can be used to better understand control of lysine catabolism. In summary, the Lys concentration and an additional energy source did not affect the AASS gene expression. The increase in the Lys concentration shown is the reduction in the AASS gene expression. Better understanding of Lys

metabolism and AASS involvement is still needed to fully employ MDBK cells as a model system for understanding lysine catabolism in bovine.

2.3. ACKNOWLEDGEMENTS

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2.4. REFERENCES

Arriola Apelo, S.I., A.L. Bell, K. Estes, J. Ropelewski, M.J. de Veth, and M.D. Hanigan. 2014a. Effects of reduced dietary protein and supplemental rumen-protected essential amino acids on the nitrogen efficiency of dairy cows. J. Dairy Sci. 97:5688–5699. doi:10.3168/jds.2013-7833.

Arriola Apelo, S.I., A.L. Bell, K. Estes, J. Ropelewski, M.J. de Veth, and M.D. Hanigan. 2014b. Effects of reduced dietary protein and supplemental rumen-protected essential amino acids on the nitrogen efficiency of dairy cows. J. Dairy Sci. 97:5688–5699.

Benevenga, N.J., and K.P. Blemings. 2007. Unique aspects of lysine nutrition and metabolism. J. Nutr. 137:1610S-1615S.

Bionaz, M., R.E. Everts, H.A. Lewin, J.K. Drackley, and J.J. Loor. 2008. Bovine kidney (MDBK) cells and liver tissue of periparturient cows share remarkable similarity in gene expression profiles. J. Dairy Sci 91:178.

Blemings, K.P., T.D. Crenshaw, R.W. Swick, and N.J. Benevenga. 1994. Lysine-α-ketoglutarate reductase and saccharopine dehydrogenase are located only in the mitochondrial matrix in rat liver. J. Nutr. 124:1215–1221.

Blouin, J.P., J.F. Bernier, C.K. Reynolds, G.E. Lobley, P. Dubreuil, and H. Lapierre. 2002. Effect of supply of metabolizable protein on splanchnic fluxes of nutrients and hormones in lactating dairy cows. J. Dairy Sci. 85:2618-2630.-2630.

Cantor, J.R., M. Abu-Remaileh, N. Kanarek, E. Freinkman, X. Gao, A. Louissaint Jr, C.A. Lewis, and D.M. Sabatini. 2017. Physiologic medium rewires cellular metabolism and reveals uric acid as an endogenous inhibitor of UMP synthase. Cell 169:258–272.

Cleveland, B.M., A.S. Kiess, and K.P. Blemings. 2008. α-Aminoadipate δ-semialdehyde synthase mRNA knockdown reduces the lysine requirement of a mouse hepatic cell line. J. Nutr. 138:2143–2147.

Elgendy, R., M. Giantin, and M. Dacasto. 2017. Transcriptomic characterization of bovine primary cultured hepatocytes; a cross-comparison with a bovine liver and the Madin-Darby bovine kidney cells. Res. Vet. Sci. 113:40–49.

Foster, A.R., P.W.D. Scislowski, C.I. Harris, and M.F. Fuller. 1993. Metabolic response of liver lysine α-ketoglutarate reductase activity in rats fed lysine limiting or lysine excessive diets. Nutr. Res. 13:1433–1443.

Gatrell, S.K., L.E. Berg, J.T. Barnard, J.G. Grimmett, K.M. Barnes, and K.P. Blemings. 2013. Tissue distribution of indices of lysine catabolism in growing swine. J. Anim. Sci. 91:238–247.

Hristov, A.N., and F. Giallongo. 2014. Feeding protein to dairy cows-what should be our target? Pages 75–84 in Proceedings of the 23rd Tri-State Dairy Nutrition Conference, Fort Wayne, Indiana, USA, 14-16 April 2014. Ohio State University.

Kiess, A.S., B.M. Cleveland, M.E. Wilson, H. Klandorf, and K.P. Blemings. 2008. Protein-induced alterations in murine hepatic α-aminoadipate δ-semialdehyde synthase activity are mediated posttranslationally. Nutr. Res. 28:859–865.

Klasing, K.C. 2008. Minimizing amino acid catabolism decreases amino acid requirements. J. Nutr. 139:11–12.

Livak, K.J., and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25:402–408.

Markovitz, P.J., D.T. Chuang, and R.P. Cox. 1984. Familial hyperlysinemias. Purification and characterization of the bifunctional aminoadipic semialdehyde synthase with lysine-ketoglutarate reductase and saccharopine dehydrogenase activities.. J. Biol. Chem. 259:11643–11646.

Moraes, L.E., E. Kebreab, J.L. Firkins, R.R. White, R. Martineau, and H. Lapierre. 2018. Predicting milk protein responses and the requirement of metabolizable protein by lactating dairy cows. J. Dairy Sci. 101:310–327.

Muramatsu, K., R. Takada, and K. Uwa. 1984. Adaptive responses of liver and kidney lysine-ketoglutarate reductase and lysine oxidation in rats fed graded levels of dietary lysine and casein. Agric. Biol. Chem. 48:703–711.

NRC. 2001. Nrc. 2001.. Nutr. Requir. Dairy Cattle. 7th rev. ed. Natl. Acad. Press , Washington, DC 1–333.

Papes, F., E.L. Kemper, G. Cord-Neto, F. Langone, and P. Arruda. 1999. Lysine degradation through the saccharopine pathway in mammals: involvement of both bifunctional and monofunctional lysine-degrading enzymes in mouse.. Biochem. J. 344:555.

Pink, D.B.S., S.K. Gatrell, R. Elango, J. Turchinsky, A.S. Kiess, K.P. Blemings, W.T. Dixon, and R.O. Ball. 2011. Lysine α-ketoglutarate reductase, but not saccharopine dehydrogenase, is subject to substrate inhibition in pig liver. Nutr. Res. 31:544–554.

Robinson, P.H. 2010. Impacts of manipulating ration metabolizable lysine and methionine levels on the performance of lactating dairy cows: A systematic review of the literature. Livest. Sci. 127:115-126

Spek, J.W., J. Dijkstra, G. van Duinkerken, W.H. Hendriks, and A. Bannink. 2013. Prediction of urinary nitrogen and urinary urea nitrogen excretion by lactating dairy cattle in northwestern Europe and North America: A meta-analysis. J. Dairy Sci. 96:4310–4322.

St-Pierre, N.R., and J.T. Sylvester. 2005. Effects of 2-hydroxy-4-(methylthio) butanoic acid (HMB) and its isopropyl ester on milk production and composition by Holstein cows. J. Dairy Sci. 88:2487–2497.

Tucker, H.A. 2013. Impacts of protein and amino acid supply on milk production, intermediary metabolism, and mRNA expression in liver of early lactation dairy cows.

Tucker, H.A., M.D. Hanigan, J. Escobar, P.H. Doane, and S.S. Donkin. 2017. Hepatic expression of aminoadipate semialdehyde synthase is unchanged by postruminal lysine supply in lactating dairy cows. J. Dairy Sci. 100:1009–1018.

Voet, D., J.G. Voet, and C.W. Pratt. 2016. Fundamentals of Biochemistry: Life at the Molecular Level. John Wiley & Sons.

White, H.M., S.L. Koser, and S.S. Donkin. 2012. Gluconeogenic enzymes are differentially regulated by fatty acid cocktails in Madin-Darby bovine kidney cells1. J. Dairy Sci. 95:1249–1256. doi:10.3168/jds.2011-4644.

Table 0-1 Amino acid concentrations (mM) of the extracellular media provided to MDBK cells

Components	Treatments											
	Control	BLPr	BLL	BLA	2xL	2xLPr	2xLL	2xLA	3xL	3xLPr	3xLL	3xLA
Amino Acids												
Glyc	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400
Arg	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398
Cys	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201
Glu	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
His	0.200	0.200	0.200	0.200	0.200	0.200	0.200	0.200	0.200	0.200	0.200	0.200
Iso	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802
Leu	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802	0.802
Lys	0.798	0.798	0.798	0.798	1.596	1.596	1.596	1.596	2.393	2.393	2.393	2.393
Met	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201
Phe	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400
Ser	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400
Thr	0.798	0.798	0.798	0.798	0.798	0.798	0.798	0.798	0.798	0.798	0.798	0.798
Try	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.078
Tyr	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398	0.398
Val	0.803	0.803	0.803	0.803	0.803	0.803	0.803	0.803	0.803	0.803	0.803	0.803
Energy source												
D-Glucose (Dextrose)	5.556	5.556	5.556	5.556	5.556	5.556	5.556	5.556	5.556	5.556	5.556	5.556
Sodium Pyruvate	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Propionate		1.000				1.000				1.000		
Lactate			1.000				1.000				1.000	
Ala				1.000				1.000				1.000

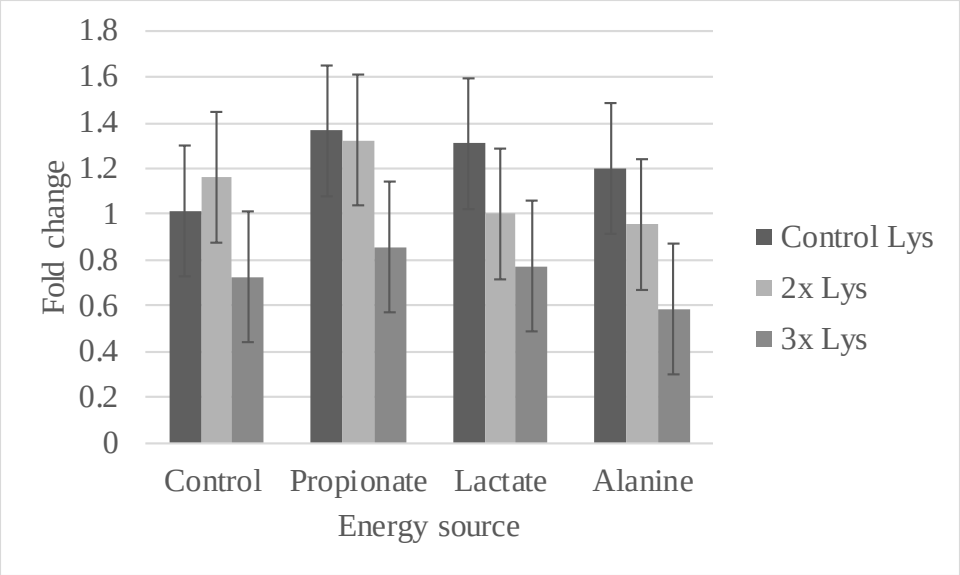

Figure 0-1 Expression of aminoadipate semialdehyde synthase (AASS) in MDBK cells after 24h of exposure to a media with different Lys concentrations (control, 2x Lys, 3x Lys) and energy source additional to media (propionate, lactate or alanine). Cells were cultured in media for 24 h, RNA was extracted and analyzed for AASS and GAPDH mRNA. Data are LSmeans and standard errors for 3 independent cell preparations.

APPENDIX

The appendix presented in this thesis is part of the first work in which I was involved as a member of Dr. Shawn S Donkin's laboratory. The data and sample collection were made by the other students and workers from Dr. Donkin's lab. I was responsible for extracting the cow performance data from Purdue Dairy files, preparing and organizing the blood analysis data in the laboratory, performing the statistical analysis, preparing the tables and drafting the first version of the manuscript. This study was important in training and developing my skills to work with data sets and statistical analysis and develop the skills needed for the main studies of this thesis. In the IVGTT study, data analysis tools and methodologies were used, such as the calculation of the area under the curve, clearance rate and half-life, which were used in the development and analysis of data from the lysine infusion study.

Short communication: Effect of glucose infusion dose and stage of lactation on glucose tolerance test kinetics in lactating dairy cows.

ABSTRACT

The objective for this study was to determine the effects of glucose dose, and stage of lactation during an intravenous glucose tolerance test (IVGTT) on plasma glucose, insulin, and NEFA kinetics in lactating dairy cattle. Six lactating Holstein dairy cows, (3 primiparous and 3 multiparous) were assigned to 2 squares and to receive 0.092, 0.15, or 0.3 g glucose/kg BW during IVGTT during early- (74 ± 8.5 DIM) and mid-lactation (221±8.5 DIM) in a replicated Latin Square design. Treatments were applied in a Latin square design using 21-d periods at each stage of lactation. Periods within stage of lactation were seven days in duration. Milk production and DM intake were determined daily during the first 6 d of each period and the last day of each period was

used for IVGTT. For IVGTT, cows were prepared with indwelling catheters in each jugular vein and blood samples were collected at -15, -10, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min relative to the glucose infusion and analyzed for plasma glucose, serum insulin, and plasma NEFA. Increasing the glucose dose during IVGTT increased ($P < 0.05$) plasma glucose area under the curve (AUC), decreased glucose half-life, and increased maximal glucose concentrations in plasma during IVGTT. Greater glucose dose during IVGTT elevated serum insulin AUC and increased nadir NEFA concentrations. Maximal glucose during IVGTT was lower whereas maximum NEFA, NEFA AUC, and NEFA clearance rate were greater during early lactation compared with mid-lactation. Only glucose half-life was responsive ($P<0.05$) to stage of lactation × glucose dose effects during IVGTT and the decrease in glucose half-life with increasing glucose dose was greater during early lactation compared to mid-lactation. Glucose AUC was greater and NEFA AUC was lower ($P <0.05$) during mid-lactation compared to early-lactation. A glucose dose in excess of 0.092 g/kg BW resulted in peak blood glucose concentration that exceeded the previously reported renal glucose threshold of 8.3 mmol /L. Based on maximal peak glucose concentrations, insulin response criteria and effects on glucose half-life we identify 0.092 g/kg BW (0.46 g/kg $BW^{0.75}$) as a preferred glucose dose for IVGTT in early- and mid-lactation dairy cows.
Keywords: Glucose tolerance, insulin, dose, clearance, IVGTT.

SHORT COMMUNICATION

The insulin resistance is a natural homeorhetic adaptation that occurs in dairy cows and other mammals during the late gestation and persists into early lactation. During this period, tissues that are normally insulin responsive acquire reduced sensitivity to the effects of insulin to promote glucose clearance from blood. This adaptation serves to make more glucose available for non-insulin responsive glucose uptake by other tissues including the growing fetus and mammary gland (Bell and Bauman, 1997). Although the metabolic adaptations to divert glucose to fetal and mammary tissue are important during late gestation, the lack of appropriate timing and degree of adaptation may be associated with metabolic diseases like ketosis, fatty liver, and may be associated with a reduction in milk production potential (Hayirli, 2006).

Intravenous glucose tolerance test (IVGTT) is frequently used to assess systemic glucose metabolism and insulin sensitivity in dairy cattle (De Koster and Opsomer, 2013) and humans as an indicator of health (Singh and Saxena, 2010). Plasma glucose concentration is measured during the IVGTT at predetermined intervals prior to, and following, the intravenous infusion of a bolus dose of glucose. Glucose and insulin kinetics in blood are used to assess physiological status and insulin resistance. A rise in blood glucose in response to intravenous glucose infusion results in a commensurate release of insulin from the pancreas, which in turn acts to clear glucose by insulin sensitive tissues to preinfusion levels. The clearance rate of glucose from blood is dependent on the amount of insulin released from the pancreas and the peripheral response to insulin. Although the capacity for insulin release and responsiveness are confounded during the test, the technique provides a technically simple and inexpensive assessment of insulin responsiveness compared with other techniques (Defronzo et al., 1979). Despite its shortcomings the IVGTT remains as valuable clinical assessment of insulin resistance. In addition, insulin–inhibited adipose lipolysis is also a key feature of IVGTT that can be used to assess insulin responsiveness (Boston et al., 2008). Although the technique has been standardized for other species (Ayala et al., 2010) similar standardization does not yet exist for cattle.

Despite frequent use, there is little standardization of the IVGTT in characterizing glucose kinetics in dairy cattle. Glucose doses of 0.15 (Holtenius et al., 2003), 0.25 (Wheelock et al., 2010), 0.30 (Subiyatno et al., 1996) and 0.50 (Bigner et al. 1996) g/kg BW have all been reported in the literature. It has been suggested that an insufficient dose of glucose during IVGTT may fail to elicit maximal pancreatic insulin release whereas a dose that is too high may overwhelm the ability of the kidney to resorb glucose and result in excretion of glucose in urine (González-Grajales et al., 2018). Both scenarios could result in an inaccurate evaluation of glucose kinetics and estimate of insulin resistance. A direct comparison of glucose dose during IVGTT in lactating dairy cattle is lacking and evaluation of the impact of dose and physiological status is needed to establish a standardized method.

We hypothesize that there is an optimal glucose dose for IVGTT in lactating dairy cattle and that the parameter estimates of insulin, glucose and NEFA kinetics determined from IVGTT are responsive to the dose of glucose administered during the test. Furthermore we hypothesized that the effect of glucose dose during IVGTT would differ with stage of lactation. Therefore, the objective for this experiment was to determine the effect of dosing either 0.092, 0.15, or 0.3 g

glucose/kg BW during IVGTT on glucose, insulin and NEFA kinetics during early- and mid-lactation. The doses selected were based on previous work using 0.15 (Holtenius et al., 2003) and 0.30 (Subiyatno et al., 1996) with inclusion of a lower dose of glucose of 0.092 glucose /kg BW. The doses selected also represent approximately one-half and one-third of the values, per metabolic body weight of those explored to optimize IVGTT in rats (Frangioudakis et al., 2008).

Six lactating Holstein dairy cows, 3 primiparous and 3 multiparous, weighing 640 kg (SD = 116 kg) were blocked and assigned by parity to one of two 3x3 Latin squares. Cows selected for the experiment were housed in a freestall facility and managed as a group and were relocated to a tie-stall facility for two 21-d intensive data collection intervals during early- (74 DIM, SD = 8.5) and mid-lactation (221 DIM, SD = 8.5 DIM). Cows were fed a corn silage based diet throughout the experiment. Feed was delivered once daily as a TMR fed to meet or exceed all nutrient requirements according to NRC (2001). Cows had access to feed at all times including the period prior to and during IVGTT. All animal care and handling protocols were approved by the Purdue University Animal Care and Use Committee.

Each intensive data collection interval was of 21-d in duration and consisted of 3 periods of 7 d each. Each period included 6 d of adjustment and 1 d of IVGTT. Body weight was measured at the beginning of each 21-d interval. The BW for cows during early- late mid-lactation was 618 and 626 kg respectively with a standard error of least squares means for stage of lactation of 59.7 kg. Individual feed intake and milk production was determined for days 1 through 6 of each period and milk composition was determined on day 3 of each period. Milk samples, collected from consecutive milkings, were analyzed for fat, protein, lactose, total solids, MUN, and somatic cells by Dairy One (Ithaca, NY).

Cows were assigned within square to receive either 0.092, 0.15, or 0.3 g glucose/kg body weight (BW) during the IVGTT. On day 7 of each period, approximately 1 hour prior initiation of IVGTT, cows were fitted with indwelling catheters (16-gauge, Abbocath-TAL catheter; Abbott Laboratories, North Chicago, IL) in the left and right jugular veins. One vein was used for infusion and the other for sample collection. Cows were given a sterile 50% glucose solution (Huvepharma, Inc, St Joseph, MO) to deliver the assigned dose of glucose. Infusions were timed and all were completed with 2 minutes of initiation. Following glucose infusions and sample collection catheters were flushed and blocked with heparinized saline (2 I.U./ml).

Blood samples were collected from indwelling catheters using 12 cc syringes at -15, -10, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min relative to the glucose infusion. Samples (10 ml) for glucose analysis were collected into BD Vacutainer tubes (Franklin Lakes, NJ) containing sodium fluoride and potassium oxalate, samples for NEFA contained K_3EDTA. Samples (10 ml) for insulin analysis did not contain any additive and were allowed to clot at room temperature for 30 minutes. Within 30 minutes of collection, plasma and serum were separated by centrifugation at 1,000 x g for 15 min and stored at -20º C or 1-3 months pending analysis.

Plasma glucose concentration was determined by an enzymatic method using commercially available kit (Autokit Glucose, Catalog nr 997-03001, Wako Diagnostics™, Richmond, VA). Plasma NEFA concentration was determined using a commercially available kit (HR Series NEFA-HR, Catalog nr 999-34691, Wako Diagnostics™, Richmond, VA). Serum insulin concentration was determined using a commercially available ELISA kit (ALPCO, Salem, NH). Intra-assay and interassay CV for insulin analysis was 4.71 % and 6.50%, respectively and was less than 5% for all other blood analytes. A single baseline value each insulin, glucose and NEFA excursion curve was calculated as the average of samples collected at -15 and -10 min relative to glucose infusion. The AUC of glucose, NEFA, and insulin were calculated using the trapezoidal rule (Cardoso et al., 2011) and ratios of glucose to insulin, glucose to NEFA, and insulin to NEFA ratios were calculated to estimate systemic insulin sensitivity (Hosseini et al., 2015) . The clearance rate (CR) and half-life of glucose, NEFA, and insulin were calculated as described by Pires et al. (2007).

Data were analyzed using the Mixed Procedure of SAS 9.4 with random effects of the cow nested within treatment by stage of lactation. The normality of data was determined using the UNIVARIATE procedure in SAS and the Shapiro-Wilk test. The model for milk production, DMI, milk composition, blood metabolites, and glucose, NEFA and insulin kinetics accounted for the effects of glucose dose, square, period, and the random effect of cow nested within square. Effects of parity were not tested due to confounding within square. Data are LSmeans and standard errors unless otherwise indicated. Means were considered different when $P < 0.05$ and tended to differ if $0.05 \leq P \leq 0.10$. Tukey-Kramer studentized adjustments were used for multiple comparisons.

Data for BW, DMI, milk production and composition are shown in Table 1. As expected milk production was greater in early lactation and milk fat and milk protein percentages were greater in mid-lactation. There were no other differences in milk composition or body weight

between the two stages of lactation. There were no effects of dose of glucose during IVGTT observed for milk protein, fat, and lactose. And no glucose dose x stage of lactation effects for milk production or composition.

Concentrations of glucose, insulin, and NEFA (prior to IVGTT) did not differ between assigned glucose infusion dose (Table 2). Basal NEFA tended ($P = 0.09$) to be lower for cows in mid-lactation but there were no glucose dose x stage of lactation effects for basal insulin, glucose, or NEFA. There was a significant effect of glucose dose during IVGTT on glucose, insulin and NEFA kinetics (Figure 1; Table 2). Maximal glucose achieved during IVGTT was increased with increasing dose of glucose with greater maximal concentrations observed for mid-lactation cows however there were no stage of lactation by dose of glucose effects for maximal glucose concentration. Glucose AUC increased with increasing quantity of glucose and was greater for mid-lactation cows. There were no stage of lactation × glucose dose effects during IVGTT on glucose AUC. The clearance rate of glucose was increased ($P<0.05$) and half-life was decreased ($P<0.05$) with increasing glucose dose and there was a tendency ($P=0.06$) for decreased glucose half-life for mid-lactation cows. There was a significant ($P<0.05$) stage of lactation × dose of glucose during IVGTT effect for glucose half-life. The longest half-life was observed for the 0.092 g/kg BW glucose dose during early lactation and increasing the glucose dose had a more pronounced effect to reduce glucose half-life during early lactation compared with mid-lactation.

There was no effect of dose of glucose given during IVGTT on peak insulin concentrations or insulin clearance rate but insulin half-life tended ($P=0.06$) to increase with glucose dose. The insulin AUC increased with increasing glucose dose during IVGTT. There was a tendency for greater insulin half-life for mid lactation cows. There were no dose of glucose during IVGTT × stage of lactation effects for any insulin kinetics.

All glucose doses selected for our study (0.092, 0.15, or 0.3 g glucose/kg BW) increased blood glucose concentrations and serum insulin in both lactation phases (Figure 1). In all cases glucose and insulin concentration returned to the baseline after 60 minutes and there was no stage of lactation effect. Others have reported when using 0.3 g/kg BW of glucose in lactating dairy cows that 160 min were required for plasma glucose to return to baseline concentration (Sano et al., 1993; Roche et al., 2008; Oliveira et al., 2016). Reasons for these differences in return to baseline between studies are not apparent but may warrant further investigation in assessing insulin resistance.

Plasma NEFA responded to IVGTT with a biphasic response characterized by decreasing concentration from 5 to 30 minutes post infusion and increasing concentrations from 30 through 120 minutes post infusion (Figure 1). There was a greater depression in plasma NEFA with increasing glucose dose that was not affected by stage of lactation (Table 2). Cows in early lactation displayed a greater increase in NEFA from nadir through 120 min post infusion resulting in a stage of lactation effect for NEFA AUC for 30 to 120 min post glucose infusion. There were no interaction effects of glucose dose during IVGTT or stage of lactation effects for NEFA kinetics.

Because of the physiological relationships between NEFA, insulin, and glucose we evaluated the effect of glucose dose during IVGTT and stage of lactation on the ratio of their AUCs. The AUC for glucose to AUC for NEFA through 60 minutes post glucose infusion increased ($P<0.05$) with increasing glucose dose and was greater ($P<0.05$) for cows in mid-lactation. There was no effect of glucose dose or stage of lactation on ratio of AUC for glucose AUC to insulin or insulin to NEFA.

Despite being a widely utilized assessment tool for glucose metabolism and insulin resistance the IVGTT test for dairy cattle has not been extensively evaluated and standardized. Previous reports of the dose of glucose used during IVGTT in cattle indicate a range of levels across age, physiological status, and BW (González-Grajales et al., 2018). Experiments in growing heifers using doses ranging from 0.5 to 3 kg glucose/kg $BW^{0.75}$ indicate an optimal dose for dairy heifers between 0.5 to 1 g of glucose/kg of $BW^{0.75}$ for IVGTT in order to maximize insulin response and avoid plasma glucose concentrations that approach the renal threshold of 5.5 to 8.3 mmol /L (Blum et al., 1999; Stämpfli and Oliver-Espinosa, 2015; González-Grajales et al., 2018). Based on the average BW for animals used in the present experiment the equivalent glucose doses were 0.46, 0.75 and 1.5 g/kg $BW^{0.75}$. Contrary to work in dairy heifers (González-Grajales et al., 2018) our lowest dose of glucose (0.092 g/kg BW, 0.46 g/kg $BW^{0.75}$) was enough to trigger a maximal response in insulin concentrations in our lactating dairy cows and there was no effect ($P>0.05$) of dose on maximum insulin concentration during IVGTT. In addition, all doses except 0.092 g/kg (0.46 g/kg $BW^{0.75}$) resulted in blood glucose concentrations in excess of the upper reported values for renal threshold of 8.3 mM (Blum et al., 1999). Furthermore a dose of 0.092 g/kg permitted observation of differences in glucose half-life between early and mid-lactation dairy cows, and observation that is obscured with glucose doses of 0.15 and 0.30 0.092 g/kg. Based

on maximal peak glucose concentrations and insulin response criteria and lack of dose × stage of lactation effects with IVGTT we identify 0.092 g/kg BW (0.46 g/kg $BW^{0.75}$) as the preferred target glucose dose for IVGTT in early- and mid-lactation dairy cows.

Cows in this study produced more milk during early lactation and had reduced maximal glucose, glucose AUC. It is generally accepted that dairy cows are insulin resistant in early lactation (De Koster and Opsomer, 2013) which helps to prioritize glucose supply for lactating mammary gland. Because glucose uptake by the mammary gland is insulin-independent (Laarveld et al., 1981) the level of milk production relative to dose of glucose during IVGTT may have been a factor in our experiments. The observed effect of glucose dose on glucose half-life during IVGTT is greater during early lactation which suggests an effect of level of milk production on glucose clearance rate. Greater milk production potentially provides for greater insulin independent mammary uptake and metabolism and may result in more rapid glucose clearance. However, the lack of effect of stage of lactation on the ratio of AUC glucose to AUC insulin would suggest the proportions of insulin and noninsulin mediated glucose uptake are similar between these stages of production. These findings parallel previous observations that the capacity of insulin to clear glucose is similar between physiological states in dairy cattle (dry versus lactating) and that the primary observed differences in glucose metabolism are due to ability to release insulin in response to changes in blood glucose (Sano et al., 1993) and that although the response of insulin-sensitive tissues may change with stage of lactation, whole body responsiveness to insulin does not appear to be altered (Marrett et al., 2015).

Because plasma NEFA is sensitive to changes in blood insulin through the inhibition of hormone-sensitive lipase by insulin (Ferrannini et al., 1997) the use of IVGTT has been explored as a tool to characterize the sensitivity of adipose tissue to changes in insulin concentrations (Boston et al., 2008). Current models represent the characteristic initial down-slope of NEFA concentrations in response to IVGTT, a nadir, and then rebound of NEFA to exceed pre IVGTT test levels (Boston et al., 2008). Our data follow this pattern and indicate a change in NEFA with glucose dose and stage of lactation. This is consistent with the greatest AUC for glucose at the highest glucose dose and greater AUC with advancing lactation. Consequently, there is a significant effect of glucose dose and stage of lactation on the ratio of glucose AUC to NEFA AUC however these effects are not mirrored by the ratio of AUC for insulin to AUC for NEFA. The latter suggest a codependency of insulin and NEFA that is not shared by glucose which may

represent an effect of glucose clearance that is dose dependent but not associated with insulin mediated clearance and may include renal clearance.

Our results reinforce the importance of standardization of IVGTT and justification of dose of glucose infused. For many IVGTT parameters, the dose and stage of lactation impact glucose, insulin and NEFA kinetics but only glucose half-life was responsive to both factors. Given the physiological connection between changes in insulin, glucose and NEFA it is important to minimize a bias in data resulting from excessive glucose dosing during IVGTT and the resulting impact of renal clearance on glucose kinetics. A glucose dose that exceeds the correct target dose for IVGTT may result in an overestimate of glucose clearance rate and inability to detect insulin resistance. Although we did not directly evaluate the glucose urinary excretion it appears that a dose of 0.092 g/kg BW is a desired target for IVGTT in early- and mid-lactation dairy cows based on ability to evoke an increase in plasma glucose that is below renal threshold, effects to stimulate an increase plasma insulin and NEFA concentrations and effect to reveal differences is glucose half-life that would be occurred with a higher glucose dose.

REFERENCES

Alves-Nores V., C. Castillo, J. Hernandez, A. Abuelo. 2017. Comparison of surrogate indices for insulin sensitivity with parameters of the intravenous glucose tolerance test in early lactation dairy cattle. Domest. Anim. Endocrinol. 61:48-53.

Ayala, J.E., V. T. Samuel, G. J. Morton, S. Obici, C. M. Croniger, G. I. Shulman, D. H. Wasserman, and O. P. McGuinness, 2010. Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. Dis. Model. Mech. 3:525–534. doi: 10.1242/dmm.006239

Bell, A. W. and D. E. Bauman, 1997. Adaptations of glucose metabolism during pregnancy and lactation. J. Mammary Gland Biol. Neoplasia. 2:265-278.

Bigner, D. R., J. P. Goff, M. A. Faust, J. L. Burton, H. D. Tyler, and R.L Horst. 1996. Acidosis effects on insulin response during glucose tolerance tests in Jersey cows. J Dairy Sci. 79:2182-2188.

Blum, J. W., R. M. Bruckmaier, and P. Y. Vacher. 1999. Insulin-dependent whole-body glucose utilization and insulin-responses to glucose in week 9 and week 19 of lactation in dairy cows fed rumen-protected crystalline fat or fat free fatty acids. Domest. Anim. Endocrinol. 16:123–134

Bossaert, P., J. L. M. R. Leroy, S. De Campeneere, S. De Vliegher, and G. Opsomer, 2009. Differences in the glucose-induced insulin response and the peripheral insulin responsiveness between neonatal calves of the Belgian Blue, Holstein-Friesian, and East Flemish breeds. J. Dairy Sci., 92:4404-4411.

Boston, R. C., J. R. Roche, G. M. Ward, and P. J. Moate. 2008. A novel minimal model to describe non-esterified fatty acid kinetics in Holstein dairy cows. J. Dairy Res. 75:13-18.

Cardoso, F. C., W. Sears, S. J. LeBlanc, and J. K. Drackley. 2011. Comparison of 3 methods for analyzing areas under the curve for glucose and nonesterified fatty acids concentrations following epinephrine challenge in dairy cows. J. Dairy Sci. 94:6111-6115.

De Koster, J. D., G. Opsomer. 2013. Insulin resistance in dairy cows. Vet. Clin. North Am. Food Anim. Pract. 29:299-322.

DeFronzo, R. A., J. D. Tobin, and R. Andres. 1979. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Amer. J. of Physiol.-Endo. Metab. 237:E214-E223.

Ferrannini E. S., S. Camastra, S. W. Coppack, D. Flisher, A. Golay, A. Mitrakou. 1997. Insulin action and non-esterified fatty acids. Proc. Nutr. Soc. 56: 753–761.

Frangioudakis, G. 1., A. C. Gyte, S. J. Loxham, and S. M. Poucher. 2007. The intravenous glucose tolerance test in cannulated Wistar rats: a robust method for the in vivo assessment of glucose-stimulated insulin secretion. J Pharmacol Toxicol Methods 57:106-13

González-Grajales, L. A., L. Pieper, S. Mengel, and R. Staufenbiel. 2018. Evaluation of glucose dose on intravenous glucose tolerance test traits in Holstein-Friesian heifers. J Dairy Sci. 101:774-782

Hayirli, A. 2006. The role of exogenous insulin in the complex of hepatic lipidosis and ketosis associated with insulin resistance phenomenon in postpartum dairy cattle. Vet Res Commun. 30:749-74.

Holtenius, K, S. Agenäs, C. Delavaud, and Y. Chilliard, 2003. Effects of feeding intensity during the dry period. 2. Metabolic and hormonal responses. J Dairy Sci. 86:883-891.

Hosseini, A., M.R. Tariq, F. Trindade da Rosa , J. Kesser, Z. Iqbal, O. Mora, H. Sauerwein, J.K. Drackley, E. Trevisi, and J.J. Loor. 2015. Insulin Sensitivity in Adipose and Skeletal Muscle Tissue of Dairy Cows in Response to Dietary Energy Level and 2,4-Thiazolidinedione (TZD). PLoS One. 10:e0142633. doi: 10.1371/journal.pone.0142633.

Kahn, C. R. 1978. Insulin resistance, insulin insensitivity, and insulin unresponsiveness: a necessary distinction. Metabolism. 27 (Suppl 2):1893-1902.

Laarveld, B., D. A. Christensen, and R. P. Brockman. 1981. The effect of insulin on net metabolism of glucose and amino acids by the bovine mammary gland. Endocrinology 108: 2217-2221.

Marett, L. C., M. J. Auldist, P. J. Moate, W. J Wales, K . L. Macmillan, F. R. Dunshea, and B. J. Leury. 2014. Response of plasma glucose, insulin, and nonesterified fatty acids to intravenous glucose tolerance tests in dairy cows during a 670-day lactation. J Dairy Sci.;98:179.-189.

National Research Council. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC

Oliveira, L. H., A. B. Nascimento, P. L. J. Monteiro, M. M. Guardieiro, M. C. Wiltbank, and R. Sartori. 2016. Development of insulin resistance in dairy cows by 150 days of lactation does not alter oocyte quality in smaller follicles. J. Dairy Sci. 99:9174–9183.

Pires J. A., A. H. Souza, and R. R. Grummer. 2007. Induction of hyperlipidemia by intravenous infusion of tallow emulsion causes insulin resistance in Holstein cows. J. Dairy Sci. 90:2735–2744.

Roche, J. R., A. J. Sheahan, L. M. Chagas and R. C. Boston. 2008. Short communication: Change in plasma ghrelin in dairy cows following an intravenous glucose challenge. J. Dairy Sci. 91:1005–1010.

Sano, H., S. Narahara, T. Kondo, A. Takahashi, and Y. Terashima. 1993. Insulin responsiveness to glucose and tissue responsiveness to insulin during lactation in dairy cows. Dom. Anim. Endo. 10:191-197.

Singh B. and A. Saxena. 2010. Surrogate markers of insulin resistance: A review. World J Diabetes. 15: 36-47.

Stämpfli, H. and O. Oliver-Espinosa. 2015. Clinical chemistry tests. Pages 350-373 in Large Animal Internal Medicine (5th ed.), B.P. Smith (Ed.), Elsevier Mosby, St. Louis, MO.

Subiyatno, A., D. N. Mowat, W. Z. Yang. 1996. Metabolite and hormonal responses to glucose or propionate infusions in periparturient dairy cows supplemented with chromium. J Dairy Sci. 79:1436-1445.

Wheelock, J. B., R. P. Rhoads, M. J. Vanbaale, S. R. Sanders, and L. H. Baumgard. 2010. Effects of heat stress on energetic metabolism in lactating Holstein cows. J Dairy Sci. 93:644-655.

Table 0-1 Effect of stage of lactation and glucose load during intravenous glucose tolerance test on mean DMI, body weight, daily milk yield, and milk composition

	Treatment[1] Glucose dose g/kg BW									
	0.092	0.15	0.3	0.092	0.15	0.3		P Value		
Item	Early lactation			Mid lactation			SEM	Dose[2]	Stage[3]	Stage x Dose[4]
DMI, kg/d	22.2	22.9	21.9	21.5	22.0	22.0	2.03	0.95	0.77	0.96
Milk yield, kg/d	39.5	39.4	40.3	32.4	36.23	33.6	3.18	0.83	<0.05	0.8
Fat, %	3.17	2.80	3.09	3.84	3.85	3.82	0.319	0.83	<0.05	0.8
Fat yield, kg/d	1.31	1.07	1.27	1.28	1.36	1.35	0.192	0.85	0.42	0.68
Protein, %	2.51	2.30	2.49	2.92	2.87	2.96	0.210	0.74	<0.05	0.91
Protein, kg/d	1.04	0.88	1.02	0.98	1.00	1.05	0.157	0.8	0.79	0.82
Lactose, %	4.45	4.10	4.42	4.87	4.78	4.82	0.321	0.72	0.05	0.86
Lactose, kg/d	1.84	1.55	1.81	1.64	1.67	1.72	0.268	0.81	0.77	0.81

[1]Treatments: Glucose dose of 0.092, 0.15, or 0.3 g glucose/kg body weight during IVGTT during early and mid-lactation.
[2]Main effect of glucose dose.
[3]Stage of lactation effect
[4]Stage of lactation x treatment effect
5

Table 0-2 Effect of stage of lactation and glucose dose during intravenous glucose tolerance test on plasma glucose, insulin, and nonesterified fatty acid kinetics

	Treatment[1] Glucose dose g/kg BW									
	0.092	0.15	0.3	0.092	0.15	0.3		P Value		
Item	Early lactation			Mid lactation			SEM	Dose[2]	Stage[3]	Stage x Dose[4]
Glucose										
Basal (mM)	3.90	3.86	3.66	3.74	3.48	3.83	0.16	0.67	0.35	0.27
Maximum (mM)	6.20	8.31	12.57	7.64	9.44	13.87	0.49	<0.05	<0.05	0.95
CR 5 to 60 (%/min)	0.95	1.40	2.19	1.25	1.61	2.07	2.19	< 0.05	0.15	0.14
Half life 5 to 60	74.35[a]	50.32[a]	31.87[a]	57.49[b]	44.22[bc]	36.04[c]	3.82	<0.05	0.06	<0.05
AUC 0 to 60 (mM/min)	84.66	102.54	143.49	94.90	114.28	158.52	4.63	< 0.05	<0.05	0.87
Insulin										
Basal (ng/mL)	0.13	0.07	0.10	0.13	0.05	0.11	0.02	0.07	0.80	0.87
Maximum (ng/mL)	1.38	1.78	2.11	1.60	1.34	1.86	0.28	0.20	0.52	0.50
CR 5 to 60 (%/min)	6.07	4.97	4.25	4.92	3.83	2.87	0.98	0.17	0.14	0.99
Half life, 5 to 60 min	16.15	15.76	18.20	14.66	21.84	31.62	3.76	0.06	0.06	0.16
AUC 0 to 60 (ng/min)	28.04	42.23	67.27	31.60	37.17	67.98	8.96	<0.05	0.97	0.88
NEFA										
Basal (mM)	0.27	0.22	0.27	0.21	0.18	0.20	0.27	0.49	0.09	0.91
Minimum (mM)	0.14	0.13	0.10	0.12	0.10	0.09	0.01	<0.05	0.12	0.79
Maximum (mM)	0.49	0.44	0.35	0.27	0.24	0.24	0.06	0.45	<0.05	0.67
AUC, 0 to 60 (mM/min)	4.41	3.56	4.25	3.15	3.02	2.92	0.57	0.69	<0.05	0.74
AUC, 0 to 120 (mM/min)	34.84	32.63	24.28	25.05	20.05	18.71	3.79	0.11	<0.05	0.65
CR, 5 to 30 (%/min)	1.89	2.06	3.52	2.38	2.06	2.18	0.49	0.06	0.14	0.41
CR, 30 to 120 (%/min)	-0.90	-1.16	-1.19	-0.73	-0.54	-0.82	0.18	0.53	<0.05	0.46
Half life, 5 to 30 min	86.02	34.77	37.66	74.18	101.81	39.86	32.18	0.43	0.19	0.57
Half life, 30 to 120 min	-22.51	-61.76	-65.65	-105.69	-58.30	-122.56	41.37	0.67	0.19	0.57
Ratio of AUC 0 to 60 min										
Glucose : Insulin	3.09	2.73	2.27	3.77	3.30	2.71	0.47	0.15	0.15	0.96
Glucose : NEFA	22.86	29.23	37.43	35.54	41.78	58.51	4.85	<0.05	<0.05	0.61
Insulin : NEFA	7.84	12.64	19.24	12.31	13.63	28.53	5.49	0.26	0.58	0.75

[1]Treatments: Glucose dose of 0.092, 0.15, or 0.3 g glucose/kg body weight during IVGTT during early and mid-lactation.
[2]Main effect of glucose dose.
[3]Stage of lactation effect
[4]Stage of lactation x treatment effect
[a,] Means with different superscripts differ P < 0.05

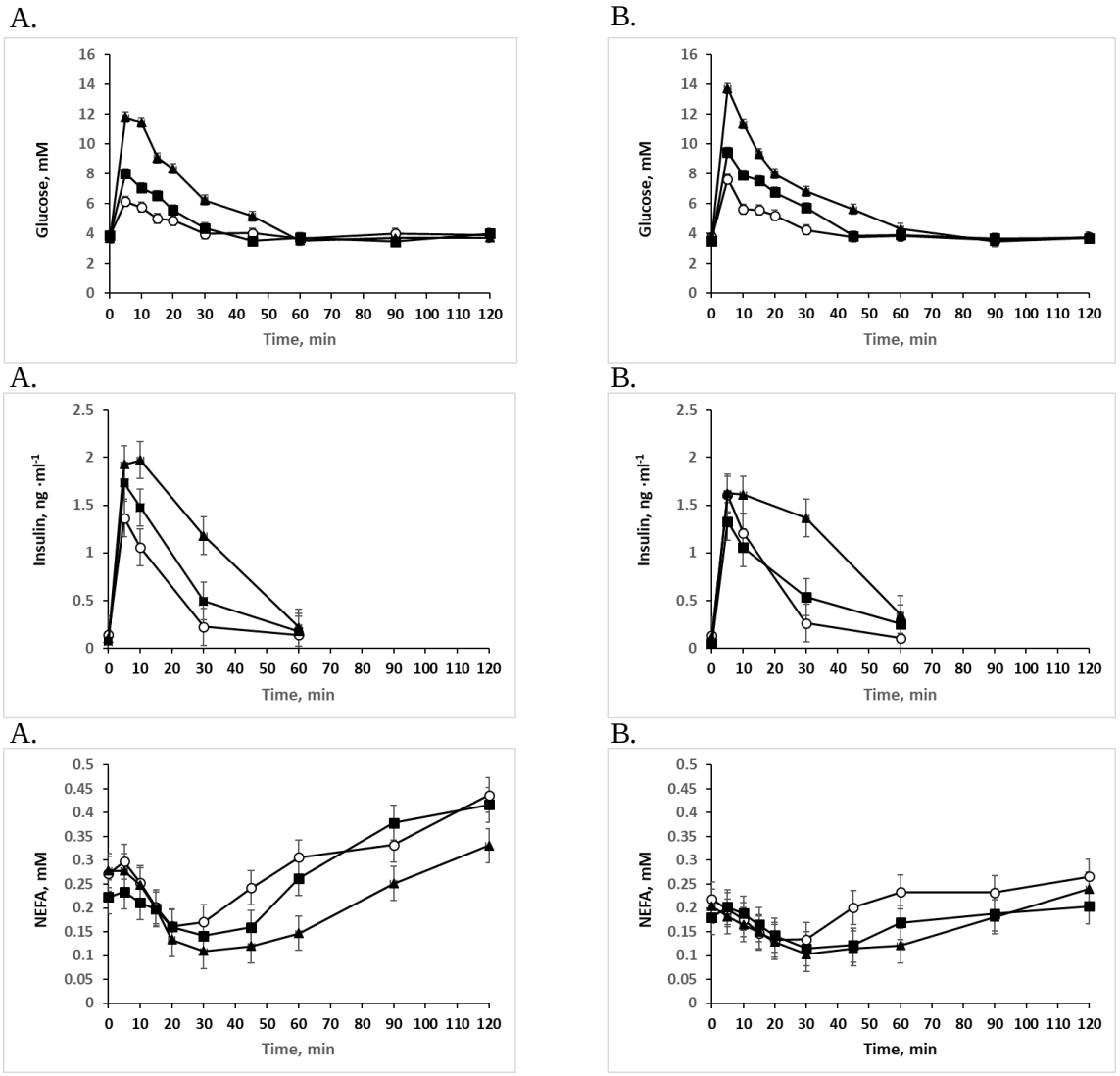

Figure 0-1 Plasma glucose, insulin and nonesterified fatty acids in response to glucose dose of 0.092 g glucose/kg BW (○), 0.15 g glucose/kg BW (■) or 0.3 g glucose/kg BW (▲) during IVGTT during early (A panels) and mid-lactation (B panels). Data are least-squares means and standard errors.