The Effect of Heat Stress and Essential Amino Acids on Production and Metabolism of Lactating Dairy Cattle

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DEDICATION

Dedicated in memory of Nicole Bangart, one of the brightest and most beautiful people inside and out I have ever had the pleasure of knowing. Also, I dedicate this thesis to my Mother who always pushed me to become the best I could be, to Cody Schmidt for being my rock to lean on, and to my dog Lucy for being my companion.

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ABSTRACT

Heat stress (**HS**) causes decreases in production of lactating cattle that is only partially explained by decreases in intake. Evidence suggests that changes in energy and protein metabolism occur to cope with the impact of HS. The objective of this thesis was to determine if the effect of jugular infusion of essential amino acids (**AA**) ameliorated the negative effects of HS in milk production and metabolism. Twelve multiparous lactating Holstein cows were used in a crossover design to evaluate the effect of thermoneutral (**THN**) and HS environments along with the absence (**CTL**) or presence (**ML+BCAA**) of essential AA infusion. Infusions consisted of methionine (12 g), lysine (21 g), leucine (35 g), isoleucine (15 g), and valine (15 g) per day. Thermal treatments were imposed from days 1 to 14 and jugular infusion of AA from days 7 to 14. Milk and blood samples were collected on days 5 to 7 and 12 to 14. Data were analyzed using the Mixed procedure of SAS and reported as least square means ± [plus or minus] standard error of the mean. Temperature humidity index (**THI**) values during THN never exceeded 66, whereas THI values during HS peaked at 76 and were above 68 for 14 h/d. Compared with the CTL treatment, ML+BCAA treatment increased rectal and vaginal temperatures in the HS treatment by 0.5 and 0.4°C respectively, but did not increase temperatures in the THN treatment (interaction $P < 0.05$). Heat stress decreased ($P < 0.05$) DMI (17.4 vs 18.9±0.41 kg/d), milk yield (29.3 vs 32.1±1.09 kg/d), milk protein percentage (2.95 vs 3.06±0.06%), and milk protein yield (0.87 vs 0.98 ±0.05 kg/d). The ML+BCAA treatment had no effect on milk and milk protein yield but increased ($P <0.001$) milk protein percent (3.04 vs 2.96±0.06%). Heat stress elicited expected

decreases in production, while the infusion of AA increased milk protein percent indicating a possible improvement of protein synthesis. However, the rise in rectal temperatures due to infusion is a cause for concern.

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CHAPTER I: LITERATURE REVIEW

Introduction

In the livestock industry improving N efficiency, the conversion of dietary N into a protein product, is a vital objective in sustainable agriculture. Ruminants, particularly lactating dairy cattle, are not efficient at converting dietary N into saleable products. Dairy cattle convert only 25-30% of dietary N into milk protein (Bequette et al., 1998), which leaves an average of 72% of the N intake to be excreted in waste (Castillo et al., 2000). Although the low N efficiency is an issue by itself, environmental factors such as heat stress (**HS**) can compound this issue.

Nitrogen efficiency declines during periods of HS through increases in N excretion via urine and decreases in milk production and milk protein synthesis (Schneider et al., 1988; Rhoads et al., 2009; Wheelock et al., 2010; Bernabucci et al., 2015). A lower intake is partially responsible for this loss in production, however, intake accounts for only 50% of the loss (Rhoads et al., 2009; Wheelock et al., 2010). The unexplained portion could possibly be due to changes in metabolism that are present during HS. The catabolism of lean tissue and the production of urea consistently increase in dairy cattle exposed to HS (Schneider et al., 1988; Kamiya et al., 2006; Shwartz et al., 2009). The absorption of urea in the gut is reduced in HS cattle (Obitsu et al., 2011); thus, the increase in urinary urea is associated with the breakdown of muscle tissue. In order to reduce catabolism of proteins and improve production of HS cows, the implementation of different nutritional management strategies should be investigated.

Infusions of the AA Met, Lys, and the branched-chain amino acids (**BCAA**) in lactating dairy cattle increased milk protein percent and decreased urea N excretion

through milk urea nitrogen (Rulquin and Pisulewski, 2006; Appuhamy et al., 2011). Armentano et al. (1997) reported a 4 g increase in milk protein yield for every supplemented g of AA. These studies were conducted under thermoneutral (**THN**) conditions, however, AA supplementation has the potential to elicit similar effects under HS conditions (Chen et al., 1993; Huber et al., 1994). Therefore, the main objective of this research is to investigate the use of M8et, Lys, and BCAA to improve production and metabolism of lactating dairy cattle experiencing HS.

Essential Amino Acids in Lactating Cattle

Nitrogen Efficiency and Amino Acids

Cattle inefficiency to convert dietary N into milk protein can be illustrated by the assumed constant (0.67; NRC, 1989) for the efficiency of metabolizable protein. Using the efficiency of metabolizable protein constant, Rius et al. (2010) calculated that for every gram of milk protein produced 1.5 g of metabolizable protein is required. When compared to other livestock species, dairy cattle are among the least efficient at converting dietary N into protein products. A lactating sow is 6% more efficient in milk protein production than a lactating cow (Huber et al., 2015). A growing broiler is 19% more efficient in producing consumer protein products than the lactating cow (North and Bell, 1990). The increasing concern of public on agricultural practices environmental impact mandates improvements in N efficiency.

Implementing diet manipulations may correct poor N inefficiency in cattle. Low crude protein (**CP**) diets and lower than NRC (2001) recommended levels of rumen undegraded protein (**RUP**) have both shown the potential to improve N efficiency

(Broderick, 2003; Cyriac et al., 2008). In addition to manipulating the protein fractions of the diet, feeding rumen-protected Met in a low CP diet with a highly digestible RUP fraction was 3.3% more efficient compared with diets lacking Met supplementation (Noftsger and St-Pierre, 2003). Amino acids have the potential to improve N efficiency by feeding the exact profile necessary because when excess an AA is fed it is deaminated and N is excreted in waste.

Amino Acid Requirements of Lactating Dairy Cattle

All animals require certain AA in their diet. These AA are classified as essential because the animal is unable to synthesize these AA themselves. The essential AA for ruminants include Met, Lys, Arg, His, Ile, Leu, Thr, Trp, Phe and Val. Ruminants do not receive all essential AA through their diet like monogastric animals. However, the RUP fraction of a ruminant's diet is similar to the protein in monogastric diets in that it undergoes gastric digestion and absorption in the small intestine. The RUP fraction of the diet is not the only source of AA that are absorbed in the small intestine. A large contributor to absorbed AA is the protein that is synthesized by the rumen microbe population, which is known as microbial CP.

Microbial CP synthesis complicates the ability to quantify the exact AA requirements for ruminants. Mathematical modeling has been implemented in order to estimate these requirements. The Cornell Net Carbohydrate and Protein System has been a widely adopted model in the dairy industry to help predict the AA requirements of ruminants. This model uses dry matter intake (**DMI**), the composition of AA supplied by the diet, and the metabolizable protein requirements to predict the flow of AA to the

small intestine. The predicted AA flow is combined with tissue and milk composition to determine the essential AA requirement for a ruminant (Fox and Tedeschi, 2003).

The complexities of predicting the essential AA requirements due to the contribution of the rumen microbial population presents challenges when researching specific effects of an AA on the performance of lactating ruminants. Often the infusion of AA or protein through the abomasum or jugular vein is used in research because these infusions bypass the rumen (Rulquin and Pisulewski, 2006; Appuhamy et al., 2011). In the case of the jugular infusion, it allows AA to bypass the absorption process in the small intestine and increases milk yield 0.8 kg/d more than abomasal infusions (Thivierge et al., 2002). These methods are most often used when investigating protein metabolism in the peripheral tissues.

Methionine and Lysine

Although the ruminant requirements for essential AA have not been clearly defined, Met and Lys are considered to be the first most limiting AA in the dairy cattle (NRC, 2001). Use of common feedstuffs used in dairy cattle diets lead to deficiencies in Met (corn, soybeans, and animal byproducts) and Lys (corn; NRC, 2001). Although predicting the exact requirements of AA is a challenge in ruminants, the authors of the NRC (2001) used mathematical models and data from several studies to determine the estimated requirement of 2.4% of metabolizable protein for Met and 7.2% of metabolizable protein for Lys. Determining the supply of metabolizable protein based on diets is not easily predictable due to the interference of the rumen microbial population.

Multiple studies have reported the positive effect of Met and Lys supplementation on milk production (Robinson, 2010). The supplementation of Met in total mixed ration (**TMR**) consisting of alfalfa, corn silage, and soybeans increased milk production by 4 g for every g of Met supplemented (Armentano et al., 1997). Additionally, jugular infusions of Met and Lys increased milk protein yield by 0.13 kg/d (Appuhamy et al., 2011). These results indicate that diets commonly fed to lactating cattle are lacking in Lys and Met. Thus, supplementation may be a beneficial solution to increase milk protein output.

The function of Met is not limited to protein synthesis alone. It is also important in the synthesis of the antioxidant glutathione. Glutathione is a tripeptide consisting of the AA cysteine, glycine, and glutamate, and it functions to protect the cell from oxidative damage by inactivating free radicals through hydrogen donation (Donovan, 2007). Methionine is not one of the three AA that make up glutathione, however, the *in vitro* addition of Met to hepatocyte culture media increases the amount of glutathione two-fold compared to freshly harvested hepatocytes (Wang et al., 1997). Methionine contributes to the increase in glutathione production because Met can be interconverted into the AA cysteine (Donovan, 2007).

In addition to contributing to antioxidant production in the body, methionine also acts as a methyl donor. Methionine along with adenosine triphosphate (**ATP**) form the universal methyl donor S-adenosyl methionine (**SAM**) through an enzymatic reaction (Donovan, 2007). S-adenosyl methionine can contribute to the *de novo* synthesis of choline, which plays a significant role in increasing milk fat (Pinotti et al., 2002). The

supplementation of 20 g/d of rumen-protected methionine increased milk fat yield by 1.76 kg/d (Pinotti et al., 2003). Choline is highly degraded in the rumen, therefore, the *de novo* synthesis from SAM helps maintain milk fat even if small amounts of choline are available in the diet (Dawson et al., 1981; Pinotti et al., 2002).

Branched-Chain Amino Acids

Leucine, isoleucine, and valine are classified as BCAA based on their branched side chain containing a central carbon with two to three additional carbons attached. BCAA are critical for the synthesis of milk protein production. When the AA content of dairy milk is analyzed, BCAA make up 43% of the essential AA found in milk proteins (NRC, 2001). Furthermore, the absence of BCAA in an infusion caused the upregulation of the protein factors eIF2Bε and eIF2α, which when upregulated inactivate the mammalian target of rapamycin (**mTOR**) signaling cascade (Doelman et al., 2015). The mTOR signaling cascade regulates the translation step of protein synthesis throughout various body tissues and the mammary gland. The evident effect of BCAA signaling cascade regulation further illustrates the importance of BCAA in the milk protein synthesis.

Dietary supplemented BCAA are similarly recognized for their role in increasing muscle protein synthesis. Neonatal BCAA supplemented swine diets upregulated several protein factors involved in the regulation of protein synthesis in various muscle tissues (Escobar et al., 2006). Muscle protein synthesis is exemplified in decreased muscle soreness post-exercise in humans that consumed supplemental BCAA (Shimomura et al., 2006). The involvement of the BCAA in protein synthesis demonstrates the possibility of

BCAA as a beneficial protein supplement during situations when muscle catabolism occurs. The catabolism of most AA transpires in the liver or kidney; however, catabolic enzymes required for the breakdown of BCAA are distributed through the body. The distribution of enzymes allows for the body wide catabolism of BCAA, which initiates their release from muscle stores. Branched-chain AA can then be utilized in alternative ways, such as to produce energy (Goodwin et al., 1987).

The BCAA are decarboxylated in the rumen to formulate branched-chain volatile fatty acids: isobutyric, isovaleric, and 2-methylbutyric (Allison and Bryant, 1963, Allison, 1969, Miura et al., 1980). Branched-chain volatile fatty acids are an important substrate for rumen bacteria and have been found to increase the growth of cellulolytic bacteria (Dehority et al., 1967), which ferment cellulose in the rumen. Feed intake increased and milk production improved throughout lactation on average 2.5 kg/d when branched-chain volatile fatty acids were supplemented with ammonia salts (Hungate and Dyer, 1956, Papas et al., 1984). Since branched-chain volatile fatty acids are highly utilized by the rumen microbiome it is important to consider that if branched-chain volatile fatty acids requirements are not met in the diet that there is an adequate supply of branched-chain volatile fatty acids in the rumen degradable protein fraction of CP (Tedeschi et al., 2000).

Literature suggests that the cattle fed corn-based diets are possibly deficient in BCAA (Appuhamy et al., 2011). When Leu was infused into the duodenum at the rate of 40 g/d, milk protein yield increased by 82 g/d and milk protein percent by 0.43%, however, overall milk production did not increase (Rulquin and Pisulewski, 2006).

Although there have been reports of positive effects on milk production, BCAA infusions lack consistent increases in production similar to reports on Met and Lys infusions. Four treatments of AA mixtures, containing His and various amounts of BCAA, were infused in lactating cattle. All four infusions reported a lack in increased milk or milk protein production when compared with the no infusion control (Korhonen et al., 2002). The jugular infusion of BCAA plus Met and Lys also did not increase the milk production of cattle as compared with an infusion only containing Met and Lys (Appuhamy et al., 2011). Inconsistency between results indicate that BCAA should be considered as a second limiting AA that is highly dependent upon the diet and stage of lactation.

Ketogenic and Glucogenic Amino Acids

Leucine and Lys are exclusively ketogenic essential AA because they are converted into the ketones acetyl-CoA or acetoacetyl-CoA. Ketones are alternative energy sources that are converted to acetyl-CoA and oxidized in the mitochondrion; however, they cannot be converted to glucose. Glucogenic AA are directly converted to glucose to maintain glucose homeostasis or fully oxidized to produce energy. The essential AA that are glucogenic include His, Met, Thr, and Val. The glucogenic AA, when catabolized, will be able to yield the formation of pyruvate through the TCA cycle (D'Mello, 2003). These AA can be converted to the TCA cycle intermediate succinyl-CoA and from there follows the cycle and oxaloacetate is produced. Oxaloacetate can be converted into pyruvate, which is the beginning substrate of gluconeogenesis. This pathway through succinyl-CoA and oxaloacetate is the same pathway that used to convert

propionate, the major glucogenic precursor for ruminants, to pyruvate for gluconeogenesis (D'Mello, 2003).

Effects of Heat Stress

Heat stress occurs when an animal is unable to dissipate the heat load placed upon them by increased environmental temperature and humidity (Bernabucci et al., 2010). The temperature humidity index (**THI**) is used in order to account for both these factors. The equation for THI uses the linear relationship with humidity and temperature, increasing as the dry bulb temperature (**Tdb**) and relative humidity (**RH**) increase. The equation used for this research is taken from NRC (1971) and also reported in Dikmen and Hansen (2009) the equation is as follows:

$$THI = (1.8 \times Tdb + 32 - [(0.55 - 0.0055 \times RH) \times (1.8 \times Tdb - 26.8)]).$$

A minimum threshold THI of 72 was set in past literature to induce HS (Armstrong, 1994; Ravagnolo et al., 2000). However, since producers have been genetically selecting for higher producing animals, recent research suggests that the threshold could now be as low as THI of 68 (Zimbelman et al., 2009).

Heat stress has a large economic impact on the dairy industry that will only continue to increase in cost. Estimates have set the economic cost at 900 million dollars annually due to losses in production and fertility as well as increases in illness and mortality (St-Pierre et al., 2003). In 2010, the estimated cost was set at 1.2 billion dollars, increasing the economic cost by three million dollars in less than a decade (Key et al., 2014). Expenses from HS will only continue to rise as temperatures rise due to climate change (Key et al., 2014). The mean global surface temperature is expected to continue to

rise by 0.3-0.7°C between the years 2016 and 2035 compared with the mean global temperature between the years of 1986 and 2005 (Klimont, 2013). Besides rising global temperatures, the cost of HS will also continue to increase due to the trend of selecting higher producing animals. In the U.S. since 1950, the amount of milk produced annually per cow has increased by 16,500 lbs (Blayney, 2002). Although this achievement has been positive for the industry, HS has a consistently larger negative impact on high producing cattle (Bernabucci et al., 2010). The need to determine solutions for ameliorating the negative effects of HS in cattle will be vital to the success of the dairy industry in the future.

Rumen Health Effects

Dairy cattle respond to HS by increasing panting, sweating, and vasodilation to increase blood flow to the skin. These changes in physiology attempt to reduce the body temperature of the animals (West, 1999). Cattle experiencing HS will on average increase respiration rates by 50 breaths/min and rectal temperatures by 2.06°C compared to their THN counterparts (West et al., 2003; Rhoads et al., 2009; Wheelock et al., 2010). When responses such as sweating and increased respiration rates occur to induce evaporative cooling, a 17% increase in water consumption occurs (Schneider et al., 1988). Responses that induce evaporative cooling alter the blood buffering system, which can have serious impacts on the health of cattle.

Changes in respiration rates can alter the blood acid-base chemistry. Panting increases the loss of CO_2 which reduces the blood concentrations of carbonic acid and increase the blood pH resulting in respiratory alkalosis (Costanzo, 2014). Compensation

for the respiratory alkalosis involves an increase in excretion of urinary HCO_3-. The reduction in HCO_3- concentration in the blood will reduce the amount present in the saliva. The main buffering component of ruminant saliva is HCO_3- and by reducing the concentration of HCO_3- the buffering capacity of saliva is also reduced (Collier et al., 1982; Kadzere et al., 2002). The amount of saliva entering the rumen is also reduced through less ruminating. For example, a negative correlation ($r = -0.32$; $P < 0.05$) was reported between rumination and max THI (Soriani et al., 2013). The reduction of saliva produced and quality of buffer will lead to decreases in rumen pH. The decrease in rumen pH is a serious consequence for rumen health causing acidosis (Church, 1988).

Milk Production Effects

A large portion of the cost attributed to HS results from losses in milk production. During periods of HS decreases in milk production by as much as 35% occurs (Bernabucci et al., 2010). These losses will continue to increase in the future due to the increases in global temperature (Key et al., 2014). Milk production has an inverse relationship with THI where milk production decreases as THI increases. Ravagnolo et al. (2000) reported that starting at a THI of 72, every unit increase in THI caused a decrease in milk production of 0.2 kg. This reduction in milk volume is a product of the changes in HS cattle intake and physiology.

Heat stress not only decreases the total volume of milk produced by lactating cattle, it also decreases milk component production, such as milk protein. When milk protein percent was measured over a period of 10 d, it was reported that there was a significant decrease in milk protein percent through time for the HS cattle (Rhoads et al.,

2009). Furthermore, at a THI greater than 68 milk protein percent was reported to decrease 0.1% in Holstein cattle (Smith et al., 2013). In addition Wheelock et al., 2010 reported a similar decrease of 0.3% in milk protein percentage due to HS (Wheelock et al., 2010). Analogous with reduction in overall production, protein production results from physiological coping mechanisms put into place due to HS.

In addition to milk protein, lactose is affected because of HS conditions. When dairy cattle are experiencing HS, they exhibit decreases in lactose yields. Lactose yield is lower by 200-400 g/d in HS cattle when compared with their pair-fed THN counterparts (Rhoads et al., 2009; Wheelock et al., 2010; Baumgard and Rhoads, 2013). Decreases in lactose are likely due to reductions in intake, thus reducing the amount of glucose available to produce lactose. Interestingly, decreases in lactose will cause reductions in the volume of milk produced because lactose is the main osmotic factor in the mammary gland (Kronfeld, 1982; Zhao, 2014). These decreases in milk components can reduce the value of the product produced.

Although lactose and protein yields decrease due to HS, milk fat production does not have the same results. Heat stress conditions have not had consistently altered milk fat. Several studies have reported that HS did not significantly affect milk fat percentage (Rhoads et al., 2009; Wheelock et al., 2010) and in fact, there have been increases in milk fat percentages of 0.4% reported (Smith et al., 2013). These differences in component production may be reflected in the metabolism changes of HS cattle, specifically the energy source utilized by the body during HS.

Heat Stress and Energy Metabolism

Nutrient Intake

Intake of nutrients dramatically changes under stress-inducing conditions, such as high environmental temperatures. During periods of HS, dairy cattle can experience a decrease in DMI by as much as 29% (Shwartz et al., 2009). Intake most likely decreases due to metabolic heat production that occurs during digestion. By decreasing intake, cattle are able to partially protect against increasing body temperature (West, 1994). In addition to metabolic heat production, ruminants also produce heat during fermentation of feed which further would increase body temperature due to intake (Beede and Collier, 1986). These decreases in intake can have severe implications, such as causing various metabolic disorders.

Dairy cattle under HS not only decrease their overall intake, but also they change their feeding patterns. A cow under HS will reduce the amount of meals consumed per day to around 3 to 5 meals instead of 12 to 15 meals under THN conditions (Bernabucci et al., 2010). The quantity of feed consumed during HS will tend to be greater than the typical meal (Bernabucci et al., 2010). Over consumption of feed can cause decreases in rumen pH, which causes acidosis. Acidosis negatively affects the cellulolytic bacteria population, the main fermenters of forage in the rumen. When pH levels are below 6.0 these bacteria are not as active and therefore, a decrease in the amount of feed fermented occurs (Russell and Wilson, 1996).

Heat stress causes a negative energy balance in lactating cattle. Negative energy balance occurs when an animal's input of energy does not meet the body's demand for

energy output. In the instance of HS cattle, the combination of decreases in nutrient intake and increases in the amount of energy required for maintenance results in Negative energy balance (NRC, 1989, Shwartz et al., 2009). The increase in maintenance requirements for cattle experiencing HS has been estimated to be up to 30% (Fox and Tylutki, 1998). The increase in energy for maintenance energy is utilized by HS cattle to sustain their body temperature in a normal range. Consequently, this leads to the diversion of energy to coping mechanisms, resulting in decreases milk production.

Diet manipulation has shown promise in reducing the effect of HS animals. One such area that has been investigated in swine is the reduction of CP in the diet. Multiple studies have found that reducing the CP in the diet to around 14% resulted in increases in performance of HS lactating swine due to reduced N excretion and increased average daily feed intake (Renaudeau et al., 2001, Silva et al., 2009). In dairy cattle the supplementation of rumen protected γ-aminobutyric acid (**GABA**) at 2.4 g/d increased the DMI of cattle by 0.8 kg/d (Wang et al., 2013). As a neurotransmitter, GABA controls body temperature regulation and at increased levels will reduce overall body temperature (Yakimova et al., 1996, Frosini et al., 2000). This effect has also been reported in HS cattle where increasing levels of GABA by 40, 80, and 120 g/kg of DM in the diet reduced body temperature throughout the day by 0.18, 0.22, and 0.09°C respectively (Cheng et al., 2014). The decrease in rectal temperature may allow for the increase in DMI due to there being a lower metabolic heat.

Decreased intake that results from HS cannot fully explain decreased milk production of lactating cattle. Two studies, Rhoads et al. (2009) and Wheelock et al.

(2010), exposed one group of cows to HS and measured their intake, and fed the second group of cows the same amount the HS group had consumed. The cows under HS decreased their milk production 10.6 kg/d in the first study and 9.56 kg/d in the second study. The pair fed cows only decreased their production 5 kg/d and 4.79 kg/d. This results in only 50% reduction in production explained by decreases in nutrient intake (Rhoads et al., 2009; Wheelock et al., 2010). The other 50% is not clearly defined but evidence suggests that it can possibly be attributed to changes in energy and protein metabolism. It is possible to explore some of these changes in metabolism through the analysis of blood metabolites.

Non-Esterified Fatty Acids

When animals are experiencing negative energy balance, they are apt to mobilize adipose tissue for energy through. Negative energy balance occurs in early lactation cattle due to their inability to consume enough energy to meet their production demands and will often mobilize their adipose tissue for energy in the form of non-esterified fatty acids (**NEFA**). When circulating NEFA levels are compared between the week before parturition and the week after, there is a three-fold increase (Murondoti et al., 2004). The response for HS cattle, however, is opposite of what is seen in animals that are experiencing other forms of negative energy balance. Plasma concentrations of NEFA have not shown increases in HS cattle (Wheelock et al., 2010). Interestingly, when THN cows are pair fed to match HS cattle there is a 120% increase in basal NEFA levels (Rhoads et al., 2009). The absence of an increase in NEFA levels suggests that HS

animals are not mobilizing their fat stores and are using another source, possibly protein stores in muscle, in order to correct for negative energy balance.

Glucose

Decreases in basal glucose levels of HS has been observed to be as much as 11% (Wheelock et al., 2010). The decreases in nutrient intake coupled with the higher demand of energy exhibited by HS cattle will decrease the concentrations of plasma glucose. A portion of this decrease in plasma glucose is possibly due to the decrease in volatile fatty acids (**VFA**) produced by cattle experiencing HS. Overall production of VFA decreased from 119 mmol/L under THN to 108.2 mmol/L in cattle kept in a HS chamber (Schneider et al., 1988). The reduction in DMI that HS cattle exhibit can partially explain the decrease in VFA production. However, when orts from cannulated cattle exposed to differing levels of temperature were placed into the rumen to eliminate differences in intake, VFA concentrations continued to decrease from the lowest temperature treatment to the highest temperature treatment at a level of 153.05 to 66.27 mmol/L (Kelley et al., 1967). Another study also illustrated this same point, at a temperature of 35°C, rumen concentration of acetate and propionate decreased compared to cattle that were housed at 18°C despite similar DMI (Gengler et al., 1970). Since propionate is the major precursor for glucose in ruminants, decreases in propionate cause decreases in glucose levels (Church, 1988).

Insulin

Heat stress cattle have exhibited increases in plasma insulin concentrations, which may explain the reduction in glucose plasma concentrations due to increased maintenance requirements, tissues may be more apt to uptake higher levels of glucose. Basal insulin level increases in HS cattle have been reported to between 29-37% (Itoh et al., 1998; Wheelock et al., 2010). Whole body insulin sensitivity has been reported to increase due to HS in a porcine model and in Holstein bull calves (O'Brien et al., 2010; Sanz Fernandez et al., 2015). In lactating cattle the response to glucose tolerance tests have not been consistent to show increased sensitivity (Baumgard and Rhoads, 2013), however it has been reported that lactating cattle exhibited a larger increase ($P < 0.05$) in insulin in response to glucose compared with cattle that were pair fed and housed in THN conditions (Wheelock et al., 2010). The increase in insulin due to administering glucose indicates an increase in pancreatic sensitivity. The increased pancreatic sensitivity may be a metabolic coping mechanism to deal with increased energy demands by trying to maximize glucose uptake into the tissues.

β-Hydroxybutyric Acid

Negative energy balance in animals will usually lead to an elevated level of ketones in the blood. Ketones originate from the incomplete oxidation of fatty acids in the liver. β-hydroxybutyric acid (**BHBA**) is the ketone most often measured in the blood due to its stability (Oetzel, 2007). Lactating HS cattle do not show increases levels BHBA compared to THN cows (Lamp et al., 2015). The absence of an increase in BHBA is expected because circulating NEFA levels are related to ketone levels and as

mentioned previously, NEFA levels do not increase during HS. In addition to ketones coming from incomplete oxidation of fatty acids, they can also originate from butyrate, a VFA produced in the rumen. Heat stress reduces the amount of VFA produced in cattle (Kelley et al., 1967; Schneider et al., 1988) thus, the absence of increase in BHBA follows what has been reported for VFA production. The lack of increased ketone levels further indicates that mobilizing adipose is not the preferred source of energy for HS cattle.

Heat Stress and Protein Metabolism

Changes in Nitrogen Efficiency

In addition to the changes in carbohydrate and lipid metabolism, changes in protein metabolism due to HS may contribute to a portion of the production loss not accounted for by intake reduction. Dairy cattle are not efficient at converting dietary N to protein product under THN conditions; however, HS further compounds this issue. Urinary N excretion increased by 14% for cattle under HS (Obitsu et al., 2011). Milk protein fractions decreased by 23% in α_s-casein and 19% in β-casein concentrations during summer months when compared with production in the winter months (Bernabucci et al., 2015). These factions of protein make up a large portion of the milk protein produced. The combination of the decrease in milk protein production and increased N excretion indicates decreases in N efficiency.

Muscle Catabolism

The increase in N excretion is not the sole change in protein metabolism of HS animals, muscle protein changes also occur. These changes are evident in the decrease of carcass quality traits of livestock species such as swine. In swine that are experiencing HS, there are reductions in protein accretion and increases in adipose deposition, which are found through decreases in muscle mass of carcass and increases in back-fat thickness (Baumgard and Rhoads, 2013). The reduction in protein muscle mass supports the notion that muscle is being catabolized for energy while adipose tissue is not.

Further evidence found in blood metabolites supports the occurrence of muscle catabolism in HS cattle. Increases of 47-70% in plasma urea N and increases of 13.2% in plasma creatinine occurred compared to cows housed in THN conditions (Schneider et al., 1988; Shwartz et al., 2009; Wheelock et al., 2010). Although increases in plasma urea N may be due to increased circulating urea from the liver or muscle catabolism, increased creatinine indicates muscle catabolism. The observed increase level of plasma urea N and plasma creatinine can be a result of elevated liver metabolism of skeletal muscle AA through deamination. The deamination of AA produces carbon skeletons that can be used to produce various intermediates of the TCA, thereby contributing to energy production in the body. This may happen in an attempt to offset the negative energy balance created by HS conditions.

Conclusion

Literature has established that the supplementation of certain AA (Lys, Met, BCAA) in a ruminant diet can increase milk yield and milk protein production. This may

also be true for cattle experiencing HS. Feeding a diet with a high content of Lys improved milk production by 9% in HS cattle (Chen et al., 1993; Huber et al., 1994). The ability of Met to increase the antioxidant glutathione would benefit HS cattle due to the increased level of oxidative stress that they experience (Wang et al., 1997; Bernabucci et al., 2002; Chauhan et al., 2016). Furthermore, BCAA may also be a viable option for supplementation due to their importance in muscle metabolism and the evidence of muscle catabolism during HS (Schneider et al., 1988; Escobar et al., 2006; Wheelock et al., 2010). The purpose of this thesis is to investigate whether the infusion of the essential AA (Met, Lys, and BCAA) will benefit lactating dairy cattle exposed to HS.

CHAPTER II: THE EFFECT OF HEAT STRESS AND JUGULAR INFUSIONS OF METHIONINE, LYSINE, AND BRANCHED-CHAIN AMINO ACIDS IN LACTATING CATTLE

Abstract

Reduction in milk protein is a negative consequence of heat stress (**HS**) that may be alleviated by changes in supply of amino acid supply. The objective of this study was to assess the effect of infusing jugular infusions of an amino acid mixture on milk production in lactating dairy cattle exposed to short-term HS conditions. Twelve multiparous lactating Holstein cows were assigned to two environments (thermoneutral [**THN**] or HS) from d 1 to 14 and two levels of amino acid infusion (**control** (0 g) or **ML+BCAA** [L-methionine (12 g), L-lysine (21 g), L-leucine (35 g), L-isoleucine (15 g), and L-valine (15 g) per d]) from d 7 to 14 of the study in a crossover design. The common basal diet and the ML+BCAA treatment provided 99.8% and 104.9 % of estimated metabolizable protein requirements. Temperature humidity index (**THI**) did not exceed 66 in the THN treatment, whereas THI peaked at 76 and were above 68 for 14 h/d in the HS treatment. Milk and blood samples were collected on d 5 to 7 and 12 to 14. Cows exposed to HS had lower milk production (2.82 kg/d) and dry matter intake (1.48 kg/d) compared with cows exposed to THN. The milk protein yield decreased by 0.11 kg/d in the HS treatment but it did not change in the ML+BCAA treatment. However, milk protein percent increased by 0.08% in the ML+BCAA treatment. For the THN environment, cows receiving the ML+BCAA treatment had greater concentrations of milk urea nitrogen compared with those receiving the control treatment, but it was not affected by amino acids in the HS environment . For the HS environment, cows receiving the ML+BCAA treatment had greater rectal temperature (0.5°C) and vaginal temperature (0.4°C) compared with those receiving the control treatment, but rectal and vaginal

temperature was not affected by amino acids in the THN environment. In summary, HS elicited expected changes in production; however, infusions of ML+BCAA did not reduce its negative impact in milk and milk protein yield. The changes in body temperature in response to infusion of ML+BCAA in heat-stressed cows may contribute to the lack of response in milk production.

Introduction

Heat stress (**HS**) continues to negatively affect the dairy industry resulting in an estimated cost of 1.2 billion dollars (Key et al., 2014). Although cooling systems (i.e. fans and sprinklers) have helped to ameliorate production losses in hot months, nutritional strategies should also be developed to alleviate the negative effect of HS in dairy cattle production. Nutritional management should be investigated particularly because feed intake only accounts for 50% of the HS-induced losses in production (Wheelock et al., 2010). Although a portion of production losses remains unexplained, feed intake-independent shifts in glucose, lipid, and AA metabolism have been reported (Schneider et al., 1988; McGuire et al., 1989; Wheelock et al., 2010).

In addition to the reductions in milk yield, the synthesis of casein, the percentage of milk protein, and the yield of milk protein decline in HS cows (Bernabucci et al., 2002; Wheelock et al., 2010). Conversely, the catabolism of protein and AA increases in HS cows as indicated, in part, by the increased concentration of plasma urea N (Wheelock et al., 2010). Plasma urea nitrogen originates primarily from inefficient conversion of rumen ammonia into microbial protein or degradation of muscle protein (McGuire et al., 1989). Plasma creatinine and 3-methyl histidine are specific biomarkers

of muscle degradation, both of which are elevated in HS cows, further indicating muscle catabolism (Schneider et al 1988; Kamiya et al., 2006). Provision of essential amino acids (**EAA**) reduces muscle catabolism and increases protein synthesis in metabolically active tissues which is expected to benefit cows exposed to HS.

The infusion of Met, Lys, and branched-chain amino acids (**BCAA)** increased milk protein synthesis and milk production in dairy cows (Rulquin and Pisulewski, 2006; Appuhamy et al., 2011). Furthermore, the provision of BCAA has been reported to consistently increase the synthesis of muscle protein in pigs (Escobar et al., 2006; Shimomura et al., 2006). Leucine, a potent inhibitor of protein degradation, regulates the activity of branched-chain α-keto acid dehydrogenase complex (Harris et al., 2004). The degradation of muscle BCAA are tightly regulated by the activity of the branched-chain α-keto acid dehydrogenase complex. The supplementation of BCAA may help reduce the degradation of muscle in HS cows.

The common diets high in corn and soy products are often limiting in Met and Lys (NRC, 2001). Indeed, increasing Lys in the diet by 2.2% increased milk production in HS cattle (Chen et al., 1993). Supplementation with Met prevented oxidative stress and increases levels of the antioxidant glutathione in hepatocytes through the interconversion of Met to Cys (Wang et al., 1997). As an antioxidant, glutathione donates H atom to deactivate free radicals (Donovan, 2007). The increase in antioxidants would benefit HS cows because they experience a greater oxidative stress than THN animals (Bernabucci et al., 2002; Chauhan et al., 2016).

We hypothesized that infusion of amino acids would improve milk and milk protein production and reduce muscle protein degradation in cows exposed to HS. The objectives of this study were to assess the effect of Met, Lys, and BCAA on milk production and energy metabolism in HS lactating dairy cattle.

Materials and Methods

Animals, Treatments, and Management

Animal procedures were approved by Institutional Animal Care and Use Committee of the University of Tennessee. Twelve multiparous (mean=2.3 ± 0.5 parities) lactating Holstein cows [mean=120 ± 12 DIM; 636±40.3 kg BW and 2.4 ± 0.3 of BCS] from the East Tennessee AgResearch and Education Center-Little River Dairy (**ETREC**) were divided into two groups (n=6) and utilized in this study. Within each group, cows were randomly assigned to individual tie stalls in one of two climate chambers (3 cows/chamber) located at the University of Tennessee Johnson Animal Research and Teaching Unit (**JARTU**; Bartlett and Smith, 2003). Upon completion of treatments cows were moved to ETREC for a washout period of 19 d returned to JARTU, and assigned to the other chamber.

Environmental temperature (°C) and relative humidity (%) of the chambers were recorded every 10 minutes using HOBO Pro v2 Series probes (Onset Computer Corporation, Bourne, MA). Cows were exposed to environmental treatments in two periods of 14 d each consisting of either THN conditions [20.8°C, 52.2% relative humidity (temperature-humidity index, **THI** = 66.5; Dikmen and Hansen (2009) or HS conditions [> than 22.7° C, 63.8% relative humidity (THI = 68)] with 14-h light and 10-

dark cycles. Cows allocated to the HS treatment received cyclical variation temperatures (in an attempt to mimic daily fluctuation) ranging from 21.5°C to 29.8°C. Between 0000 and 0800 h, the THI remained below 68; thereafter the conditions became increasingly warm until peaking at THI= 76 between 1300 and 1500 h and were above 68 for 14h/d (Figure 1 in Appendix). After peak THI, temperature gradually declined until the THI reached 68 at 2300 h.

An indwelling catheter (12 Ga catheter set LA 1220; MILA International, INC., Erlanger, KY) was aseptically inserted into the jugular and maintained patent using a sterile heparinized 0.9% saline solution (Rius et al., 2010). This procedure was conducted to administer a mixture of AA. Cows received the control (**CTL**) treatment which consisted in 0g/d infusion of AA from day 1 to 6. Cow received an intravenous infusion of L-Met (12 g), L-Lys (22 g), L-Leu (35 g), L-Ile (15 g), and L-Val (15 g; Ajinomoto USA, Inc., Raleigh, NC) dissolved in 0.9% saline solution at a rate of 2 L/d from d 7 to 14 (**ML+BCAA**). The solution of AA and saline was sterile filtered 0.22-μm membrane filters (Millipore, Billerica, MA) and pH was adjusted to 7.4. Solutions were prepped two days in advanced of use and stored at 4°C. Infusions were conducted using a clinical medical pump [Abbott Plum XL IV Pump; Abott-Lifecare, San Antonio, TX; Appuhamy et al. (2011)]. Infusates were delivered to provide 103% of Met, 96.6% of Lys, 101.1% Leu, 107.6% Ile, and 106.1% of Val of the predicted MP requirements using Agricultural Modeling and Training Systems ration formulating software (AMTS LLC., Groton, NY). The use of jugular infusions was implemented in this study to bypass the effects of the

rumen microbe population as well as the small intestine due to the reduced absorption of α-amino N (McGuire et al., 1989).

Cows were milked twice daily at 0700 and 1800 h throughout the study. All animals were individually fed the same TMR diet twice daily (0600 and 1700 h) using a self-propelled mixer (American Calan Inc., Northwood, NH), and orts were recorded daily before the a.m. feeding. The diet was formulated to meet the requirements of a 635 kg lactating Holstein producing 43 kg of milk and 3.0% true protein and 3.8% milk fat (NRC, 2001; Table 1). Samples of TMR, orts, ryegrass silage, ryegrass hay, and concentrate pellet were collected twice a week. Samples were dried at 55° C for 48 h and ground using a 1 mm screen. Dried and ground samples were analyzed for chemical and nutrient composition (Dairy One, Ithaca, NY).

Assessment of Thermal Stress

Body temperatures (rectal and cutaneous) and respiration rates were collected twice daily (0700 and 1500 h). Rectal temperatures were recorded with a Sharptemp V digital thermometer (Cotran Corporation, Portsmouth, RI) and cutaneous temperatures were collected rear mammary glands with an infrared temperature gun (TG 165 FLIR Systems Inc., Willsonville, Oregon). Intravaginal temperatures were taken by inserting data loggers (DS1921G Thermochron iButton Device, Maxim Integrated, San Jose, CA) attached to a modified blank controlled internal drug release [Elanco (Dikmen et al., 2008, Burdick et al., 2012)] device. Measurements were recorded in 10 min intervals over a period of 5 d before and after each infusion. Respiration rates were determined by counting flank movements to determine breaths/min.

Collection and Analysis of Milk and Blood Samples

Milk samples from each cow were collected a.m. and p.m. on d -2 and -1 relative to allocation to tie stalls (covariate period) and at 4 consecutive milkings on d 5, 6, and 7 and d 12, 13, and 14 of each period. Samples were stored at 4°C with a preservative (bronopol tablet; D&F Control System, San Ramon, CA) and later analyzed by United Federation of DHIA Laboratory (Blacksburg, VA) by infrared analyses (Foss MilkoScan; Foss, Eden Prairie, MN) for true protein, fat, MUN, MSNF, and lactose. Calculations for energy corrected milk (**ECM**) were obtained using the equation reported by Tyrrell and Reid (1965).

Blood samples were harvested by coccygeal venipuncture using 18 G x 1 in needles into sodium heparin and EDTA-coated collection tubes on d -2 and -1 relative to allocation to tie stall (covariate period) and after each milking on d 5, 6, and 7 and d 12, 13, and 14 of each period. Samples were centrifuged at 1,200 x g and plasma was collected. Plasma was stored at -20°C until further analysis. Plasma glucose and NEFA concentrations were determined using commercially available enzymatic kits (Sigma-Aldrich, St. Louis, MO; Wako Diagnostics, Mountain View, CA, respectively; Gaverick et al, 2013). Plasma concentrations of BHBA were also determined using commercially available enzymatic kit (Sigma-Aldrich, St. Louis, MO; McCarthy et al., 2015). All enzymatic kits were measured by microplate spectrophotometer (BioTek Synergy H1 Multi-Mode Reader, Winooski, VT). Intra-assay and inter-assay coefficients of variation (**CV**) for all metabolites were less than 10%. Insulin concentrations were measured from plasma by performing a radioimmunoassay (EMD Millipore's Porcine Insulin RIA) using

Wizard2 Gamma Counter (Perkin Elmer, Waltham, MA). The intra-assay CV for insulin was below 10%. Liquid chromatograph-mass spectrometer (Orbitrap LC-MS, Thermo Scientific) was used to determine concentrations of plasma free AA using a commercially available kit (Phenomenex EZ:faast, Torrance, CA).

Net Energy Balance Analysis

In order to calculate energy balance (EBAL), BW were recorded immediately after a.m. milking on d -1 and the p.m milking on d 14. The average of these weights was used to determine net energy of maintenance (NEM) using the following equation: NEM = 0.08 × BW0.75 (NRC, 2001). The NEM was increased for HS cattle by 25% (NRC, 1989). The net energy of lactation (NEL) was calculated using milk production data and the following equation: NEL = [(0.0929 × fat %) + (0.0563 × true protein %) + (0.0395 × lactose %)] × milk yield (NRC, 2001). The values for NEM and NEL were used to calculate the net EBAL using the following equation: EBAL = net energy intake – (NEM + NEL).

Statistical Analysis

The effect of two levels of environments and EAA infusions were tested in a split plot arrangement using a crossover design with two periods. The main effects of temperature and infusion and their interaction were analyzed with covariate using MIXED model procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) for all data, with the exception of a covariate for intake. This is due to no intake measurements being possible to record during the covariate period.

$$Y_{ijkl}=\mu+ P_i+ C_j+ T_k+ P*C*T_{ijk}+ I_l+ I*T_{kl}+ \beta(\chi)_{ijkl}+ e_{ijkl}$$

where Y_{ijkl}= the response of the variable in the i^{th} period of the j^{th} cow subjected to the k^{th} environment and the l^{th} AA treatment, μ = the mean, P_i= the random effect of the i^{th} treatment period, C_j= the random effect of the j^{th} cow, T_k= the fixed effect of the k^{th} environment treatment, I_l= the fixed effect of the l^{th} AA treatment, $\beta(\chi)_{ijkl}$= the effect of the covariate period before treatments and e_{ijkl}= the random error. Milk production, milk component yield, nutrient and DMI were also analyzed with repeated measures in time (d) using an autocorrelation structure, as these variables are expected to change over time. Results are reported as least squares means and were considered significant if $P < 0.05$. Interactions of environment treatment × AA treatment were separated using the LSD method $P < 0.05$.

Results

The time of day significantly increased ($P < 0.01$) rectal, vaginal, and udder temperature and respiration rates during the p.m. measurement. Heat stress caused increased ($P < 0.01$) udder temperatures by 2.7°C in the p.m. compared to the THN. Heat stress increased rectal and vaginal temperatures but this increase was greater for ML+BCAA treatment (39.2 vs. 39.7°C and 39.4 vs. 39.8, respectively; interaction $P < 0.05$). Similarly, HS increased respiration rates but this increase was greater for ML+BCAA treatment (68.2 vs. 76.4 breaths/min; interaction $P < 0.05$). Heat stress decreased ($P < 0.01$) DMI by 1.5 kg/d compared to THN conditions (Table 3). The ML+BCAA treatment decreased ($P < 0.05$) DMI by 0.70 kg/d. Energy balance for both

temperature treatments was negative, however HS induced a greater negative EBAL of -7.12 compared to -4.36 for THN conditions.

Heat stress reduced ($P < 0.05$) overall milk yield by 2.82 kg/d and also reduced daily yields of lactose, true protein, and MSNF by 0.22 kg/d, 0.10 kg/d, and 0.26 kg/d, respectively (Table 3). The ML+BCAA treatment reduced ($P < 0.01$) the daily yield of lactose by 0.11 kg/d and MSNF by 0.17 kg/d. The ML+BCAA treatment did not affect ($P > 0.05$) true protein yield, however true protein percent increased ($P < 0.01$) by 0.08%. The ML+BCAA treatment had no effect on milk fat yield during THN but decreased under HS conditions (1.25 vs. 1.15 kg/d; interaction $P < 0.04$). The ML+BCAA treatment increased MUN under THN (10.9 vs. 11.7 mg/dL) but no change occurred under HS conditions (12.8 vs. 12.2 mg/dL; interaction $P < 0.01$)

Treatments did not affect ($P > 0.05$) the blood metabolites, NEFA, BHBA, and insulin plasma concentrations (Table 4). The ML+BCAA treatment reduced ($P < 0.01$) plasma concentrations of glucose by 0.17 mmol/L but temperature treatments had no effect ($P > 0.05$). The ML+BCAA treatment increased ($P < 0.01$) Met plasma concentrations by 8.27 µM (Table 5). The ML+BCAA treatment did not affect plasma concentration of Lys during THN conditions but decreased under HS by 12.8 µM (interaction $P < 0.05$). Similarly, the ML+BCAA treatment did not affect plasma concentration of Arg and Trp during THN conditions but decreased under HS (77.5 vs 56.1 µM and 40.4 vs. 32.9 µM, respectively; interaction $P < 0.05$). Treatments had no effect on ($P > 0.05$) Val, Ile, or any of the other remaining essential AA. The ML+BCAA treatment decreased ($P < 0.05$) plasma concentrations of the nonessential AA Ala, Asp,

Glu, and Pro by 67.5, 2.17, 15.0, 15.8 µM, respectively. Heat stress decreased the plasma concentration of Glu by 11.7 µM. The ML+BCAA treatment did not affect plasma concentration of Asn and Tyr during THN conditions but decreased under HS (28.3 vs. 22.3 µM and 58.1 vs. 42.8 µM, respectively; interaction $P < 0.05$). Treatments did not affect ($P > 0.05$) Glu, Ser, and 3-methylhistidine plasma concentrations. The ML+BCAA treatment decreased γ-aminobutyric acid (**GABA**) by 0.03 µM.

Discussion

Although many studies have looked at the positive influence of Met, Lys, and BCAA on milk production in lactating cattle, there is little known about how these AA effect production in HS cattle. The current study's aim was to determine if the jugular infusion of Met, Lys, and BCAA would improve the production and metabolism of lactating dairy cows. The results of this study however, did not show improvements in milk production and there was incidence of greater HS when infusions took place.

It has been well established that HS reduces intake and production in lactating dairy cattle (Collier et al. 1982; West et al., 1994; Schwartz et al., 2009). In the current study HS conditions reduced in the range of 4.3-8.6% reported for cattle experiencing moderate levels of HS (THI > 72; Ominski et al., 2002). Ominski et al. (2002) also reported a decrease in milk production of 2.4 kg/d which is similar to the 2.82 kg/d decrease in the present study. The reductions in intake and milk production indicate that negative effects of HS were induced allowing for the further investigation of the effect of Met, Lys, and BCAA on lactating HS cattle.

Under THN conditions the infusions of Met, Lys, and BCAA have been reported to improve milk protein yield between 0.82 and 1.13 kg/d (Rulquin and Pisulewski, 2006; Appuhamy et al., 2011). The infusions of Met, Lys, and BCAA, in the current study, did not improve milk or milk protein production under HS or THN conditions. A possible explanation for our results may be due to the fact that our cattle were 120 DIM producing 40.5 kg/d versus the cattle in the study done by Appuhamy et al. (2001) that were high producing, early lactation cows at 43 DIM producing 53.5 kg/d, which have a higher demand for dietary protein. Although production did not increase as expected, the increase in milk protein percent of 0.08% observed due to the infusion of Met, Lys, and BCAA is slightly lower but still consistent with other infusion studies that report increases between 0.12-0.43%, indicating improved protein synthesis (Rulquin and Pisulewski, 2006; Appuhamy et al., 2011).

The lack of response in milk yield of HS cattle to the infusion of Met, Lys, and BCAA may also have occurred because the effect of HS was greater during infusions. Heat stress conditions increased rectal temperatures, however, this increase was greater when Met, Lys, and BCAA were being infused. The results for vaginal temperatures and respiration rates followed the greater increase during Met, Lys, and BCAA infusions for cattle experiencing HS. The increasing temperatures are a negative result of the present study and indicate that supplementation of Met, Lys, and BCAA may not be a viable option to alleviate the consequences of HS in lactating cattle.

The body temperature results were unexpected and not fully understood, however, these results are consistent with studies infusing AA under hypothermic conditions.

Several studies have reported that the infusions of AA increased the body temperatures between 0.34 and 1.3°C in rats (Yamaoka et al., 2006), canines (Jin et al., 2012), and humans (Wu et al., 2015) under anesthesia, which lowers core body temperature, compared to those who did not receive infusions. It is known that protein does have a high heat increment and when swine were fed increasing amounts of crude protein (**CP**) the amount of metabolic heat produced significantly increased as well (Le Bellego et al., 2001; Noblet et al., 2001). The added AA from this experiment could have also contributed to increasing metabolic heat production in the cattle. Interestingly, in another swine study feeding a diet with 12% CP plus the supplementation of Lys, Trp, and Thr reduced overall heat production when compared to swine fed 12% and 16% CP diets by approximately 7 kcal*d−1*kg−0.75 (Kerr et al., 2003). It is possible that the AA selected for the current study (Met, Lys, and BCAA) were not the best possible selection and Trp and Thr should be further explored in future studies using HS lactating cattle.

Results from this study also coincide with the decreased plasma concentrations of GABA due to the infusion of Met, Lys, and BCAA in this study. The addition of GABA to the diet of cattle increased circulating levels of GABA in the blood and also decreased rectal temperatures during HS (Cheng et al., 2014). A two-fold increase in plasma GABA concentrations decreased rectal temperatures by 0.23°C. Thus, the 0.03 μM reduction in plasma GABA concentrations caused by infusion of Met, Lys, and BCAA in the present study would contribute to increased body temperature. The higher body temperatures are associated with the decrease in GABA concentrations because GABA is a

neurotransmitter that is involved in regulating body temperature in the hypothalamus (Yakimova et al., 1996, Frosini et al., 2000).

Conclusion

The lack of response in milk yield coupled with the increasing body temperatures and respiration rates due to the infusion of Met, Lys, and BCAA that supplemental AA may not be a viable option for reducing the losses in milk yield caused by negative energy balance in HS cattle. However, THN cattle also did not respond which is abnormal according to previous literature. Due to the inconsistencies with other literature in regards to THN cattle's repose to Met, Lys, and BCAA infusions more research should be done, focusing on investigating AA uptake in the tissues.

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APPENDIX

Table 1. Ingredient composition of diet

Item, % of DM	Value
Ingredient	
Ryegrass silage	22.8
Ryegrass hay	22.3
Corn Grain, fine ground	32.6
Corn Distillers Grain	9.10
Soybean Meal, Solv. 48%CP	8.38
BergaFat[1]	1.77
Sodium Bicarbonate	0.91
Calcium Carbonate	0.63
Urea	0.33
Salt	0.36
Calcium Phosphate (mono-)	0.28
Magnesium Oxide	0.18
Calcium Propionate	0.02
Trace mineral and vitamin mix[2]	0.35
Chemical analysis	
DM, %	70.9
CP, % of DM	16.2
NDF, % of DM	33.9
ADF, % of DM	20.1
Potassium, % of DM	1.48
Sodium, % of DM	0.48
Calcium, % of DM	0.58
Phosphorus, % of DM	0.43
Magnesium, % of DM	0.27
NE_L, Mcal/kg	1.59

[1] Berg+Schmidt Feed (Libertyville, IL); Rumen-stable fat powder.

[2] AgCentral Cooperative (Athens, TN); formulated to provide (per kg of dietary DM): 12.4×10^6 IU of vitamin A, 3.1×10^5 IU of vitamin D, and 5.7×10^4 IU of vitamin E, 1200 mg of Co, 2.6×10^4 mg of Cu, 2100 mg of I, 8.3×10^4 of Fe, 1.2×10^5 of Mn, 2.1×10^5 mg of Zn, and 600 mg Se.

Table 2. Rectal, vaginal, and udder temperatures and respiration rates in lactating Holstein cows under heat stress or thermoneutral conditions

	Experimental Treatment[1]					Effect (*P*-value)		
	THN		HS					
Item	CTL	ML+ BCAA	CTL	ML+ BCAA	SEM	Temp[2]	AA[3]	Temp x AA
Rectal, °C								
a.m.	38.2	38.1	38.4	38.7	0.13	0.01	0.48	0.07
p.m.	38.4[c]	38.4[c]	39.2[b]	39.7[a]	0.31	<0.01	0.01	<0.01
Vaginal, °C								
a.m.	38.6	38.8	38.9	38.9	0.01	0.07	0.07	0.53
p.m.	38.8[c]	38.9[c]	39.4[b]	39.8[a]	0.13	<0.01	<0.01	0.05
Udder, °C								
a.m.	36.6	36.6	36.8	37.5	0.27	0.11	0.04	0.06
p.m.	37.0	36.9	39.6	39.6	0.71	<0.01	0.67	0.74
Respiration rates, breaths/min								
a.m.	37.3	40.0	37.7	42.0	2.3	0.49	0.02	0.56
p.m.	46.8[c]	46.8[c]	68.2[b]	76.4[a]	6.0	<0.01	0.03	0.03

[a-d] Means with differing superscripts differ ($P < 0.05$) within row

[1] THN= thermoneutral, HS= heat stress, CTL= no infusion of amino acids, ML+BCAA= infusion of methionine, lysine, and branched-chain amino acids

[2] Temp= temperature

[3] AA= amino acid infusion

* Time of day had a significant effect ($P < 0.001$) on rectal, vaginal, and udder temperatures and respiration rates increasing during the p.m.

Table 3. Least squares means of intake, milk production and composition for temperature and infusion treatment

Item	Experimental Treatment[1]							
	THN		HS			Effect (*P*-value)		
	CTL	ML+ BCAA	CTL	ML+ BCAA	SEM	Temp[2]	AA[3]	Temp x AA
Intake, kg/d								
DM	19.1	18.6	17.9	17.0	0.53	<0.01	0.04	0.53
CP	3.08	3.08	2.87	2.72	0.18	<0.01	0.04	0.48
NDF	6.44	6.27	6.01	5.71	0.15	<0.01	0.04	0.53
ADF	3.96	3.86	3.69	3.50	0.10	<0.01	0.04	0.52
Milk production								
Milk yield, kg/d	32.6	31.7	30.0	28.6	1.12	<0.01	0.27	0.77
Lactose, kg/d	1.59	1.50	1.48	1.35	0.10	0.03	0.01	0.65
True protein, kg/d	0.99	0.96	0.89	0.84	0.05	<0.01	0.07	0.51
Fat, kg/d	1.28[a]	1.29[a]	1.25[a]	1.15[b]	0.09	0.05	0.15	0.04
MSNF[4], kg/d	2.86	2.73	2.64	2.44	0.18	<0.01	0.59	0.45
ECM[5], kg/d	34.8	34.9	32.5	31.3	2.44	0.02	0.03	0.14
Lactose, %	4.89	4.83	4.84	4.81	0.04	0.16	0.01	0.23
True protein, %	3.02	3.10	2.91	2.98	0.06	0.04	0.01	0.43
Fat, %	4.12	4.33	4.30	4.19	0.15	0.94	0.61	0.11
MSNF, %	8.77	8.79	8.62	8.64	0.06	<0.01	0.26	0.92
MUN[6], mg/dL	10.9[b]	11.7[a]	12.8[a]	12.2[ab]	0.75	0.07	0.51	<0.01
EBAL[7], Mcal/d	-4.06	-4.66	-7.15	-7.41	3.55	<0.01	0.39	0.74

[a-d] Means with differing superscripts differ ($P < 0.05$) within row

[1] THN= thermoneutral, HS= heat stress; CTL= no infusion of amino acids, ML+BCAA= infusion of methionine, lysine, and branched-chain amino acids

[2] Temp= temperature

[3] AA= amino acid infusion

[4] Milk solids nonfat.

[5] Energy corrected milk calculated in equation derived from Tyrrel and Reid (1965) and converted to kg/d: ECM = [0.327*Milk (lbs) + 12.95*Milk Fat (lbs) + 7.65*Milk True Protein (lbs)]/2.205

[6] Milk urea nitrogen.

[7] Energy balance calculated from NRC (2001): Energy balance= net energy intake – {(0.08 × $BW^{0.75}$) + [(0.0929 × fat %) + (0.0563 × true protein %) + (0.0395 × lactose %) × milk yield]}

Table 4. Least squares means of metabolite and insulin plasma concentration for temperature and infusion treatments

Item	Experimental Treatment[1]					Effect (*P*-value)	
	THN	HS	CTL	ML+ BCAA	SEM	Temp[2]	AA[3]
Insulin µU/mL	12.48	12.08	12.26	12.30	0.59	0.64	0.96
NEFA, µEq/L	159.6	163.6	162.1	161.1	22.4	0.81	0.93
Glucose, mmol/L	2.91	2.88	2.98	2.81	0.05	0.50	<0.01
BHBA, mg/dL	5.42	5.49	6.03	5.31	0.82	0.66	0.47

[1]THN= thermoneutral, HS= heat stress; CTL= no infusion of amino acids, ML+BCAA= infusion of methionine, lysine, and branched-chain amino acids
[2]Temp= temperature
[3]AA= amino acid infusion

Table 5. Plasma AA concentrations (μM) of cows experiencing temperature and infusion treatments.

Item	Experimental Treatment[1]				SEM	Effect (*P*-value)		
	THN		HS					
	CTL	ML+ BCAA	CTL	ML+ BCAA		Temp[2]	AA[3]	Temp x AA
Essential AA, μM								
Arg	80.5[a]	85.6[a]	77.5[a]	56.1[b]	7.13	0.01	0.05	0.03
His	38.9	43.2	33.5	35.3	6.28	0.18	0.38	0.65
Ile	104	95.4	85.0	85.9	25.4	0.10	0.57	0.49
Leu	140	143	124	129	22.4	0.09	0.54	0.95
Lys	92.1[ab]	103[a]	97.6[a]	84.7[b]	10.8	0.30	0.86	0.01
Met	14.1	23.2	13.6	21.1	0.88	0.15	<0.01	0.35
Phe	47.4	47.8	51.5	43.7	2.78	0.99	0.09	0.06
Thr	81.8	120	60.1	61.0	18.4	0.07	0.38	0.43
Trp	40.6[a]	40.3[a]	40.4[a]	32.9[b]	3.59	0.14	0.03	0.05
Val	204	158	137	155	44.6	0.17	0.39	0.06
Nonessential AA, μM								
Ala	240	133	176	148	47.6	0.33	0.002	0.06
Asn	26.3[ab]	29.5[ab]	28.3[a]	22.3[b]	4.12	0.44	0.51	0.03
Asp	7.18	6.00	6.87	3.70	2.47	0.16	<0.01	0.13
Gln	159	179	157	142	25.7	0.14	0.79	0.06
Glu	79.6	73.0	76.3	52.8	8.48	0.01	<0.01	0.06
Pro	100	78.8	89.6	79.5	38.0	0.61	0.05	0.47
Ser	59.2	62.5	59.1	48.8	10.0	0.24	0.44	0.13
Tyr	55.6[a]	53.4[a]	58.1[a]	42.8[b]	3.34	0.25	<0.01	0.01
3-Methylhistidine	1.97	2.22	2.58	2.01	0.37	0.77	0.52	0.12
GABA[4]	0.12	0.09	0.12	0.08	0.01	0.78	<0.01	0.27
Citruline	48.3[ab]	52.2[ab]	55.1[a]	42.8[b]	9.56	0.73	0.24	0.03
Ornithine	50.0[a]	53.9[a]	53.5[a]	39.6[b]	5.29	0.05	0.06	<0.01

[a-d] Means with differing superscripts differ ($P < 0.05$) within row

[1] THN= thermoneutral, HS= heat stress; CTL= no infusion of amino acids, ML+BCAA= infusion of methionine, lysine, and branched-chain amino acids

[2] Temp= temperature

[3] AA= amino acid infusion

[4] GABA= γ-aminobutyric acid

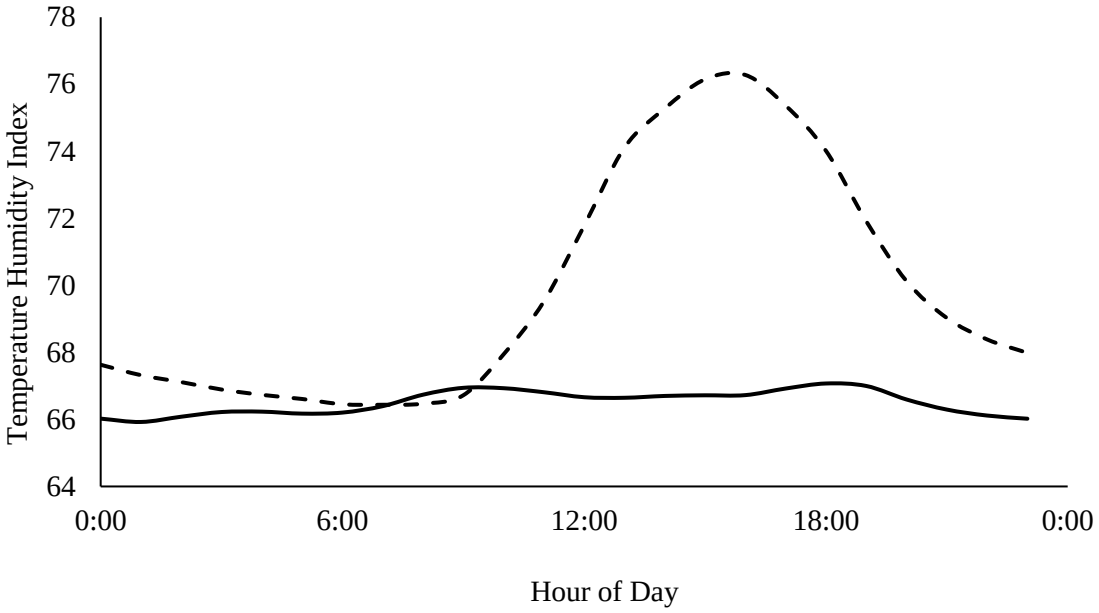


Figure 1. The hourly averages of the temperature humidity index (THI) under thermoneutral (solid line) or heat stress (dashed line) conditions. The THI was calculated using the equation from Dikmen and Hansen (2009): THI = (1.8 × Tdb + 32 − [(0.55 − 0.0055 × RH) × (1.8 × Tdb − 26.8)]).

VITA

Kimberly Rose Kassube was born on the Davis-Monthan Air Force base in Tucson, Arizona on January 18, 1992. In 2010, she graduated from Wittenberg-Birnamwood High School in Wittenberg, WI. Upon completing high school she attended the University of Wisconsin-Madison majoring in Animal Science and graduated in May 2014 with a Bachelor's of Science in Natural Science. In August 2016, she graduated from the University of Tennessee- Knoxville with a Master's of Science in Animal Science.