

THE EVALUATION OF DIETARY BETAINE, PRE AND PROBIOTICS, TRANSITIONAL
SUBSTRATES, AND B-MERCAPTOACETATE ON PHYSIOLOGICAL, METABOLIC, HORMONAL
AND PRODUCTION RESPONSES IN LACTATING HOLSTEIN COWS SUBJECTED TO THERMAL
STRESS.

By

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TABLE OF CONTENTS

LIST OF TABLES.....	8
LIST OF FIGURES.....	9
LIST OF ABBREVIATIONS.....	13
ABSTRACT.....	15
INTRODUCTION.....	18
CHAPTER ONE: LITERATURE REVIEW.....	21
Thermoneutral Zone.....	21
Upper and Lower Critical Temperatures and Temperature Humidity Index.....	21
Effects of Heat Stress.....	23
Facilities and Heat Abatement.....	28
Acclimation and Adaptation.....	30
Impact of Heat Stress on Rumen, Metabolism and Nutrient Requirements of Dairy Cattle.....	31
Nutritional Strategies.....	34
β -Hydroxybutyrate.....	39
SUMMARY.....	44
CHAPTER TWO: EVALUATION OF DIETARY BETAINE IN HEAT-STRESSED HOLSTEIN COWS IN LACTATION	51
Abstract.....	51
Introduction.....	52
Materials and Methods.....	55
Results.....	61
Discussion/ Conclusion.....	62
CHAPTER THREE: THE EFFECTS ON PERFORMANCE, PHYSIOLOGICAL RESPONSES AND CHANGES IN MICROBIAL POPULATIONS OF CALSPORIN TREATED LACTATING HEAT-STRESSED HOLSTEIN COWS.....	81
Abstract.....	81
Introduction.....	81
Materials and Methods.....	84
Results and Discussion.....	88
Conclusion.....	93

CHAPTER FOUR: AN EVALUATION OF OMNIGEN-AF™ IN HEAT-STRESSED LACTATING
HOLSTEIN COWS: PHYSIOLOGICAL RESPONSES, PRODUCTION RESPONSES, MILK
QUALITY, MEASURES OF IMMUNITY AND HORMONES OF THE ADRENAL

AXIS.....	107
Abstract.....	107
Introduction.....	108
Materials and Methods.....	110
Results and Discussion.....	114
Conclusion.....	117

CHAPTER FIVE: IMPACT OF β -HYDROXYBUTYRATE AND MERCAPTOACETATE ON FEED
INTAKE SERUM METABOLITES, AND SERUM INSULIN IN LACTATING HOLSTEIN

COWS.....	135
Abstract.....	135
Introduction.....	136
Materials and Methods.....	139
Results.....	143
Discussion.....	145

SUMMARY AND CONCLUSIONS.....	163
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REFERENCES.....	165
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LIST OF TABLES

Table 2.1. Ingredients and chemical composition of the diet ¹ during the betaine study.....	71
Table 2.2. Rectal temperature and respiration rate with different doses of betaine at 1800 h.....	74
Table 2.3. Milk production and intake for lactating cows fed varying levels of betaine..	77
Table 3.1. Ingredients and chemical composition of the diet.....	95
Table 3.2. Effect of Calsporin, heat stress and thermoneutral conditions on milk composition of lactating Holstein cows.....	100
Table 3.3. Effect of Calsporin, heat stress and thermoneutral conditions on respiration rate and rectal temperatures of lactating dairy cows.....	101
Table 3.4. Effect of feeding Calsporin to lactating dairy cows on selected microbial cell populations while at the dairy, during acclimation.....	106
Table 4.1. Ingredients and chemical composition of the diet.....	121
Table 4.2. Dry matter intake, milk yield and milk composition of CON and OG fed animals housed under TN or HS conditions in environmentally controlled rooms.....	122
Table 4.3. Mean respiration rates and rectal temperatures from cows fed OG and exposed to TN and HS.....	129
Table 5.1. Ingredients and chemical composition of the diet.....	153
Table 5.2. The change in production and physiological responses with BHB and MAA.....	155

LIST OF FIGURES

Figure 1.1 Thermal Heat Index Adapted for Dairy Cows (Zimbleman et al, 2009).....	48
Figure 1.2. Mean vaginal temperatures in lactating dairy cows for the first two days of HS in the environmental rooms at the University of Arizona.....	49
Figure 1.3. Summary of production, physiological and metabolic changes associated with response to HS in lactating dairy cows.....	50
Figure 2.1. Environmental rooms (1 and 2) conditions by day during the betaine study in room 1 (R1) and room 2 (R2).....	68
Figure 2.2. Diurnal conditions during TN during the betaine study in the environmental rooms at the University of Arizona. Red line indicates the threshold THI (68) for HS in lactating dairy cows.....	69
Figure 2.3. Diurnal conditions during HS. Red line indicates the threshold THI (68) for HS in lactating dairy cows.....	70
Figure 2.4. Images of primary bovine mammary epithelial cells (BMECs) in collagen after 8 h of exposure at 42°C treated with betaine.....	72
Figure 2.5. Expression of HSP27 and HSP70 in BMEC cells with 0mM or 25mM betaine.....	73
Figure 2.6. Hours spent at or above specific vaginal temperatures with betaine supplementation during TN (a) and HS (b).....	75
Figure 2.7. Mean diurnal vaginal temperatures by environment with betaine during TN (a) and HS (b).....	76
Figure 2.8. Mean plasma glucose with different doses of betaine in TN and HS.....	78
Figure 2.9. Mean plasma insulin with different doses of betaine in TN and HS.....	79
Figure 2.10. Fold change in HSP27 (a) and HSP70 (b) during TN and HS at different levels of betaine. Values are mean gene expression in buffy coat fraction of the blood...80	80
Figure 3.1. Room environments during the ARC phase of the Calsporin study.....	94
Figure 3.2. Change in on-dairy milk production with treatment of Calsporin by week...96	96
Figure 3.3. Dry matter intake (ARC) in Calsporin fed cows.....	97

LIST OF FIGURES (continued)

Figure 3.4. Milk yield during ARC portion with supplemental Calsporin during TN and HS.	98
Figure 3.5. Effect of Calsporin, heat stress and thermal neutral conditions on somatic cell count in milk from lactating Holstein cows.....	99
Figure 3.6. Effect of HS and Calsporin on mean rectal temperatures at 0500, 1400 and 1700 hours in lactating dairy cows.....	102
Figure 3.7. Effects of HS and Calsporin on respiration rates at 0500, 1300 and 1700 hours in lactating dairy cows.....	103
Figure 3.8. Effect of HS and Calsporin on HSP27 gene expression in leukocytes of lactating dairy cows.....	104
Figure 3.6. Effects of feeding Calsporin to lactating dairy cows on circadian expression of HSP27 in the buffy coat fraction of the blood.....	105
Figure 4.1. Environmental conditions in the environmental rooms during the 21-day ARC study.....	119
Figure 4.2. Environments in the controlled rooms during the ARC portion of the OG trial.	120
Figure 4.3. Mean milk production at the start of the study and on day 45 at the dairy.....	123
Figure 4.4. Milk Somatic cell count by day in CON and OG Fed cows during TN (d 1-7), HS (8-17) and recovery (18-20) periods.....	124
Figure 4.5. Dry matter intake by day during the ARC phase.....	125
Figure 4.6. Mean daily water consumption in CON and OG fed cows during TN, HS and recovery periods.....	126
Figure 4.7. Plasma glucose concentrations in CON and OG Fed cows during TN, HS and recovery periods.....	127
Figure 4.8. Plasma Insulin (ARC) concentrations with OG during TN and HS.....	128

LIST OF FIGURES (continued)

Figure 4.9. Mean serum cortisol in CON and OG fed cows during TN, HS and recovery periods.....	130
Figure 4.10. Serum ACTH concentrations in CON and OG fed animals during TN, HS and recovery periods.....	131
Figure 4.11. Ionized serum calcium in CON and OG fed cows during TN, HS and recovery periods.....	132
Figure 4.12. IL-8R Gene Expression at dairy and ARC with OmniGen-AF.....	133
Figure 4.13. The change in expression of HSP27 with treatment of OG by period.....	134
Figure 5.1. Serum B-OH Butyrate and non-esterified fatty acid concentrations 1, 3, and 10 hours following exposure to temperature humidity index (THI) > 68.....	151
Figure 5.2. Treatment schedule of MAA and BOHB bolus studies with times of treatment, sampling, feeding and milking.....	152
Figure 5.3. HS conditions during the MAA portion of the study.....	154
Figure 5.4. The change from baseline (time 0) in; (a) serum BHB, (b) serum glucose, (c) serum NEFA, and (d) serum insulin and (e) glucose : insulin in cows treated with a bolus of β -hydroxybutyrate.....	156
Figure 5.5. The change in temperature from baseline (0 h) by treatment. Skin temperature (a) and vaginal temperature (b).....	157
Figure 5.6. The mean change in dry matter intake (a) between measurements, and the cumulative total in DMI (b) by time point in BHB treated cows.....	158
Figure 5.7. The change from baseline (time 0) in; (a) serum BHB, (b) serum glucose, (c) serum NEFA, and (d) serum insulin and (e) glucose : insulin in cows treated with a bolus of Mercaptoacetate.....	159
Figure 5.8. Changes in mean (a) skin temperature and (b) vaginal temperature with MAA.....	160

LIST OF FIGURES (continued)

- Figure 5.9. The correlation of vaginal and rectal temperatures in MAA treated cows at times 0, 15m, 30m, and 1h relative to bolus. Saline cows are not represented in this figure as there was no change in vaginal temperatures.....161
- Figure 5.10. Cumulative dry matter intake by hour with saline or MAA treatment....162

LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
ADH	antidiuretic hormone
ANOVA	analysis of variance
ARC	Agricultural Research Center
ATP	adenosine triphosphate
BCC	betaine-choline-carnitine
BCS	body condition score
BHB	β -hydroxybutyrate
β -MA	β -mercaptoacetate
BMEC	bovine mammary epithelial cells
BPM	breaths per minute
BSMC	bovine abomasum smooth muscle cells
BW	body weight
cDNA	complementary deoxyribonucleic acid
CON	Control
CV	coefficient of variation
d	day
DFM	direct fed microbial
DIM	days in milk
DMEM	Dulbecco's Modified Eagle Medium
DM	dry matter
DMI	dry matter intake
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
FAO	fatty acid oxidative
G	gram
GIT	gastrointestinal tract
GPR109a	G-protein-coupled receptor 109a
H	hour
HI	high dose
HLI	heat load index
HPA	hypothalamic-pituitary-adrenal axis
HPLC	high-performance liquid chromatography
HS	heat stress
HSP	heat shock protein
IL8R	interleukin-8 receptors
kg	Kilogram
LCT	lower critical temperature
MAA	β -mercaptoacetate
MID	mid dose
mRNA	messenger ribonucleic acid

MY	milk yield
NEFA	non-esterified fatty acid
OG	OmniGen-AF®
PCR	polymerase chain reaction
REC	recovery
RIA	radioimmunoassay
RNA	ribonucleic acid
RR	respiration rate
RT	rectal temperature
SCC	somatic cell count
THI	temperature-humidity index
TMR	total mixed ration
TN	thermoneutral
UCT	upper critical temperature
VFA	volatile fatty acids

ABSTRACT

This dissertation evaluated nutritional approaches such as the addition of betaine, prebiotics, probiotics, transitional metabolic substrates, and β -mercaptoacetate (MAA; a compound which inhibits β -oxidation) to the diet of lactating dairy cows to determine their impact on physiological, metabolic, hormonal and production responses during thermal stress. The first objective was to evaluate the use of an organic osmolyte, betaine to reduce the impact of heat stress (HS). Cows were fed either 0 (control; CON), 57 mg/kg BW (mid) or 114 mg/kg (high; HI) body weight (BW) betaine and subjected to thermoneutral (TN) and HS conditions. There was an increase in milk yield during TN with HI betaine over controls ($P < 0.01$), but the advantage was lost during HS. Plasma glucose increased during HS in HI dose cows compared to control ($P < 0.01$) as did plasma insulin ($P = 0.01$). Betaine increased milk production during TN and plasma glucose in HS, but did not improve the HS response. Objective two evaluated the use of a probiotic or direct fed microbial (DFM), Calsporin (*Bacillus subtilis* C-3102) to decrease the effects of HS in dairy cows. We hypothesized that feeding Calsporin prior to and during HS would reduce pathogenic strains of bacteria, maintain commensal microbes, and improve ruminal anaerobic fermentation resulting in improved milk yield (MY). Milk yield was numerically increased (1.26 kg, $P = 0.11$) in cows fed Calsporin during TN but was reduced under HS (-2.67 kg, $P < 0.01$) and milk protein content was decreased ($P = 0.05$). The DFM tended to decrease somatic cell count (SCC) across periods ($P = 0.07$). Calsporin addition to the diet did not affect

respiration rates and was associated with higher rectal temperature at 1800 in HS ($P = 0.02$). The expression of heat shock protein 27 (HSP27) was decreased with Calsporin treatment ($P = 0.03$) and in both HS and TN. The fecal microbial count did not change with the exception of the Calsporin strain in treated animals ($P < 0.01$). The third objective was to feed OmniGen-AF (**OG**) to dairy cows before and during thermal stress. We hypothesized that feeding OG to HS dairy cows will improve the immune response, and decrease production losses associated with HS. Cows fed OG maintained lower SCC compared to control ($P < 0.01$) during the recovery period. We did not detect differences between groups in serum calcium while serum non-esterified fatty acid (NEFA) concentrations ($P = 0.10$) tended to be greater in OG fed cows across the Agricultural Research Center (ARC) portion including HS. Serum Adrenocorticotrophic hormone (ACTH) levels were greater in OG cows ($P < 0.0001$) across all sample days. Feeding OG reduced the HS response including serum Cortisol. The final study measured the effects of the metabolic substrate β -hydroxybutyrate (BHB) during HS on feed intake and metabolites. Under TN conditions the cows received a bolus dose of BHB and dry matter intake (DMI) and metabolites were measured. The second part of this study used a bolus of MAA to limit the up-stream production of acetyl-CoA available for ketogenesis by inhibiting β -oxidation. We proposed that dosing lactating dairy cows with BHB would decrease DMI, increase plasma insulin, decrease NEFAs and increase skin temperature by vasodilatation. The same cows were then subjected to HS and dosed with saline and MAA on different test days. The infusion of BHB increased skin temperature (time 0.5, 1, 2, 3 and 4°C $r^2=0.98$ with serum BHB) and decreased serum

NEFA levels ($P < 0.01$). There was no change in mean DMI, glucose or insulin. The bolus of MAA decreased feed intake, vaginal temperature, and insulin. There was an increase in serum BHB with the initial dose of MAA and an initial decrease in serum glucose ($P < 0.0001$) with MAA. Serum glucose increased as insulin decreased with MAA. The infusion of BHB did not alter feed intake in this study despite high plasma levels of BHB.

INTRODUCTION

The objective of this dissertation was to address nutritional management tools as a method to improve responses to HS in lactating dairy cows. Heat stress has a tremendous economic impact on the dairy industry. It was estimated that there are about \$900 million lost annually in US dairies (St. Pierre et al., 2003). There have been advancements in facilities from shade to advanced cooling systems (Collier et al., 2006; Anderson et al., 2013) that reduce exposure and improve performance during HS. The implications of HS in dairy cows includes production losses (Bohmanova et al., 2007), reduced reproductive performance (Cavestany et al., 1985) from conception to embryonic and fetal survival (Badinga et al., 1985; Tao and Dahl, 2013), and general health and mortality (Crescio et al., 2010).

There were advantages to feeding all three of the supplements tested in this dissertation. Calsporin numerically increased milk yield under TN conditions and reduced SCC compared to controls. Betaine increased milk production during TN and increased plasma glucose levels during HS. The Calsporin and Bet treatments both lost the milk yield response and displayed elevated physiological responses to HS. The OG treatment numerically increased milk in TN and did reduce the physiological response to HS, but did not eliminate all production losses during HS. There were other factors not understood during HS so we infused BHB into lactating dairy cows to help expose the mechanism of these production and physiological losses.

The threat of climate change worsens the potential intensity of hot and humid environments and likelihood for episodes of HS in less affected areas (Gauly et al., 2013). The aims of reducing the carbon footprint of dairy systems reduces greenhouse gases and improves efficiencies by reducing waste and improving crop yields and nutrient availability (Henriksson et al., 2014). There is also a continued push to improve nutrient utilization in ruminants.

Plant extracts have demonstrated antimicrobial effects (Cowan, 1999), and inhibit pathogens while improving animal health (Jouany and Morgavi, 2007). Prebiotics such as oligosaccharides have demonstrated the ability to promote favorable gut microbes, improve immune response and help cellular function (Laparra et al., 2014).

Diet formulation can have tremendous impacts on gut microbial populations (Edwards et al., 2008). Additionally, the addition of probiotics including yeast and bacteria can improve nutrient utilization and health in ruminants (Oeztuerk and Sagmanligi, 2009; Peng et al., 2012). Under non-HS conditions, probiotics have improved propionate production, increased MY and improved feed efficiency (Peng et al., 2012).

Rumen and hindgut health can be jeopardized under HS like conditions (Gressley et al., 2011; Soriani et al., 2013). Our objective was to reduce the impact of HS in lactating dairy cows using micronutrients (OG), plant extracts (betaine) and probiotics (Calsporin and OG). This was accomplished by feeding betaine, Calsporin and OG to lactating cows and measuring changes in production, physiological changes, hormonal

alterations, and changes in microbial populations and immune response in HS lactating Holstein cows.

The second objective of this study was to measure the impact of β -hydroxybutyrate (BHB) on feed intake, metabolites, changes in temperature (skin and core body) and changes in insulin levels. Heat stress reduces feed intake, milk yield, serum NEFA concentrations, and glucose while increasing vasodilation and serum insulin levels in lactating dairy cows (Rhoads et al., 2009; Di Costanzo et al., 1997). It has also been shown that during HS hepatic production of BHB increases (Abeni et al., 2007). Increased levels of serum BHB have similar effects to HS. Elevated levels of BHB can decrease feed intake (Rossi et al., 2000), reduce mammary (mouse) cell proliferation (Kennedy et al., 2002) and inhibit lipolysis and circulating NEFAs (Taggart et al., 2005). Additionally, increased insulin are facilitated by acting on pancreatic β -cells (Itoh et al., 1998; Biden and Taylor, 1983), decrease circulating glucose with elevated levels of insulin, and increase vasodilation by binding to GPR109a (Zimbelman et al., 2010).

The aim of this objective was to determine if a bolus of BHB during TN will mimic HS responses with FI, MY, NEFA, glucose, insulin, and vasodilation seen during HS. We also tested mercaptoacetate (MAA), a fatty acid oxidative (FAO) inhibitor in HS lactating cows. This was an attempt to control the hepatic production of BHB by reducing available acetyl CoA, a product of β -oxidation of fatty acids and precursor for the synthesis of the ketone BHB.

CHAPTER ONE: LITERATURE REVIEW

Impact of Thermal Environment on Dairy Cattle

Thermoneutral zone

The range in temperature for the TN zone is about 1.7° to 21° C in lactating dairy cows (Johnson and Vanjonack, 1976). When environmental conditions, specifically temperature and humidity do not significantly interfere with thermoregulation, homeostasis and production in dairy cows, cows are at TN (St. Pierre et al., 2003). The average milk yield per US dairy cow has increased, and the cattle have become increasingly sensitive to HS and more tolerant of cold (Collier et al., 2006).

Upper and lower critical temperatures and temperature humidity index

The lower and upper critical temperatures can vary depending on stage of lactation, gestation and production (Johnson and Vanjonack, 1976; Collier et al., 1982). The TN zone is contained within the upper critical temperature (**UCT**) and the lower critical temperature (**LCT**) of dairy cows. Temperatures below the LCT and above the UCT alter feed intake. Cows below the LCT increase feed intake (Young, 1981) as metabolic needs (including heat) rise, though production decreases (Hamada, 1971). Feed intake at temperatures above the UCT decreases despite increased energy needs during HS (Rhoads et al., 2009). The UCT for Holstein cows is between 25 to 26°C, and causes an increase in rectal temperature (**RT**; Berman et al., 1985). The UCT is the threshold for HS.

HS results in disruption of homeostasis, elevated basal metabolism and initiation of physiological acclimation responses (Rhoads et al., 2009). Cattle experiencing HS have

an elevated body temperature, an increased respiration rate, decreased feed intake, reduced milk and reproduction efficiencies. Insulin production increases and circulating NEFAs decrease (Rhoads et al., 2009). Many additional physiological pathways are compromised, resulting in a loss of production (growth, reproduction, lactation). Dairy cows are especially susceptible to HS with compounding factors such as high dry matter intake (DMI), lactation, stage of gestation and limited genetic adaptation to hot and humid environments (Collier et al., 1982). The ability of animals to adjust to the environment is known as acclimatization and is essential to long-term success of dairy operations (Collier et al., 2006).

The temperature humidity index (**THI**) has been used for five decades by the dairy industry (Berry et al., 1964) and was recently revised (figure 1.1) to account for the increased production levels of US dairy cows (Zimbelman et al., 2009). The THI minimum threshold for HS was reduced from 72 (Armstrong, 1994) to 68 (Zimbelman et al., 2009) to account for the higher production of modern dairy cows. This index is a combined measure of the effect of ambient heat and relative humidity, provided to indicate the degree of thermal stress that dairy cows will undergo (Berman, 2005). The THI does not include the influence of solar radiation, wind speed, hair coat, or interactions between temperature and humidity for evaporative heat loss (Berman, 2005). Gaughan et al. (2008) developed the heat load index (**HLI**) to account for solar radiation, wind and stage of production in beef cattle, later revised the HLI for dairy cattle (Gaughan et al., 2010), but THI is predominantly used for dairy cattle. Currently, the dairy industry

utilizes THI and not HLI. Providing shade for dairy cows reduces the direct impact of solar radiation (Collier et al., 1981) and wind remains unaccounted for with THI.

Effects of Heat Stress

Animal produced proteins represent one-third of the proteins consumed by humans globally and predicted climate change threatens production (Godber and Wall, 2014). Dairy cows are more susceptible to HS than most other animals due to the high metabolic heat and increased heat of rumen fermentation associated with lactation (Liu et al., 2014). The dairy industry today is capable of producing more milk from fewer cattle by increasing production and efficiency within an individual animal. Metabolic heat is greater with increasing milk yields (Purwanto et al., 1990). The average milk production has doubled in the US over half a decade (Collier et al., 2006). Through genetic selection, nutrition, supplementation, and more efficient management the dairy industry is producing more protein from each animal (Feuz and Umberger, 2003; Capper and Bauman, 2008). Dairy production occurs in different climates with varying levels of resources and different environmental conditions. Dairy cattle with higher production (Frank et al., 2001) and increased parity (West, 1994) are more susceptible to HS.

The culmination of hot ambient conditions and metabolic heat can disrupt homeostasis and cause HS. When HS occurs the body temperature of a lactating dairy cow is $> 38.5^{\circ}\text{C}$ (Zimbelman et al., 2009; Burfeind et al., 2012). This type of stress is a direct result of high heat and humidity resulting in an unfavorable temperature gradient from cow to environment. This is complicated by the large amount of metabolic heat being produced by the animal (West, 2003). The HS response in dairy cattle is well

defined in the areas of physiological, production and reproduction (Armstrong et al., 1993). Additionally, feed intake and milk yield have a predictable decline during HS from 10 to 50% depending on exposure (Schneider et al., 1984; Rhoads et al., 2009). The changes in metabolism, immune function, hypophagic control and the fate of metabolites are less understood. Heat loss by radiation, conduction, and convection can differ between cattle of different breeds due to differences in hair coat (color, thickness, length, density and sweating rate) among animals (Gebremedhin and Wu, 2001).

Evaporation is an additional route of heat loss, which is driven by a vapor pressure gradient. Routes of natural evaporative heat loss in cattle include panting and sweating. Cattle have an apocrine sweat gland with one sweat gland associated with each hair fiber. Therefore, hair density alters the ability of animals to sweat. Likewise, hair coat factors such as thickness of the hair shaft and length of the fiber alter airflow at the skin surface which alters evaporative heat loss (McManus et al., 2011). Sweating rate and rate of evaporative heat loss varies between breeds of cattle and especially between *Bos taurus* and *Bos indicus* (Gebremedhin and Wu 2001). Additional factors that impact heat tolerance on beef cattle include age, fat thickness, and stage of production. Different stages of production include period of lactation, or stage of breeding and gestation. Both sweating rate and the use of evaporative cooling are limited by relative humidity. As the percent humidity increases the effectiveness of evaporation inversely decreases (Maia et al. 2005).

Six physiological stages have been defined in HS of cattle, (Silanikove, 2000, Gaughan et al. 2000, Mader et al. 2006). During the first stage the cow will spend

increased time standing and have an increased respiration rate. The respiration rate increases with the onset of the second stage along with minor drooling. The cattle will continue to stand and start to become restless. Stage three continues with drooling that becomes frothy. The mouth opens during the fourth stage. The tongue will protrude and the flanks will move in and out in the fifth stage. The last stage is accomplished when a slower, deeper respiration rate is reached. The head will drop and the animal may not be drooling. These outward symptoms cause a cascade of internal imbalances.

The conditions of HS vary globally due to differences in solar radiation and level of humidity. Cattle in arid and semi-arid environments can have prolonged exposure to high heat and low humidity. Tropic and subtropical zones can have exposures of high heat and humidity. More temperate areas may have short durations of HS that can result in losses as well. St. Pierre et al. (2003) estimated that the dairy industry alone lost \$900 million per annum to negative effects of HS on production and reproduction in dairy animals. The effects of HS can be both chronic and acute and have factors that limit the advancements in heat abatement. The initial effects of HS on production can be seen within hours of exposure. However, Collier et al. (1982) demonstrated that the full impact of HS on milk yield in lactating Holstein cows was not observed for 36-48 hours following the initial heat exposure. When cows are subjected to environmental conditions that do not permit them to dissipate their entire heat load they are forced to store heat, for example, in figure 1.2, the mean body temperatures of 8 dairy cows in lactation under HS conditions for the averaged first two days is illustrated over a 24 h

timeline. Cows finished each day hotter than the previous day. The accumulation of heat occurred despite the $THI < 67$ for over 5 hours each night.

During periods of HS, facilities can reduce solar exposure. Solar exposure can increase the heat load of cows and can also increase body temperature (Collier et al., 1981). The addition of shade can increase milk production by 10 to 19 % compared to exposed cows (Roman-Ponce et al., 1977; Collier et al., 1981). The addition of shade reduced HS by lowering respiration rates (82 to 54 BPM) and rectal temperatures (39.4°C to 38.9°C) in lactating cows (Collier et al., 2006).

High producing dairy cows experience the most unfavorable metabolic conditions during the hottest part of the day (Shehab-El-Deen et al., 2010). Elevated respiration rates, increased body temperature, decreased feed intake, losses in reproductive performance, health issues and decreased milk yield are observable in hyperthermic lactating dairy cows (Rhoads et al., 2009). Elevated respiration rate leads to increased salivary bicarbonate loss associated with excessive drooling and respiratory alkalosis as the animal exhales excessive amounts of carbon dioxide. Supplemental sodium bicarbonate and potassium can increase feed intake and milk production in dairy cows exposed to HS (Schneider et al., 1984). The cow responds to respiratory alkalosis by dumping excess bicarbonate into the urine, which subsequently leads to metabolic acidosis because of reduced buffering capacity in the rumen from both salivary and urinary bicarbonate loss. Respiratory alkalosis and metabolic acidosis occur when respiration rate increases (Sanchez et al., 1994). Saliva is lost with panting and

sodium bicarbonate is lost with excessive drooling which reduces ruminal buffering, increasing the risk of rumen acidosis.

Ruminants produce between 102 to 317 kg/d of saliva and spend at least 10-12 hour ruminating each day (Appuhamy et al., 2014). Rumination time depends on the particle size, internal factors (pH, anaerobic conditions, temperature, mixing and the microbiota), and physiological responses to surrounding conditions and stressors (Soriani et al., 2013). Increasing nutrient density during HS by increasing concentrate and reducing forage can improve energy outputs (West, 2003). Under non HS conditions, increasing fiber size of forage increased rumination time, but not ruminal fermentation (Zebeli et al., 2007).

Feed intake drops with HS and milk yield also decreases, only 50% of the decrease in milk yield can be accounted for by the drop in feed intake. This was demonstrated by pair-feeding lactating dairy cows under TN conditions the same amount of feed that the HS group consumed (Rhoads et al., 2007; Baumgard et al., 2011). There are still 50% of milk yield losses not directly linked with the drop in DMI. It is still uncertain how to account for all of the losses relative to milk synthesis during hyperthermia. Total VFA production also decreases with HS in lactating dairy cows (Schneider et al., 1988). Nutrient partitioning, the reduction in ruminal fermentation products (VFA and protein), and the energy required to dissipate heat during HS may account for milk yield losses.

Heat stress impacts reproduction in dairy cows from pre-conception through late gestation. Libido is reduced with fertility during HS, reducing conception rates during

hot periods (Collier et al., 1982). Implantation and early embryonic survival also decrease with HS, and pregnancy losses increased by a factor of 1.05 when the mean daily THI increased one unit between d 21 to 30 of pregnancy (Garcia-Ispuerto et al., 2006). Fetal growth and survival are altered during late gestation and the effects can carry over into lactation (Collier et al., 1982).

Facilities and Heat Abatement

The most effective way to reduce the severity of HS in lactating dairy cows is to modify their environment. Improving facilities can be as simple as shade and as complex as expensive cooling systems that use forced air, water and different housing and ventilation systems. The economic benefit to specific dairy production systems justifies the type and cost of abatement strategies (Armstrong, 1994). Modern dairy systems attempt to maximize high milk production, feed efficiency and reproductive efficiency (Collier, 2011). Heat stress jeopardizes all of these areas and the economic losses indicate the degree of abatement. Improvements in facilities reduces direct exposure to hot ambient conditions.

Early research supports the use of shade in lactating cows (Collier et al., 1981). Dairy cows will seek shade when it is provided (Roman-Ponce 1977; Schutz et al., 2011) and this behavior is enhanced with rising temperatures (Kendall et al., 2006). Shade is the primary source of heat abatement because it is economical, durable and effective (Armstrong et al., 1993). The exposure to solar radiation is reduced with shade and this has been demonstrated to reduce the heat load of dairy cows by 30 to 50 % (Collier et al., 2006). Feedline soakers are an additional method of cooling cows that offers an

economical method of reducing HS. These soakers are often utilized in dry lot dairies, but wind speed can challenge the effectiveness by reducing the amount of water actually being applied to the cow's back (Smith et al., 2012).

The stimulus of HS in intensively managed dairy systems may require more relief than shade can offer. Fans, soakers, sprinklers and misters can be used with shade when shade is insufficient. Brouk et al. (2003) compared cooling effects in dairy cows +/- fans, +/- soakers and high pressure misters with soakers used at different time intervals. The findings indicated that fans with high pressure misters and fans with soakers (turned on for 1 minute every 5 minutes) reduced respiration rates from over 110 bpm to 60 bpm compared to cows that received no treatment (Brouk et al., 2003). Shoulder skin temperatures decreased from about 38° C to about 29° C, and body temperature decreased over 1° C with the same respective treatments (Brouk et al., 2003).

In hot and dry environments evaporative cooling helps cool cows. This cools the air surrounding the cows creating a micro environment that reduces the impact of HS to the cows (Collier et al., 2006). There are a variety of commercial cooling systems that improve conditions for dairy cows in hot climates. Korral Kool (Korral Kool Inc, Mesa, AZ) utilizes a fixed reverse chimney and high pressure misters that are fixed to a shade structure, and have retracting curtains that help confine cooled air (Armstrong et al., 2003). Shade Trackers (Advanced Dairy Systems LLC, Chandler, AZ) also utilize fans with misting, but the fans oscillate and cover the shaded areas (Burgous et al., 2007). The Flip Fan (Schaefer, Sauk Rapids, MN) is able to rotate 180° and can adjust for wind. The

Flip Fan also uses fans combined with misters to cool cows (Anderson et al., 2013). The three mentioned cooling systems all decrease the severity of HS in lactating dairy cows (Armstrong et al., 2003; Burgos et al., 2007; Anderson et al., 2013).

The fan and misters combination is often seen with dry lot dairies that utilize Saudi barns and desert barns in hot environments. Dairy housing systems that utilize cross or tunnel ventilation allow producers control of the cow's environment during all seasons (Smith et al., 2007). Fans and evaporative cooling pads condition air passing through the barns and the structure protects cows from environmental factors including temperature, solar radiation and wind.

Improvements in facilities can greatly improve performance during HS in lactating dairy cows. These improvements vary depending on economic inputs, and depend on economic losses to justify the expenditures. In areas with acute and sporadic episodes of HS, more economical approaches should be employed. Additionally, facilities do not eliminate all the effects of HS. Using nutritional strategies to compensate for these two areas of deficit was our primary objective. Nutritional alterations could reduce the production of metabolic heat during HS, improve metabolism during periods of thermal stress and would therefore be more economical.

Acclimation and Adaptation

Management solutions to hyperthermia need to address both acute and long-term acclimation. Acclimation is a homeorhetic process which occurs over time and is composed of an acute short-term response and long-term acclimation response. The

short-term acclimation involves activation of the heat shock response, as well as rapid drops in feed intake and milk yield as well as cellular metabolic adjustments described by Baumgard and Rhoads (2011) and is the pathway response that helps the cows return to homeostasis. Over several generations the chronic response becomes “fixed” and is referred to as adaptation which results in a more fit phenotype (Collier et al. 2006) and long-term reflects the genetic adaptation that results in a more fit phenotype, over generations (Collier et al., 2006). The SLICK haplotype gene in Senepol cattle is an example of long-term adaptation and has been introduced to Holstein cows by researchers at the University of Florida (Dikmen et al., 2008; Hansen et al., 2004). The Holsteins with the slick gene have greater sweating rates and milk production in hot months compared to normal Holstein cows (Dikmen et al., 2014). The slick gene makes cattle more thermal tolerant.

Impact of Heat Stress on Rumen, Metabolism and Nutrient Requirements of Dairy Cattle

Acidosis

The excessive loss of CO₂ from panting leads to respiratory alkalosis (West 2003) and the kidneys compensate in part by excreting more bicarbonate in the urine which reduces available buffering capacity in the rumen saliva which has many important functions in ruminants, many of which are impaired by HS. When panting increases salivary buffers, minerals and water are lost from drooling. This can lead to lactic acidosis that can cause ruminitis, metabolic acidosis, laminitis, liver abscesses and sickness (Lean et al., 2000). When rumen pH goes below 6.5, NH₃ is converted to NH₄ transitioning it from a base to a weak acid. When buffering capacity is reduced, VFAs

can lower ruminal pH, and this will lead to reduced VFA production and an increase in lactic acid further decreasing pH (Minuti et al., 2014).

Rumen acidosis can occur during HS. In one study, the mean ruminal pH dropped from ~6.3 to 5.8 when environmental heat load was increased (from 18.3°C to 29.4°C, Mishra et al., 1970). There is also a reduction in rumination associated with the acidosis (DeVries et al., 2009). Acidosis will decrease digestion and increase undigested fermentable carbohydrate concentrations leaving the rumen, and these carbohydrates can reduce pH (Brzozowska et al., 2013). The availability of nonstructural carbohydrates can alter microbial populations and increase the passage of carbohydrates to the hindgut. *Streptococcus bovis* in particular will thrive when large amounts of starch are present and produces lactic acid that is 10 times stronger than VFAs (Russell and Hino, 1985). When lactic acid increases the pH drops making the rumen environment more acidic. An increase in acidity promotes growth of some microbes including *S. bovis* and *Lactobacillus* that will accelerate their growth rate further with decreasing rumen pH. Protozoa and fungi die in acidotic conditions, which reduce the outflow of nutrients from the rumen (figure 1.3).

In addition, the increase in acidity can damage rumen wall epithelium and alter permeability. Lactate can be absorbed across the rumen wall and lower the pH of blood. Bacteria can invade the rumen epithelium and cause ruminitis or make it to the liver where organisms such as *Fusobacterium necrophorum* and *Archanobacterium spp* can cause abscesses (Bolton and Pass, 1988). The liver is vital to gluconeogenesis and performance can be impaired after the occurrence of HS.

When the rumen becomes too acidic the composition of rumen microbiota can change. Coupled with the increased permeability of the rumen, vasoactive substances can alter blood flow and can increase laminitis. These substances include lactate, serotonin, histamine and endotoxins (Westwood and Lean, 2001) and can be absorbed through the compromised rumen and large intestine. Endotoxin release results from acidosis killing gram-negative bacteria in the rumen and the large intestine (Dong et al., 2011). Grain induced ruminal acidosis has been shown to increase gram-negative *Escherichia coli*, a potential source of the endotoxin LPS (Khafipour et al., 2009). Endotoxin can generate a nonspecific immune response called an acute phase response (Ametaj et al., 2010). Infusion of LPS in lactating dairy cows decreased DMI and milk yield and resulted in greater numbers of cows with metabolic disorders such as displaced abomasum compared to control cows (Zebeli et al., 2011).

Hindgut Acidosis

The hindgut fermentation provides 5-10% dietary energy under normal conditions (Gressley, 2011). Decreased rumination and digestion associated with rumen acidosis can increase the passage of fermentable carbohydrates out of the rumen. When excessive fermentable carbohydrates make it to the hindgut and are fermented, hindgut acidosis can occur (Gressley, 2011). Loose stool that is foamy with mucous indicates hindgut acidosis and possibly the sloughing of intestinal lining. Environmental stress often causes hindgut acidosis in lactating dairy cows.

Heat Shock

The occurrence of HS can damage proteins within a cell by causing them to unfold (denature). The up-regulation of the cytoprotective heat shock proteins (HSP) also called chaperones during HS is essential to cell survival because HSPs repair proteins required for normal cellular function by refolding them (Sharma et al., 2009). The cytoskeleton and transport function within epithelial mammary cells is also impaired (Collier et al., 2008). Even though the HSPs benefit the cell there is a nominal cost to repair misfolded proteins. Sharma et al. (2010) under in vitro conditions determined that it cost about 5 ATPs for HSP 70 to repair one protein. However, large scale production of heat shock proteins within mammary cells during HS likely contributes to the decline in protein concentration of milk observed during warm summer months (Bernabucci et al., 2002). The protein synthetic factory of the mammary cell is diverted to increased synthesis of protective HSP's leading to reduced production of milk caseins (Han et al., 2011).

Metabolism

During HS, cellular and whole body metabolism shifts. Glucose becomes a key fuel source that HS cows rely on to remain euthermic and milk synthesis becomes less important (Rhoads et al., 2011). During HS dairy cows have a greater glucose disposal rate and the amount of glucose dedicated to milk synthesis is about 400g glucose/d less in HS cows compared to their pair-fed cohorts (Baumgard et al., 2011). Plasma insulin levels increase with HS which prevent mobilization of NEFAs from adipose tissues despite the negative energy balance. Currently, research indicates that supplying additional glucose alone is not sufficient to prevent these metabolic changes and does

not improve milk yield in HS lactating dairy cows (Itoh et al., 1998). The shift in post absorptive metabolism includes decreases glucose levels, lower circulating NEFAs and reduced insulin-like growth factor-1 (Rhoads et al., 2009).

Nutritional Strategies

Pre and Pro biotics

Prebiotics are compounds in foods and supplements that are not digested but can stimulate growth or influence microbial populations in the gastrointestinal tract (GIT; Ford et al., 2014). This is done by providing a substrate that can be utilized by the microbes to or alter growth of specific strains (Goh and O'Morain, 2003). Supplemental yeast can act as a pre and probiotic. The addition of both inulin and yeast cell wall altered fecal microbial populations and functioned as a prebiotic in dogs (Beloshapka et al., 2013). The addition of a yeast cell wall (beta-glucan) extract altered the immune response and increased feed intake in transport-stressed calves (Eicher et al., 2010). Yeast can improve the immune response in lactating dairy cows and can improve the HS response in lactating dairy cows (Liu et al., 2014) and can increase serum IgA, T and B lymphocytes and adjust neutrophil function in dairy cows (Sanchez et al., 2014). Yeast cultures fed to lactating dairy cows under HS have reduced rectal temperatures (RT: Liu et al., 2014), increased milk yield (MY) (Bruno et al., 2009), down regulated HSPs (Liu et al., 2014) and an altered immune response (Magalhaes et al., 2008) in dairy cows.

The use of probiotics in lactating dairy cows is a widely accepted method to maintain a healthy rumen and improved production (Nocek and Kautz, 2006). Probiotics are microbes that are included into diets to promote favorable microbial populations,

improve immunity and digestion, and are direct-fed microbials (DFMs). Most DFMs include active cultures or vegetative forms of bacteria and yeast. Additionally, DFMs can improve anaerobiosis, maintain pH, and improve feed efficiency and blood metabolites (Nocek and Kautz, 2006; Nocek et al., 2002). The rumen microbiota is responsible for the majority of energy requirements. Rumen fermentation supplies gluconeogenic precursors, microbial proteins, and other gasses such as methane (Qiao et al., 2010; Berthiaume et al., 2010).

The effectiveness of a probiotic may depend on the flora from individual cows or dairies. Changes of host microbial populations to environmental/ nutritional stimuli can be host specific. A study out of Lethbridge, AB, Canada looked at changes in ruminal variables in Holstein heifers in their first lactation fed a high concentrate or high forage diet (Mohammed et al., 2012). Each animal was classified as least, intermediate and most acidotic. Variables included changes in pH and bacterial community compositions (BCC). Production responses were similar between groups and not effected by rumen acidosis. Differences in BCC were seen in individuals and not specific to treatment or classification (Mohammed et al., 2012).

Enhancing production can be accomplished through different methods and strains of probiotics. Additionally, each product has other claims and was placed in grouping by research, observation or claims. If DFMs can reduce acidosis, promote beneficial microbial flora or elicit a favorable immune response, the impact, severity and duration of hyperthermia may be reduced.

Benefits have been documented when DMFs were fed to HS dairy cows. Shwartz et al., (2009) fed two strains of yeast with endogenous enzymes to HS lactating dairy cows. Though there were no production differences, treatment reduced rectal temperature at 1200 and 1800 h. To address the challenges of HS, the approach will likely address different aspects of HS.

Yeast extract that contain oligosaccharides can act as a prebiotic and promote favorable microbial growth. Future research with cocktails/ combinations of probiotics and other promoters may answer broader basic and applied questions. Celmanax[®] contains hydrolyzed yeast, yeast extracts and yeast culture and has a claim to improve milk production and reduce somatic cell count. Research supports the findings that yeast cultures can influence microbial metabolism (Miller-Webster et al., 2002) and can stimulate lactic acid utilizing bacteria (Nisbet and Martin, 1991).

The use of probiotic *Lactobacillus GG* (ATCC strain number 53103) in young adult mice colon (YAMC) cells induced heat shock expression. Expression was induced in TN and HS (Tao et al., 2006).

Preconditioning animals for HS needs to be further studied. DMFs can be fed during non-stressed conditions to prevent the amplification of metabolic disturbances during HS.

B-vitamins

Early findings suggest that the microbes in the rumen produce sufficient B-vitamins. More recent research has shown that cows benefit from supplementation of specific B-vitamins including niacin (Schwab et al., 2005), thiamin (Shaver and Bal, 2000)

and biotin (Zimmerly and Weiss, 2001). During HS, rumination time decreases with severity (Soriani et al., 2013); feed intake decreases and metabolic demands change (Rhodes et al., 2009). If dairy cows benefit from supplemental B-vitamins under normal production, the demand during HS could also increase. Some B-vitamins are extensively destroyed before reaching the small intestine (Santschi et al., 2005). B-vitamins include many important intermediates that act as coenzymes in normal metabolism.

Betaine (BET)

Betaine is synthesized from choline into BET aldehyde and then into glycine BET (Rathinasabapathi et al., 1994) in plants and is derived from dietary choline in animals, but is also found in feedstuffs (beets and wheat). There are three methyl groups on BET that can be used to synthesize numerous other substances, and as seen in pigs, has lipotropic (ability to mobilize NEFAs to be repartitioned for growth, or potentially lactation) effects (Eklund et al., 2005).

Betaine is an organic osmolyte that has been shown to promote favorable bacterial growth under stressed condition (Wdowiak-Wrobel et al., 2013) including fluctuations in pH (Laloknam et al., 2006). Natural BET is typically purified from beet molasses. In plants, microbes (Lai and Lai, 2011), and squid (Petty and Lucero, 1999) betaine has been shown to reduce oxidative stress, namely elevated salinity levels.

Furthermore, BET has been shown to reduce oxidative stress in liver cells (HepG2) induced by the addition of ethanol (Oliva et al., 2011). Additionally, BET has been used to reduce HS in poultry (Sayed and Downing, 2010), improve feed efficiency

in swine (Wray-Cahen et al., 2004), and increased milk yield under normal conditions in dairy cows (Peterson et al., 2009). As an osmolyte, betaine has the potential to reduce cellular strain by minimizing the need for sodium/ potassium pumping (Moeckel et al., 2002), freeing resources. This can spare ATP and promote transcription or cell proliferation (Eklund et al., 2005).

The structure and charges of BET allow it to act as an osmolyte. Betaine is a zwitterion (Lever and Slow, 2010) that has both a positive and a negatively charged region. There is no net charge with a zwitterion yet the negative region can bind to water. This ability to retain water under conditions of dehydration resulted in improved survival and growth under stress in *E. coli* (Capp et al., 2009). Intercellular betaine can hold water against a gradient yet provide living cells with usable water. The cation and anion balance can also be altered by BET as a zwitterion, as some derivatives of betaine demonstrated an affinity for strong cations (Shao et al., 2011).

The native folding of proteins can be disrupted by stress, crowding and mutations. The damaged protein can reduce cell function and cause apoptosis (Sharma et al., 2009) Betaine acts as a molecular chaperone. The HSP response is vital in maintaining cellular function in the wake of stress and damage within cells. There is a cost (ATP) associated with HSP correcting a damaged protein. Sharma et al., (2010) estimated that HSP70 uses 5 ATP to fix and denatured protein. Betaine can act as a chaperone in mammals (Roth et al., 2012), plants (Wani et al., 2013) and microbes (Morsy et al., 2010) under stress.

The use of BET in the diets of dairy cows and in cell culture of mammary tissue is a likely fit. Lactating Holstein cows are prone to HS, and BET has shown promise in other animals and in non-heat-stressed dairy cows. Betaine is a unique compound that has been classified as a chemical prebiotic involved in methyl transfer reaction in cells (Waddell et al., 2000), a methyl donor (Obeid, 2013), and BET is a micronutrient for microbial cells that increase uptake during osmotic stress (Capp et al., 2009).

β -Hydroxybutyrate

Ruminants rely on anaerobic fermentation for the majority of their caloric needs. Useable products of fermentation include volatile fatty acids, microbial protein, odd and branched chain fatty acids, and vitamins (French et al., 2012). Fermentation can also yield major losses of energy in the form of CO₂, NH₃, CH₄, and H₂S. After weaning, ruminants supply the bulk, if not all of their glucose requirements by gluconeogenesis. Microbial fermentation breaks down carbohydrates (mono di and polysaccharides) and more complex structural carbohydrates like cellulose that are not enzymatically reduced in mammals. The microbes catabolize these with other nutrients and produce microbial protein biomass and VFAs. The VFA production supplies around 70% of the energy needs for ruminants (Bergman 1990). The microbial protein biomass includes microbes and proteins that eliminate essential amino acids supplementation (assuming adequate feedstuffs). Under normal conditions, the rumen supplies the needed B vitamins for the host.

The three predominant VFAs produced in the rumen, colon, and cecum are acetate, butyrate and propionate (Richardson et al., 1976). Acetic acid is used for

energy production (ATP), typically outside of the liver, is a major source of acetyl CoA, and the acetyl CoA is the major source for lipogenesis in ruminants (Bauman et al., 1972). Propionic acid is transported from the portal system to the liver as a gluconeogenic precursor. The hepatic production of glucose is vital in ruminants as dietary glucose is not available and is not absorbed in the small intestine. The major gluconeogenic precursor in dairy cows is propionate (Aschenbach et al., 2010). The bulk of the butyric acid is synthesized by the rumen epithelium into the ketone β -hydroxybutyrate (DeFrain et al., 2004). It is difficult to understand the implications of butyrate and BHB in the ruminant with the current research.

Fatty acids cannot be used for gluconeogenesis. The glycerol from triglycerides can be used to synthesize glucose, but the balance is used for energy production in the Krebs cycle, or used to produce ketones during β -oxidation. The oxidation of fatty acids occurs in the mitochondria and peroxisome. Peroxisomal β -oxidation occurs in the liver and kidneys and is responsible for the degradation of very-long-chain fatty acids and branched chain fatty acids (Yang et al., 2014). This yields acetyl CoA and fatty acids (palmitoyl CoA). The fatty acids are then further oxidized in the mitochondria.

Mitochondrial β -oxidation is responsible for breakdown of most fatty acids. Acetyl CoA is produced from β -oxidation. Acetyl CoA is an important intermediate that can be used in the Krebs cycle to produce energy and net about 10 ATP per mole of acetyl CoA.

When malate, an intermediate in the Krebs cycle is absent, acetyl CoA cannot enter the Krebs cycle. Malate is absent when it is needed for glucose synthesis and becomes a rate-limiting factor in the Krebs cycle. Accumulations of acetyl CoA favor

ketogenesis. Acetoacetate is formed from two acetyl CoAs, and is one of three ketones produced. The synthesis of acetone or β -hydroxybutyrate from acetoacetate then occurs. β -hydroxybutyrate is the predominate ketone produced.

Also, BHB is positively correlated with maximum THI and positively correlated with rumination time during HS (Sorian et al., 2013). Butyrate is known to inhibit cell proliferation by reducing expression of cyclin D1, blocking the cell cycle at the G1 phase (Siavoshian et al., 2000). There have also been reports indicating that some cell lines (Siavoshian et al., 2000) and rumen epithelial cells (in vivo, goat) have an increased proliferation with supplemental butyrate (Malhi et al., 2013). The production of BHB is up regulated during HS. This is evident in lactating dairy cows that have decreased plasma NEFA levels despite the negative energy balance. This should reduce the acetyl CoA available for ketogenesis. Additionally, BHB levels increase during HS by enzymatic up regulation.

Niacin induces flushing by activating the GPR109a receptor resulting in the release prostaglandins (Ingersoll et al., 2012). Niacin also causes cutaneous flushing by activating the capsaicin receptor TRPV1 even without heat dependent activation (Ma et al., 2014). The vasodilation associated with the GPR109a receptor is important as BHB acts as a competitive ligand to niacin (Taggart et al., 2005). Through the activation of the GPR109a receptors on adipocytes, BHB also negatively regulates ketogenesis by blocking lipolysis (Taggart et al., 2005).

Cell proliferation is reduced by BHB *in vitro*. Cell proliferation was reduced at the G₀/G₁ phase, and apoptosis was increased in a dose dependent manner (0.1 to 10

mmol/l BHB) in HK-2 cells treated with BHB (Guh et al., 2003). Cell mortality in bovine abomasum smooth muscle cells (BSMCs) treated with BHB increased about four fold at 1.2 mM and six fold at 4.8 mM from baseline (Tian et al., 2014). The expression (mRNA) of cyclins and CDKs decreased with increasing doses of BHB in the BSMCs (Tian et al., 2014). Throughout the lactation of a dairy cow, mammary cell proliferation occurs at a rate of about 0.3%, and undergoes daily apoptosis at a rate of approximately 0.56% (Capuco et al., 2006). Increasing levels of apoptosis and decreasing cell proliferation with BHB levels seen during HS could increase milk losses during stress and after stress in lactating dairy cows.

The infusion of BHB into lactating dairy cows resulted in plasma BHB concentrations of 1.74 ± 0.02 mmol/L. There was no decrease in feed intake, milk production but did decrease plasma insulin (Zarrin et al., 2013). The cows were infused for 2 days, but feed intake typically decreases with HS over a longer period of time. The infusion of MAA in dairy cows with a bolus $300 \text{ mmol/kg body weight}^{0.75}$ MAA or saline indicated that there was no change in feeding behavior (Choi et al., 1997). The may not be high enough to elicit a feed intake response.

The aim of my dissertation was to test effects of pre and pro biotics and micronutrients to reduce the impact of HS in lactating dairy cows. I will measure changes from TN to HS in lactating dairy cows on these nutritional supplements in production, physiology, and changes in metabolites. The role of the substrate BHB on feed intake during HS will be measured by infusing a bolus of BHB to determine changes

in DMI and a bolus of MAA will be infused to test the impact of reducing the appearance of BHB during HS on DMI.

SUMMARY

The challenges associated with managing high producing dairy cows exposed to HS are formidable. As average milk production per cow in the US has increased so has their sensitivity to HS. Altering the environment by improving facilities has been the predominate approach to reducing the response to HS. However, nutritional management strategies are also key to maximizing productivity of high producing dairy cows exposed to HS.

The paradox of nutritional supplementation of high producing dairy cows exposed to heat stress is that if the supplement increases milk production under TN conditions this response may be lost when the cow is exposed to heat stress. Higher production increases metabolic heat. There were three feed additives tested for my dissertation research, and all three increased milk production on the dairy or at the ARC.

The betaine treated cows had higher milk yields during TN and the advantage was lost during HS. Calsporin fed cows had higher milk yields during TN and when exposed to thermal stress, their milk production decreased more than controls. Cows fed OmniGen-AF (OG) also had numerically higher milk yields at the dairy and ARC compared to controls. At the ARC, the OG fed cows had numerically higher milk yield in both TN and HS. OmniGen-AF fed cows also displayed a reduced stress response when exposed to HS and had a lower cortisol response during acute heat stress (first day of stress). Paradoxically, cows fed OG had significantly higher ACTH concentrations at all times tested. This suggests that the hypothalamic-pituitary-adrenal axis (HPA) in OG fed animals was altered. This finding deserves further work in elucidating the cause of the elevated ACTH concentrations in OG fed animals. If this can be confirmed it suggests that much of the reduced stress response in OG fed animals is associated with the altered HPA.

The effects of BET are also not fully understood. Milk yield was higher during TN and glucose levels were greater during HS compared to controls and this confirms previously published work. Body temperatures were higher in betaine fed cows, and the MID dose had higher body temperatures than the HI cows. The cause of the higher core temperatures in animals fed BET is not understood. It is also difficult to understand where the betaine was utilized. The rumen microbes possibly used the betaine with little to no betaine absorbed into the portal system to be used by host cells as a chaperone or an osmolyte. This may have led to a higher rumen temperature which could be confirmed in additional work with rumen temperature monitors. Future

research looking at the VFA changes with BET feeding during HS and TN could be beneficial to provide insight to the increased plasma glucose levels during HS. Changes in serum BET levels using high-performance liquid chromatography (**HPLC**) could provide important information on the levels of betaine needed to benefit GIT microbes and the host cells.

The addition of BET also improved feed to milk efficiency during HS. If BET is able to provide protection to the rumen microbes during stress, a longer recovery period at the ARC may identify benefits to HS recovery.

Calsporin supplementation benefits were seen during TN in this study. Microbial populations in the feces were measured, but not in the rumen. Changes within the rumen are important to understand the full effects of this DFM. If microbial populations are more consistent in the rumen during HS, faster recovery and better immune response may result. Larger studies in commercial dairies could provide important information on the benefits of feeding Calsporin to lactating dairy cows.

The changes seen with feeding OG are the most promising in combating the effect of HS in lactating cattle. The degree of HS was mild, but enough to elicit a physiological response. Feed intake, milk yield, cortisol levels, RR and RT were all similar during HS to the TN levels. The OG fed cows did not experience HS to the degree that the CON cows did. The role of ACTH and cortisol in OG fed cows during HS needs to be further explored. Larger studies on dairies with more pens could strengthen the findings of this study and cover cool months, then HS and follow the cows after thermal stress.

The BHB results are inconclusive. β -Hydroxybutyrate did not alter feed intake in TN cows. The elevated BHB may be responsible for minor decreases in feed intake during HS, but treating cows at TN with levels at and greater than those seen during HS did not (Zarrin et al., 2013). Changes in hormones or growth factors are possible different during HS, and this may mask the effects of exogenous BHB during TN on feed intake. Changes in pH may alter the uptake of BHB into cells, and competing substrates like pyruvate and lactate may impede BHB uptake. Ruminants could have greater tolerances to ketones as they are in a constant gluconeogenic state, and tissues may have an adaptive ability to utilize ketones for energy.

Feeding OG reduced the impact of HS on milk yield and feed intake, possibly from changes in endocrine responses. The other products tested in this dissertation research increased or had no effect on HS in lactating cows. Identifying the mechanism is fundamental to understanding the changes seen in this set of studies. The change in immune and endocrine responses during HS could alter physiological responses to HS. This would reduce the energy needed to support HS cows in mild to moderate stress.

Supplemental OG reduced the impact of HS and maintained DMI, reduced serum cortisol levels on the first day of HS, decreased RR at RT, and the cows produced more milk compared to controls. The other feed additives were not helpful to the overall reduction of HS.

Figure 1.1 Thermal Heat Index Adapted for Dairy Cows (Zimbleman et al, 2009)

Temperature		% Relative Humidity																				
°F	°C	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
72	22.0	64	65	65	65	66	66	67	67	67	68	68	69	69	69	70	70	70	71	71	72	72
73	23.0	65	66	66	66	67	67	67	68	68	68	69	69	70	70	71	71	71	72	72	73	73
74	23.5	65	66	66	67	67	67	68	68	69	69	70	70	70	71	71	72	72	73	73	74	74
75	24.0	66	66	67	67	68	68	68	69	69	70	70	71	71	72	72	73	73	74	74	75	75
76	24.5	66	67	67	68	68	69	69	70	70	71	71	72	72	73	73	74	74	75	75	76	76
77	25.0	67	67	68	68	69	69	70	70	71	71	72	72	73	73	74	74	75	75	76	76	77
78	25.5	67	68	68	69	69	70	70	71	71	72	72	73	73	74	74	75	75	76	76	77	78
79	26.0	67	68	69	69	70	70	71	71	72	73	73	74	74	75	75	76	76	77	77	78	79
80	26.5	68	69	69	70	70	71	72	72	73	73	74	74	75	75	76	76	77	78	78	79	80
81	27.0	68	69	70	70	71	72	72	73	73	74	74	75	75	76	77	77	78	78	79	80	81
82	28.0	69	69	70	71	71	72	73	73	74	74	75	75	76	77	77	78	79	79	80	81	82
83	28.5	69	70	71	71	72	73	73	74	74	75	75	76	77	78	78	79	80	80	81	82	83
84	29.0	70	70	71	72	73	73	74	74	75	75	76	77	78	78	79	80	80	81	82	83	84
85	29.5	70	71	72	72	73	74	74	75	75	76	77	78	78	79	80	81	81	82	83	84	85
86	30.0	71	71	72	73	74	74	75	75	76	77	78	78	79	80	81	81	82	83	84	84	85
87	30.5	71	72	73	73	74	74	75	76	77	77	78	79	80	81	81	82	83	84	85	85	86
88	31.0	72	72	73	74	75	75	76	76	77	78	79	80	81	81	82	83	84	85	86	86	87
89	31.5	72	73	74	75	75	76	77	78	79	80	80	81	82	83	84	85	86	86	87	88	89
90	32.0	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	86	87	88	88	89	90
91	33.0	73	74	75	76	76	77	78	79	80	81	82	83	84	85	86	86	87	88	89	90	91
92	33.5	73	74	75	76	77	78	79	80	81	82	83	84	85	85	86	87	88	89	90	91	92
93	34.0	74	75	76	77	78	79	80	80	81	82	83	84	85	86	87	88	89	90	91	92	93
94	34.5	74	75	76	77	78	79	80	81	82	83	84	86	86	87	88	89	90	91	92	93	94
95	35.0	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95
96	35.5	75	76	77	78	79	80	81	82	83	85	86	87	88	89	90	91	92	93	94	95	96
97	36.0	76	77	78	79	80	81	82	83	84	85	86	87	88	89	91	92	93	94	95	96	97
98	36.5	76	77	78	80	80	82	83	83	85	86	87	88	89	90	91	92	93	94	95	96	98
99	37.0	76	78	79	80	81	82	83	84	85	87	88	89	90	91	92	93	94	95	96	98	99
100	38.0	77	78	79	81	82	83	84	85	86	87	88	90	91	92	93	94	95	96	98	99	100
101	38.5	77	79	80	81	82	83	84	86	87	88	89	90	92	93	94	95	96	98	99	100	101
102	39.0	78	79	80	82	83	84	85	86	87	89	90	91	92	94	95	96	97	98	100	101	102
103	39.5	78	79	81	82	83	84	86	87	88	89	91	92	93	94	96	97	98	99	101	102	103
104	40.0	79	80	81	83	84	85	86	88	89	90	91	93	94	95	96	98	99	100	101	103	104
105	40.5	79	80	82	83	84	86	87	88	89	91	92	93	95	96	97	99	100	101	102	103	105
106	41.0	80	81	82	84	85	87	88	89	90	91	93	94	95	97	98	99	101	102	103	104	106
107	41.5	80	81	83	84	85	87	88	89	91	92	94	95	96	98	99	100	102	103	104	106	107
108	42.0	81	82	83	85	86	88	89	90	92	93	94	96	97	98	99	100	101	103	104	105	108
109	43.0	81	82	84	85	87	89	89	91	92	94	95	96	98	99	101	102	103	105	106	108	109
110	43.5	81	83	84	86	87	89	90	91	93	94	96	97	99	100	101	103	104	106	107	109	110
111	44.0	82	83	85	86	88	90	91	92	94	95	96	98	99	101	102	104	105	107	108	110	111
112	44.5	82	84	85	87	88	90	91	93	94	96	97	99	100	102	103	105	106	108	109	111	112
113	45.0	83	84	86	87	89	91	92	93	95	96	98	99	101	102	104	105	107	108	110	111	113
114	45.5	83	85	86	88	89	92	92	94	96	97	99	100	102	103	105	106	108	109	111	112	114
115	46.0	84	85	87	88	90	92	93	95	96	98	99	101	102	104	106	107	109	110	112	113	115
116	46.5	84	86	87	89	90	93	94	95	97	98	100	102	103	105	106	108	110	111	113	114	116
117	47.0	85	86	88	89	91	93	94	96	98	99	101	102	104	106	107	109	111	112	114	115	117
118	48.0	85	87	88	90	92	94	95	97	98	100	102	103	105	106	108	110	111	113	115	116	118
119	48.5	85	87	89	90	92	94	96	97	99	101	102	104	106	107	109	111	112	114	116	117	119
120	49.0	86	88	89	91	93	95	96	98	100	101	103	105	106	108	110	111	113	115	117	118	120

- Stress Threshold Respiration rate exceeds 60 BPM. Milk yield losses begin. Repro losses detectable. Rectal Temperature exceeds 38.5°C (101.3°F)
- Mild-Moderate Stress Respiration Rate Exceeds 75 BPM. Rectal Temperature exceeds 39°C (102.2°F)
- Moderate-Severe Stress Respiration Rate Exceeds 85 BPM Rectal Temperature exceeds 40 °C (104°F)
- Severe Stress. Respiration Rate 120-140 BPM. Rectal Temperature exceeds 41 °C (106°F)

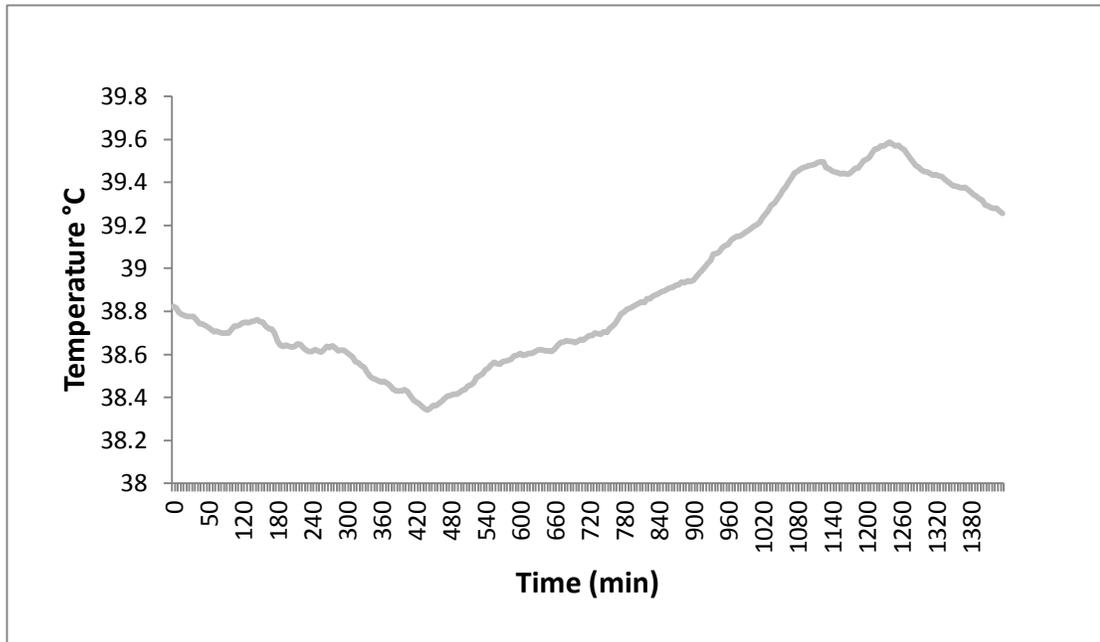
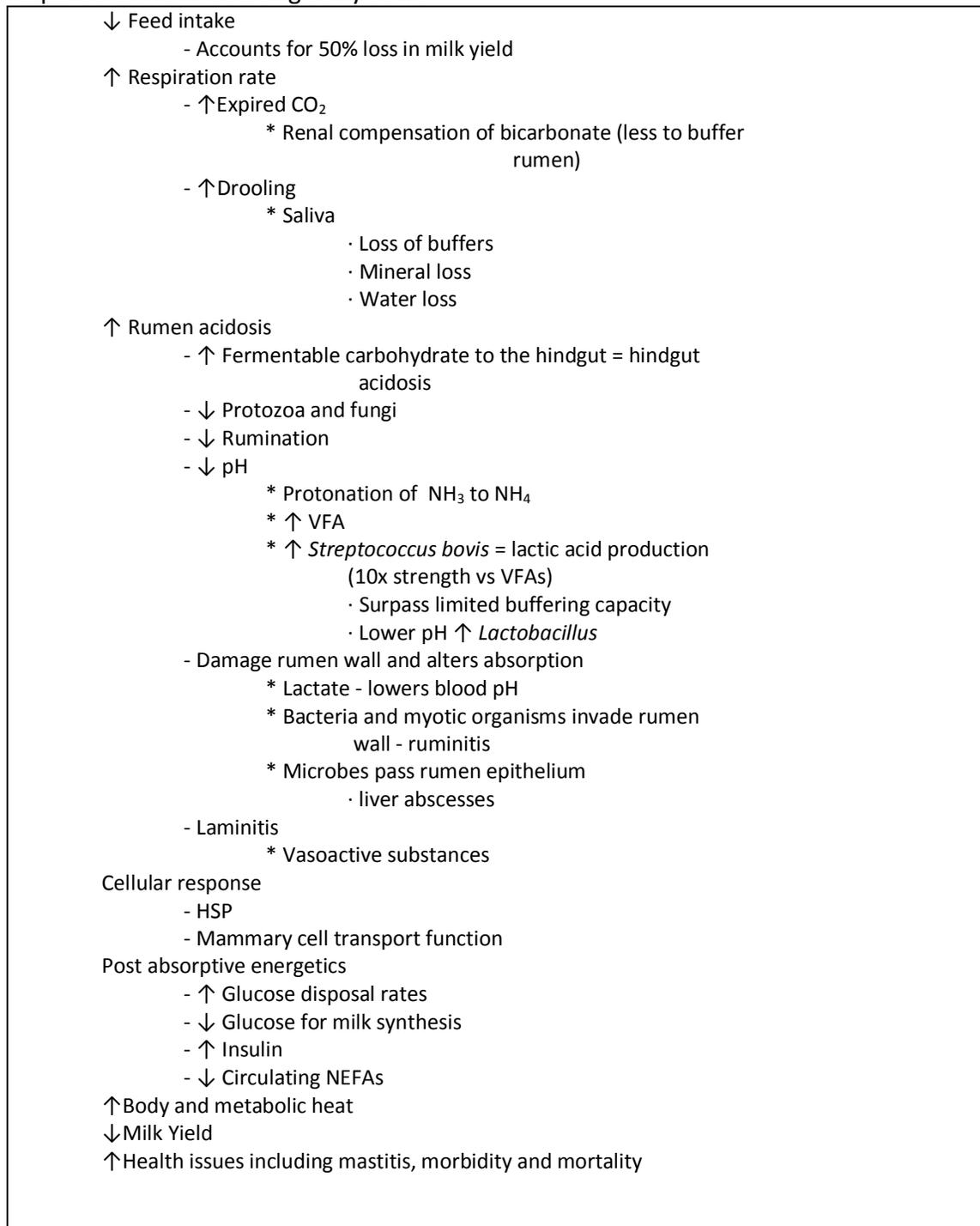


Figure 1.2. Mean vaginal temperatures in lactating dairy cows averaged the first two days of HS in the environmental rooms at the University of Arizona.

Figure 1.3. Summary of production, physiological and metabolic changes associated with response to HS in lactating dairy cows



CHAPTER TWO: EVALUATION OF DIETARY BETAINE (BET) IN HEAT-STRESSED HOLSTEIN COWS DURING LACTATION

Abstract

Betaine, a natural, organic osmolyte may improve cellular efficiency and act as a chaperone, refolding denatured proteins. To test if dietary BET reduced the effect of HS in lactating dairy cows, multiparous, lactating Holstein cows ($n = 23$) were blocked by days in milk (**DIM** 101.4 ± 8.6) and randomly assigned to one of 3 doses of dietary BET; control (**CON**) group no BET, mid dose (**MID**; 57 mg of BET / kg BW), and high dose (**HI**; 114 mg of BET / kg BW). Cows were fed twice daily and treatments were top-dressed at each feeding. Cows were milked 2 times/ d and milk samples were taken daily for analysis. Milk components, yield, feed intake, and water intake records were taken daily. Temperature and respiration rates were taken 3 times/ d at 0600, 1400, and 1800. Cows were housed in environmentally controlled rooms and following acclimation for 7 d at **TN** with a mean THI of 56.6. They were then exposed to 7 d of TN followed by 7d of **HS** represented by a THI of 71.5 for 14 d. This was followed by a recovery period of 3 days at TN. Temperature data loggers were inserted vaginally to read the last day of TN and the first 2 d of HS to follow body temperature changes. Dietary BET increased milk yield and percent protein during the TN period ($P < 0.01$). There were no differences between treatments in total milk production or percent protein during HS. Feed and water intake decreased during HS with betaine treated (CON vs. MID, and CON vs. HI) cows ($P < 0.01$) with no difference in TN. The cows on Con diets had higher pm HS respiration rates than both MID and HI treatments ($P = 0.04$, $P = 0.001$ respectively), but

lower HS body temperature compared with BET treatments ($P < 0.05$). Vaginal temperatures were higher in BET treated cows and were highest in MID. There was no difference in plasma glucose during TN, but cows given HI had elevated glucose levels during HS compared to CON ($P = 0.0074$). There were no differences in plasma insulin levels between treatments, but there was an environment effect ($P = 0.01$). The heat shock response (HSP27 and HSP70) was upregulated in BMECs and in the cows HSP27 was up regulated and HSP70 was down regulated. There was no effect seen with the MID dose with HSP 27 or HSP 70. The HI dose upregulated HSP27, and down regulated HSP70. We conclude that BET increased milk and protein production in TN conditions and was associated with reduced feed and water intake and slightly increased body temperatures during HS at the doses tested. Though the advantage in milk production was lost during HS with HI BET, plasma glucose levels increased during HS.

Introduction

Betaine is similar to a molecule of glycine with three methyl groups and is also called trimethylglycine. This molecule is a zwitterion that has both a positive and negatively charged region. Betaine anhydrous can be synthetic or a coproduct of sugar beet extraction. This compound has many activities that may reduce the impact of HS in lactating dairy cows and improve general production. These include the fact that BET is an organic osmolyte (Hammer and Baltz, 2002), a molecular chaperone (Sharma et al., 2009), has been shown to decrease susceptibility of stress to microbial populations (Lai and Lai, 2011) acts as an antimicrobial to some bacteria like *Salmonella typhimurium* (Lindstedt et al., 1990), can be utilized as a nutrient and has been demonstrated to increase milk production (Wang et al., 2010). Organic osmolytes are a class of

compounds that do not rely on ions and ion pumps to maintain cellular osmolarity though the transport of BET across cell membranes through the betaine-choline-carnitine (BCC) is Na^+ coupled (Perez et al., 2011). The osmoregulation is also seen in microbial populations and has been shown to promote favorable bacterial growth under stress conditions (Wdowiak-Wrobel et al., 2013) including fluctuations in pH (Laloknam et al., 2006). Fluctuations (pH) within the rumen (Mishra et al., 1970) and the hindgut (Gressley, 2011) have been documented during HS. The pumping of ions reduces the cellular energetic cost adenosine triphosphate (ATP) of maintaining osmolarity (Kettunen et al., 2001) during HS when electrolytes and water may be limited to cells. Betaine is a zwitterion and has a net neutral charge, but has a region of positive and negative charge. This allows BET to hold water molecules (intracellular) against a concentration gradient, yet the affinity is not so much that the water is unavailable to the cell.

Under hyperthermic conditions damage to proteins and machinery within cells occurs (Morimoto and Santoro, 1998). Molecular chaperones, namely heat shock proteins (HSP) work to refold denatured structures and thus maintain function as an adaptive response. There is a cost associated with the use of HSPs. Sharma et al. (2010) estimated the cost for one HSP70 to fix a damaged protein at 5 ATP molecules. Betaine has the ability to function as a molecular chaperone (Hall and Collier, 2013). Osmolytes can prevent this type of damage by stabilizing cellular proteins and refold unfolded peptides (Diamant et al., 2001) and allowed the disaggregation and refolding of heat damaged proteins (Diamant et al., 2003). Glycine BET increased the metabolic efficiency

of glucose in *E. coli* cells stressed with salt (Metris et al., 2014). Heat stress causes decreased DMI and there are increased energy demands due to the disruption of homeostasis (Rhoads et al., 2011), thermoregulation and repairing damaged tissues. Another advantage that organic osmolytes have over salts is that they do not interfere with native enzymatic activity within cells (Nakanishi et al., 1990).

Betaine has two potential sites of action in lactating ruminants; mammalian cells and the microbes in the gastrointestinal tract. Under different types of stress, both microbial and animal cells increase uptake of exogenous betaine (Nakanishi et al., 1990). Renal medullary cells utilize BET and other organic osmolytes to maintain cell function under different levels of hydration and fluctuating concentrations of NaCl (Nakanishi et al., 1990). Increased levels of stress can affect rumen microbes, rumen epithelial cells, liver function and metabolism. Rumination time in heat-stressed lactating dairy cattle is negatively associated with an increased respiration rate and positively correlated with milk yield (normal conditions). Additionally, rumination time decreased 2.2 min/day for every unit of THI above 76 (Soriani et al., 2013). Buffer losses from increased drooling associated with HS induced panting increase susceptibility for weaker acids like volatile fatty acids (VFAs) to decrease the pH and can increase the production of lactic acid that is 10 times stronger than VFAs (Russell and Hino, 1985). This reduces the pH further, can cause cell death, alters microbial populations, and can damage the rumen wall. Bacteria can invade the rumen epithelium and cause ruminitis and make it to the liver where organisms such as *Fusobacterium necrophorum* and *Archanobacterium spp* can cause abscesses (Bolton and Pass, 1988).

There are benefits of supplementing BET in lactating dairy cow diets that have been demonstrated, under TN conditions. These include increased milk production (Peterson et al., 2012, and Wang et al., 2010) increased VFA production and higher fat-corrected milk yield (Wang et al., 2010).

Rumen utilization and degradation of betaine would limit availability to animal cells. However, Nakai et al. (2013) indicate that fed betaine is found in the duodenal digesta indicating that some betaine passes the rumen.

The objective of this study was to evaluate the role of dietary betaine in lactating HS dairy cows. We will measure the expression of HSP27 and HSP70 *In vitro* and in the buffy coat of lactating dairy cows treated with betaine to understand the its role as a molecular chaperone. Production parameters will be compared against control to understand the effects of betaine during TN and HS conditions. We hypothesize that dietary BET will reduce the impact of HS and improve the HSP and a production responses.

We hypothesized that BET would reduce the impact of heat stress by improving milk yield, reducing the need (cost) of heat shock proteins, and increase available energy from rumen fermentation during heat stress in lactating dairy cows.

Materials and Methods

Cell culture:

Primary bovine mammary epithelial cells (BMEC) were harvested, isolated and prepped according to Collier et al. (2006). Then the cells were thawed from liquid nitrogen storage and suspended in Dulbecco's Modified Eagle Medium (DMEM)/F-12

(Gibco, Life Technologies, Grand Island, NY) and mixed in neutralized collagen and cultured (Collier et al., 2006) in 24-well plates (Falcon, BD Biosciences, San Jose, CA). The collagen was added in two steps, as the base layer was allowed to gel for 5 m and the second layer containing the cells was seeded into each well for a final volume of 500 μ l. The (BMECs) were grown in collagen that was extracted from rat-tails and allowed to grown at 37°C for 7 days (McGrath, 1987; Collier et al, 2006) with media changed every 48 h.

The serum-free DMEM/F-12 media contained 0.1 % BSA, antibiotic-antimycotic (100 U/ml penicillin, 100 μ g/ml streptomycin and 0.25 μ g/ml amphotericin B; 15240, Invitrogen Corp, Carlsbad, CA). The added growth factors included IGF-1 (recombinant human, 100 ng/ml; NIDDK, Torrance CA), EGF (recombinant human, 25 ng/ml; 13247-051, Invitrogen Corp, Carlsbad, CA) and progesterone (25 μ l of 20 μ g/ml; Zimbelman, 2008) per 100 ml media.

The seeded cells were grown in collagen for 7 d and media was changed every 48 h. On d 8 cells were treated with 0 mM betaine or 25 mM BET and one set of plates was subjected to HS (42°C) and the other set remained at 37° C for 24 h. There were six wells per treatment per environment and two wells of each subset were combined and placed in TRIzol reagent. Samples were homogenized, ribonucleic acid (RNA) isolated, purified using Qiagen RNeasy Kit (Qiagen, Valencia, CA), treated with DNase (DNase I, Invitrogen, Grand Island, NY), cDNA was synthesized (iScript cDNA Synthesis kit, Bio Rad, Hercules, CA), and quantitative real-time polymerase chain reaction (qPCR) using the Bio Rad iQ5 optical system (Bio Rad, Hercules, CA) was conducted following the procedures

described by Hernandez et al, (2009). The expression of HSP70 and HSP 27 were quantified using the calibrator gene 40S ribosomal protein S9 (RPS9) for calculating gene expression data based on the $2^{-\Delta\Delta Ct}$ method. A one-way ANOVA was conducted on ΔCt value with the PROC MIXED procedure of SAS. Data is presented in fold change.

Lactation study:

This study was conducted on a protocol approved by the Institutional Animal Care and Use Committee of the University of Arizona, Protocol Number 11-295. Cows for this study were obtained from Caballero Dairy, Eloy Arizona. Twenty four cows were randomly assigned to treatment after balancing for parity ($n \geq 2$), previous lactation record, current lactation records and DIM in a replicated factorial design. Treatments were CON (0 mg/kg BW betaine), MID betaine (57 mg/kg BW), and HI dose betaine (114 mg/kg BW). After selection, cattle were shipped to the University of Arizona, West campus agricultural center, in the William J. Parker Agricultural Research Center (**ARC**) for 31 days. Animals were housed in two controlled environment chambers (six cows each, balanced for treatments) and subjected to TN conditions and HS. The two environmental rooms had the same temperature, humidity and light/ dark schedule. The control of both rooms was identical as the different rooms were controlled by the same computer, and verified 3 times per day when respiration rate (RR) and rectal temperature (RT) were measured. The study was replicated with an additional set of 12 cows for a total of 24 cows. Upon arrival at the ARC cows were weighed, fitted with a halter and randomly assigned to a treatment, room and a tie-stall. Each room had 2 each of CON, MID and HI treatment cows. The cows were given 7 days at TN (figure 2.1)

to adapt (acclimation period) to the new diet, the tie stall and new milking procedures. The cows were then subjected to an additional 7 d of TN followed by the HS (figure 2.3) period that lasted 14 days. At the end of each study the animals were given 3 d of post HS recovery before returning to the dairy (figure 2.1).

Cows were individually fed 2 times /d at 500 and 1700. Refusals were removed and weighed at 445 daily. Individual water consumption (gal/d) was recorded. Cows were milked between 530 and 630 am, and from 1730 to 1830 daily. Milk weight was recorded at each milking. Samples were individually stored with a preservative (bronopol tablet, D&F Control Systems, San Ramon, California) at 4°C. Aliquots were analyzed by Arizona DHIA (Tempe, Arizona) by infrared for butter fat, protein, somatic cell count (SCC), lactose and solids-not-fat (SNF). Anhydrous BET, BetafinS4 (Danisco Animal Nutrition, St. Louis MO), a byproduct of sugar beet production was the source of the fed supplement. Betaine was measured out based on individual BW and divided into two equal half doses fed at each meal. The CON group received 0 mg/kg bw/d, the MID dose was fed at 57 mg/kg bw/d and the HI was 114 mg/kg bw/d. The supplement was mixed into the top one-third of all rations with cane molasses as a binder. The base total mixed ration (TMR) was alfalfa based with steam flaked corn, whole cottonseed, bypass fat (Maxxer, Tarome Inc., Eloy, AZ), water and a high producer dry mineral premix (Table 2.1). The TMR was 47% moisture with 65% concentrate and 35% concentrate. The ration was balanced to meet the energy requirements of the silage-based diet fed to the cows at the dairy prior to arrival at the ARC (table 2.1).

Physiological parameters were recorded 3 x/ d (500, 1400, 1700). Skin temperature was measured at the tail head, shoulder and rump using a Raytek® (Raytek Corporation, Santa Cruz, CA) infrared thermometer, and rectal temperature were measured using a GLA® M700 digital thermometer (GLA Agricultural Electronics, San Luis Obispo, CA). Respiration rates were recorded with temperatures. Any cow with a RT over 40.5° C was removed from the environmental chamber and soaked with cold water for 5 minutes. Additionally, HOBO® U12 stainless steel temperature data loggers (Onset Computer Corp., Bourne, MA) were used to measure vaginal temperatures in 5 min increments. The data loggers were attached to blank controlled internal drug-releasing devices (CIDR; Pfizer Animal Health, New York, NY) to keep the HOBO in place. The CIDRs with data loggers attached were inserted vaginally on day 13 for 6 days to measure the core body transition temperatures between TN and HS and removed out on day 17.

Blood sampling:

Blood samples were collected by coccygeal venipuncture. The area was wiped clean with sterile gauze, sprayed with 70% ethanol (EtOH) followed by a second wiping with a sterile gauze. Samples were collected in BD Vacutainer™ brand tubes (BD, Franklin Lakes, NJ) with an 18 gauge transfer needle. During TN and HS, samples were collected every 4 hours over a 24-hour period (0400, 0800, 1200, 1600, 2000 and 2400 h). Samples for buffy coat and plasma were collected in tubes containing sodium heparin, placed on ice and centrifuged at 1,500 x g for 15 min at 4°C. Samples for serum were collected in blank tubes, stored at 4° C for 12 hours, and centrifuged at 1,500 x g

for 15 min at 4° C. Plasma and serum aliquots were stored for analysis later into microfuge tubes, the plasma stored at -20° C and serum stored at -80° C.

The buffy coat was removed and placed into microfuge tubes containing Trizol LS® (Life Technologies, Grand Island, NY) and stored at -80° C. Buffy coat samples were thawed and homogenized. The RNA was extracted using the Trizol LS and RNA was cleaned up using the Omega clean-up columns (Omega Bio-Tek, Norcross, GA). Clean RNA was quantified using the NanoDrop® Spectrophotometer ND-1000 (NanoDrop, Wilmington, DE), treated with DNase I (amplification grade, Invitrogen) and synthesized into cDNA. Gene expression was estimated using quantitative PCR.

Plasma insulin levels were determined using a (Siemens Medical Solutions Diagnostics, Los Angeles, CA) radioimmunoassay (RIA), and plasma glucose was quantified using a colorimetric assay (Cat. # G7519, Pointe Scientific Inc., Canton, MI), both described and validated by Long and Schafer (2013). The intraassay and interassay coefficient of variation CV were 5 and 7% for glucose and less than 10% 5% for insulin (respectively).

Statistical analysis

Production, physiological, and blood data were analyzed as a 2 x 3 replicated factorial design using the MIXED procedure (SAS® Institute Inc., Cary NC) with the LSMEANS and PDIFF options. Animal was the experimental unit (n=23), one cow was unable to acclimate in the environmental chambers and was removed from the study. Days 5 to 7 were used as a covariate for production and physiological responses. Cattle

typically do not reach their on-dairy production levels, but by day 4 they acclimate and reach their peak ARC production.

Results

In vitro:

The BMECs treated with BET (figure 2.5) had greater growth and survival after HS. Gene expression of both HSP27 and HSP70 were higher in BET treated cells ($P < 0.01$). The proliferation and ductal development was visible and robust by d 7. The TN conditions were 37° C and HS was 42° C. Betaine decreased the expression of HSP27 and HSP70 during TN ($P < 0.05$), and up-regulate expression during HS ($P < 0.01$).

In vivo:

The data is presented by treatment (CON, MID, and HIGH) and environment (TN and HS). There were no differences in rectal temperature between treatments during TN at 1800 h. Physiological measurements indicated that BET treatment resulted in increased rectal temperature and decreased respirations per minute during HS (table 2.2). Rectal temperatures and vaginal temperatures were highest in the MID dose BET cows followed by HI then CON.

Respiration rates decreased linearly by dose during HS at 1800 h. Figure 2.6 shows the time spent below, at or above (incremental) 38.6° C and represents mean body temperature within a treatment group by hour. Vaginal temperatures indicated that during TN BET increased the amount of time spent over 38.6° C for HI and again the MID had the highest temperatures (figure 2.6). During HS all groups had higher vaginal temperatures compared to TN.

The averaged diurnal vaginal temperature cycle for two days of TN and HS (figure 2.7) shows that the MID dose has higher temperatures in TN and HS. During HS, the cows finish the day with higher temperatures, as they are unable to dissipate the excess heat. The mean temperatures indicate that all groups are responding to HS in a similar pattern, and all BET treated cows have higher vaginal temperatures.

Milk yield increased linearly with dose of supplemented BET in TN. Under HS conditions the MY advantage was lost and the BET cows consumed less feed. Milk protein and lactose (%) increased with the MID dose during HS compared to the controls table (2.3).

Both MID and HI groups had lower water consumption than CON during HS. However, all dietary groups had higher water consumption during HS compared to TN.

Plasma glucose and insulin levels increased during HS with BET. There were no differences between treatments during TN with glucose or insulin. The increase during HS in plasma glucose (figure 2.8) was greatest in the HI group ($P = 0.007$) compared to CON. During HS there was a treatment effect ($P = 0.01$) as insulin (figure 2.9) followed glucose and increased with higher doses of BET.

The expression of HSP27 (figure 2.10, a) was not different between treatments during HS, but in TN expression of HSP27 was down regulated ($P = 0.03$) in the HI group compared to CON. The expression of HSP70 (figure 2.10, b) was up regulated in both TN and HS. The buffy coat from HI BET cows had the greatest HSP70 response during HS.

Discussion and Conclusion

Betaine demonstrated advantages in cell culture and in dairy cows. Cows fed betaine had significantly higher milk production during TN and elevated plasma glucose during HS. The BMECs treated with betaine had a better HSP response and survival during HS.

In vitro:

Previous research shows that BMECs exposed to 42° C for 24 h have reduced growth and gene expression with increased cell death (Collier et al., 2006). Betaine appeared to protect primary BMECs from acute HS in vitro. The up regulated expression of mRNA for HSP27 and HSP70 during HS may be an extension of likely apoptotic activity that would leave fewer viable cells (0 mM BET) for gene expression. Total DNA was not measured but the photos (figure 2.4) appear to have dead clusters of cells. This suggests that BET at a dose of at 25 mM is able to protect cells for 24 hours exposure to 42° C in vitro against HS. The concentration of BET in cell culture does not represent levels in vivo. The amount of BET needed to pass the rumen and gut microbes and enter the cells in the cows is unknown. The purpose of this study was to determine the effective dose during HS. The MID group had higher temperatures than control and HI cows. A wider dose range study is needed to determine the value of higher doses. Future research should include circulating BET levels to better understand the impact on osmoregulation and function as a molecular chaperone.

Further research measuring BET concentrations in blood using a high-performance liquid chromatography (HPLC) would be a better indicator of the amount of circulating BET. This would demonstrate if BET is degraded in the rumen and what

amount needs to be fed. Additionally, measuring the uptake of BET into cells in both HS and TN via the Na⁺ dependent symporter could help identify changes in uptake with stress. Reducing the temperature for HS in the incubator microclimate and measuring total DNA in 8 h increments could measure cell death.

In vivo:

The discrepancy in treatment differences in vaginal temperatures (figure 2.7), but not in rectal temperatures at 1800 h (table 2.2) is due to the calculation of vaginal temperatures as mean temperature by treatment, and are the average of the first two days of HS; the rectal temperatures are the mean temperature of each dose for an entire period.

Betaine did not reduce the impact of HS in lactating dairy cows as we expected. The advantage in milk production during TN was lost with HS. We are particularly interested in the higher plasma glucose levels of BET fed cows with the insult of HS. When the milk yield difference was lost with BET, plasma glucose levels increased. It has been proposed that glucose availability is partially responsible for reduced milk production beyond the decrease in feed intake seen with HS (Baumgard et al., 2011). Wang et al., (2010) saw a linear and quadratic increase in VFA with increasing amounts of dietary BET. The propionate to acetate ratio did not increase, but total VFA production was higher. This does not fully explain the higher glucose levels seen in this study, but may reflect the availability of other fuels and precursors. Baumgard et al. (2011) compared the glucose rate of appearance in both HS and animals kept at TN

conditions and pair fed at the levels of the HS cows. The comparison indicated that both groups had similar decrease glucose.

The priorities of energy utilization may shift in HS (Baumgard et al., 2011). The animal consumes less feed, spends more time standing, and has decreased rumination and pants during HS. There are limitations to dissipating the increased heat load. Panting requires extra energy, reduces rumination time, increases saliva loss (including buffers), can cause respiratory alkalosis and reduces time spent at the feed bunk. Conductive heat loss is minimal due to ambient conditions and time spent standing. Reducing internal heat load and maintaining physiological function could decrease the after-effects of HS.

Betaine fed cows had better milk to feed ratios during HS with less feed compared to the controls (CON = 1.34, MID = 1.37, and HI = 1.45). It is unclear if this is due to better-feed conversion or if the cows are in a negative energy balance and would be unable to sustain that production. The correlation between increased respiration rates and decreased rumination time (Soriani et al., 2013) could help explain the better feed conversions in HS. Betaine reduced respiration rates in HS and at the same times increased body temperature (vaginal and rectal). If the rumen is maintaining a higher level of production with BET, there will be a greater heat of fermentation, less panting and thus higher core body temperatures. The increase in heat load is troubling when trying to reduce the impact of HS. Maintaining function and protecting rumen microbes, epithelial cells, and maintaining cellular homeostasis during HS may reduce some of the intensity and effects. The need to reduce HS can be accomplished by

minimizing the severity and duration of exposure while allowing a faster recovery. In both acute and chronic HS, preserving homeostasis may eliminate or reduce the post-stress issues (fetal and embryonic development, prepubertal growth, reproduction, lactation and health) seen after the occurrence of HS.

The heat shock response seen in this study is complex. The down regulated HSP 27 from the buffy coat fraction in HI cows during TN may reflect reduced need for chaperone activity and thus a lower expression of HSP 27, therefore, under TN conditions BET may function like a chaperone. During HS, HSP 70 expression increased with the HI dose. This increase does have an increased cost (ATP) but will conserve cell function. In the BMECs at TN and HS, there was an increase expression with BET. The difference between the MID and HI doses may indicate the level of BET that is utilized, metabolized or in some way degraded in the rumen. If this is the case, at 57 mg/kg BW most of the BET is rumen degraded and at 114 mg/kg BW enough BET is making it past the rumen for uptake into host cells. Improving the HSP response is a non-genetic adaptation that improves the homeorhetic response during HS.

The use of BET as an osmolyte may be important in protecting the GIT and other tissues from the effects of HS. The MID and HI cows consumed 17.5 and 16.6 liters less water per day during HS compared to the CON group. The reduction in respiration rates could impact this behavior. These differences in water consumption suggest that, additional work looking at Vasopressin is warranted. Vasopressin, also known as antidiuretic hormone (ADH) secretion may be altered with BET altering water

conservation with in the treated cows. Alternatively, the osmolyte activity of BET could reduce the water requirement of cows exposed to HS.

Changes in body condition scores (BCS) and body weight were not included in this study due to small animal numbers, short study duration and the specific stage of lactation. Lactating dairy cows that are proximal to peak lactation housed in the environmental chambers at the University of Arizona tend to lose weight and may be in a negative energy balance with HS their stage of lactation. If BET can improve or maintain BCS and body weight, the effects of HS may decrease downstream.

Figure 2.1. Environmental rooms (1 and 2) conditions by day during the BET study in room 1 (R1) and room 2 (R2).

R1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
R2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
TN														HS												TN					

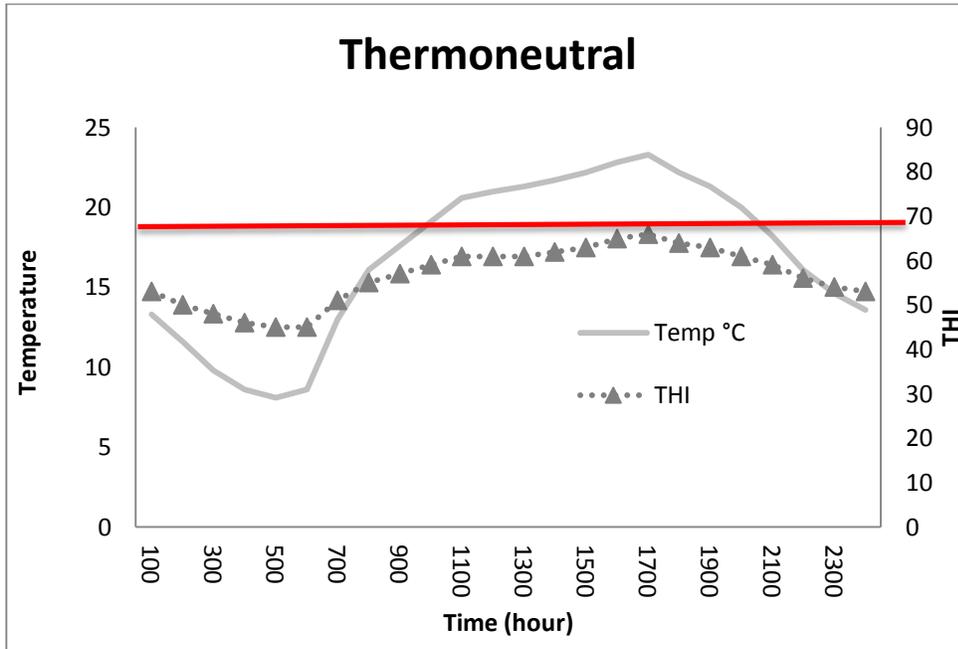


Figure 2.2. Diurnal conditions during TN during the BET study in the environmental rooms at the University of Arizona. Red line indicates the threshold THI (68) for HS in lactating dairy cows.

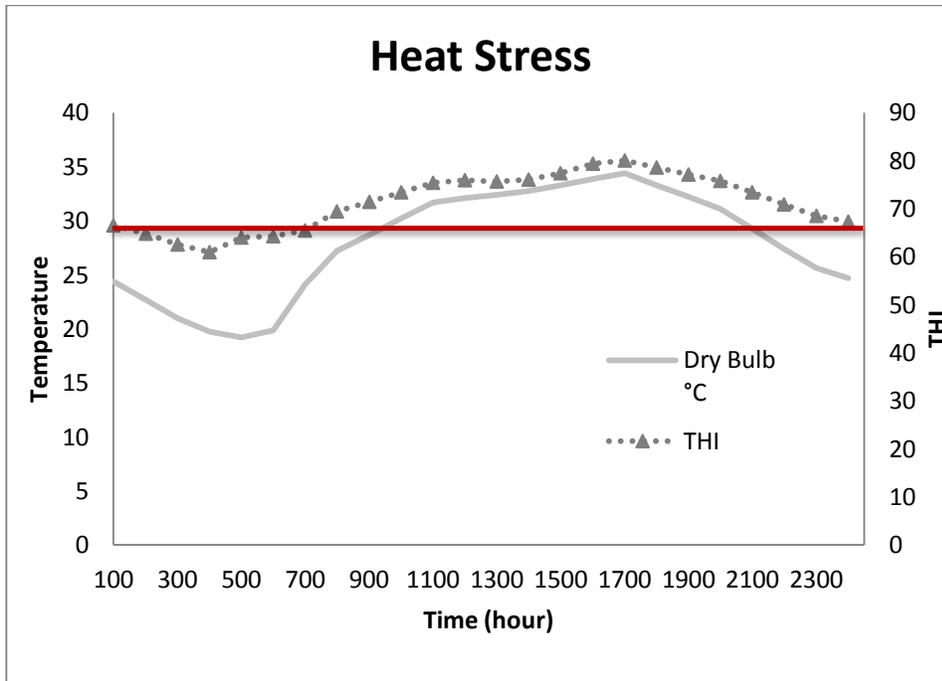


Figure 2.3. Diurnal conditions during HS. Red line indicates the threshold THI (68) for HS in lactating dairy cows.

Table 2.1. Ingredients and chemical composition of the diet¹ during the BET study

Item	% of DM
Alfalfa Hay	65.02
Corn (Steam Flaked)	22.12
Whole Cottonseed	7.28
Distillers Grains (dry)	2.58
Supplement RS-1299 ²	2.04
MAXXER ³	0.96
Chemical analysis	
CP, %	19.51
NDF, %	27.13
ADF, %	20.48
Fat, %	4.77
DM, %	53.00
NE _L , Mcal/kg	1.74

¹Diet DM averaged 53% by weight of DM and moisture including added water

²The supplement contained 1.14% fat, 10.42% Ca, 4.49% P, 3.80% Mg, 0.49% S, 0.19% K, 15.83% Na, 7.52% Cl, 2,029.06 mg/kg Zn, 1,991.82 mg/kg of Mn, 974.24 mg/kg of Fe, 583.45 mg/kg of Cu, 67.86 mg/kg of Co, 12.28 mg/kg of Se, 6.81 mg/kg of Mo, 43.68 mg/kg of I, 304.9 IU/g of vitamin A, 30.2 IU/g vitamin D, and 1.0 IU/g of vitamin E

³Calcium salts of palm oil (Tarome Inc., Eloy, AZ)

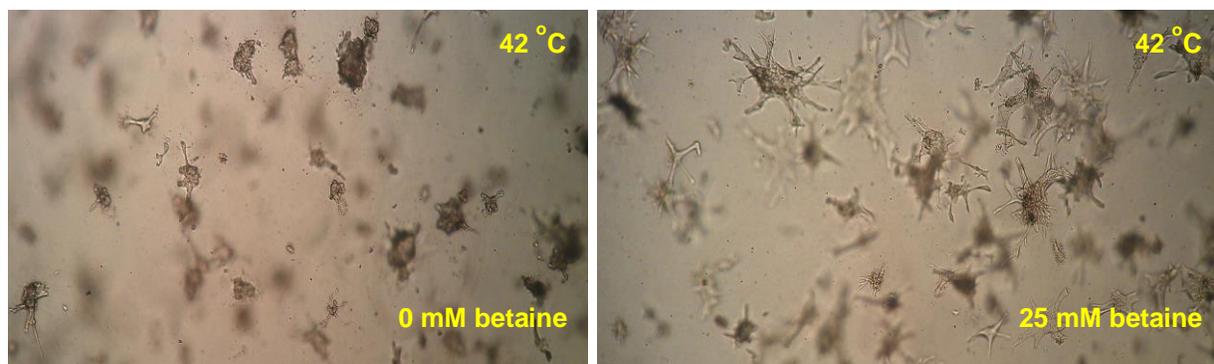


Figure 2.4. Images of primary bovine mammary epithelial cells (BMECs) in collagen after 8 h of exposure at 42°C treated with BET.

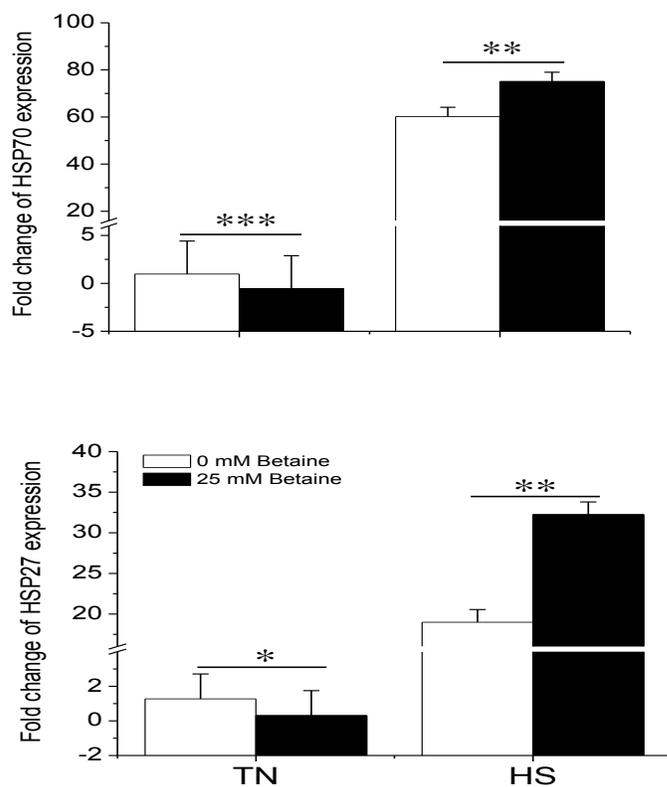


Figure 2.5. Expression of HSP27 and HSP70 in BMEC cells with 0mM or 25mM BET. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 2.2. Rectal temperature and respiration rate with different doses of BET at 1800 h

Item	Treatment						SEM
	Control		MID Betaine		HI Betaine		
	TN	HS	TN	HS	TN	HS	
Rectal temp (C)	38.5	39.4	38.6	39.8 ^{***}	38.5	39.6 [*]	0.13
Resp/ min	32.3	71.7	33.1	68.4 [*]	35.6	66.5 ^{***}	1.68

Reported *P* values are treatment vs. control. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$

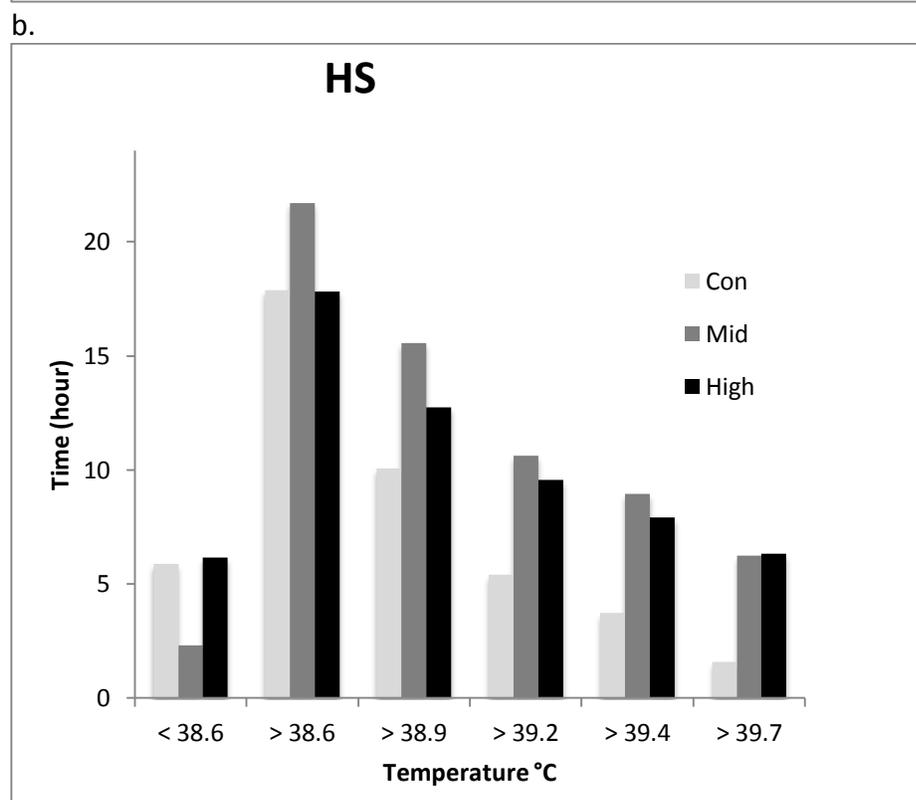
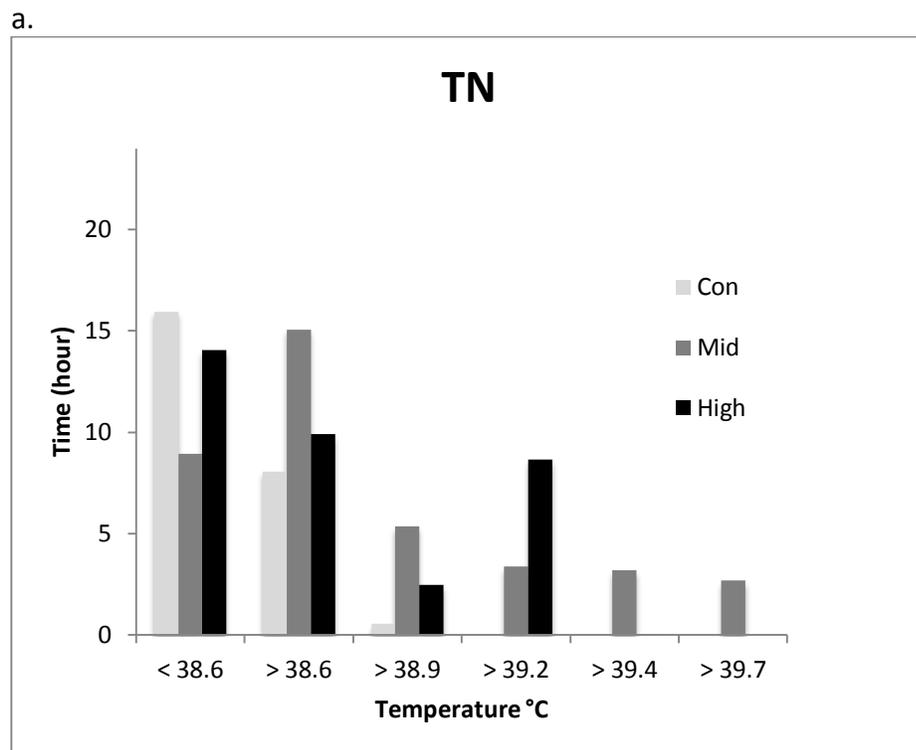


Figure 2.6. Hours spent at or above specific vaginal temperatures with betaineBET supplementation during TN (a) and HS (b).

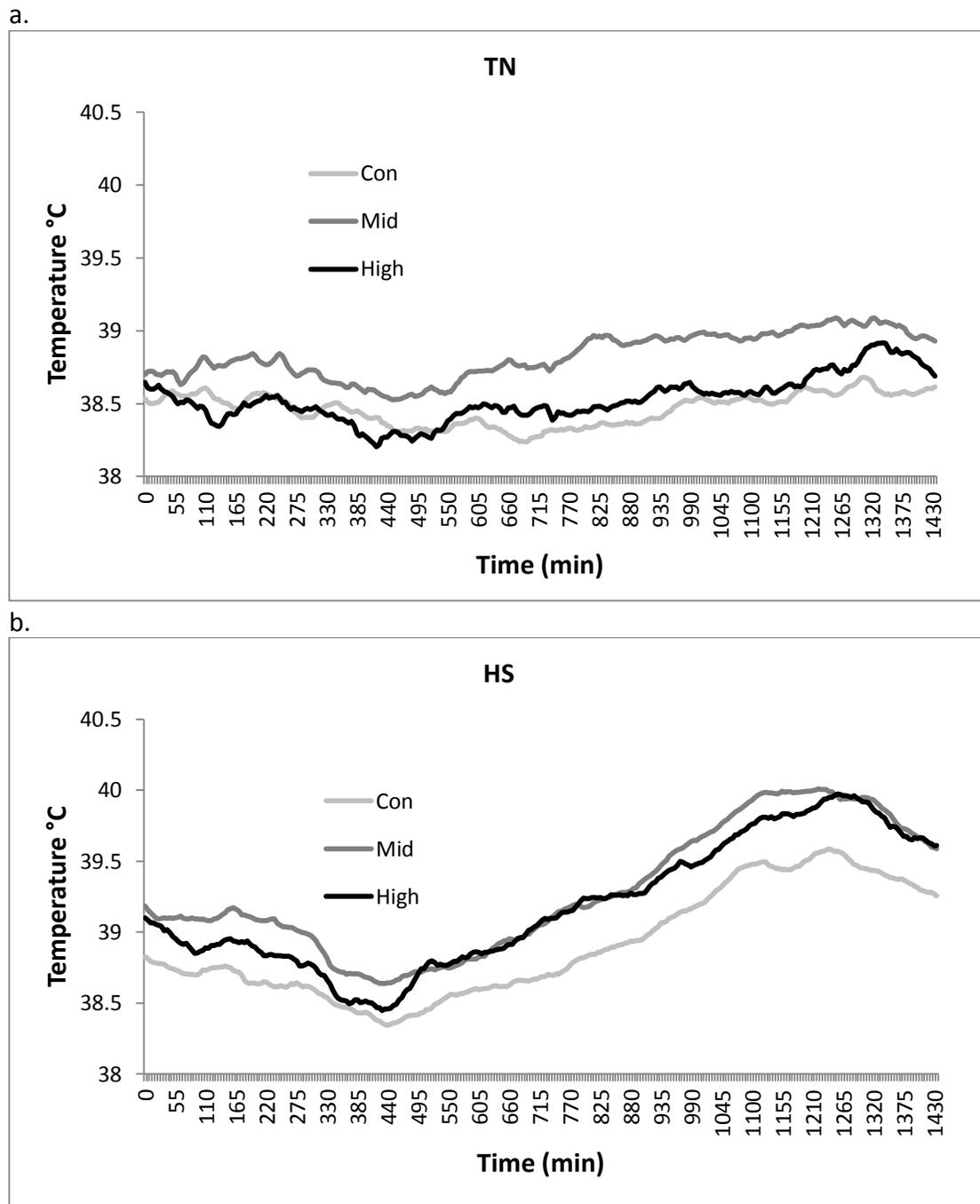


Figure 2.7. Mean diurnal vaginal temperatures by environment with betaine during TN (a) and HS (b).

Table 2.3. Milk production and intake for lactating cows fed varying levels of betaine

Item	Treatment						SEM
	Control		Mid Betaine		High Betaine		
	TN	HS	TN	HS	TN	HS	
MY (kg)	31.4	28.6	32.2	26.9	33.9*	28.8	0.68
DMI (kg)	19.9	21.3	23.9	19.9	23.5	19.9	0.48
Water (liters)	107.8	140.1	109.6	122.6**	115.3	123.5**	1.34
Protein (%)	2.77	2.71	2.83*	2.74	2.81	2.75	0.02
Lactose (%)	4.85	4.82	4.94*	4.76	4.89	4.85	0.03

Reported p values are treatment vs. control. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$. MY = milk yield, DMI = dry matter intake, TN = thermoneutral, HS = heat stress

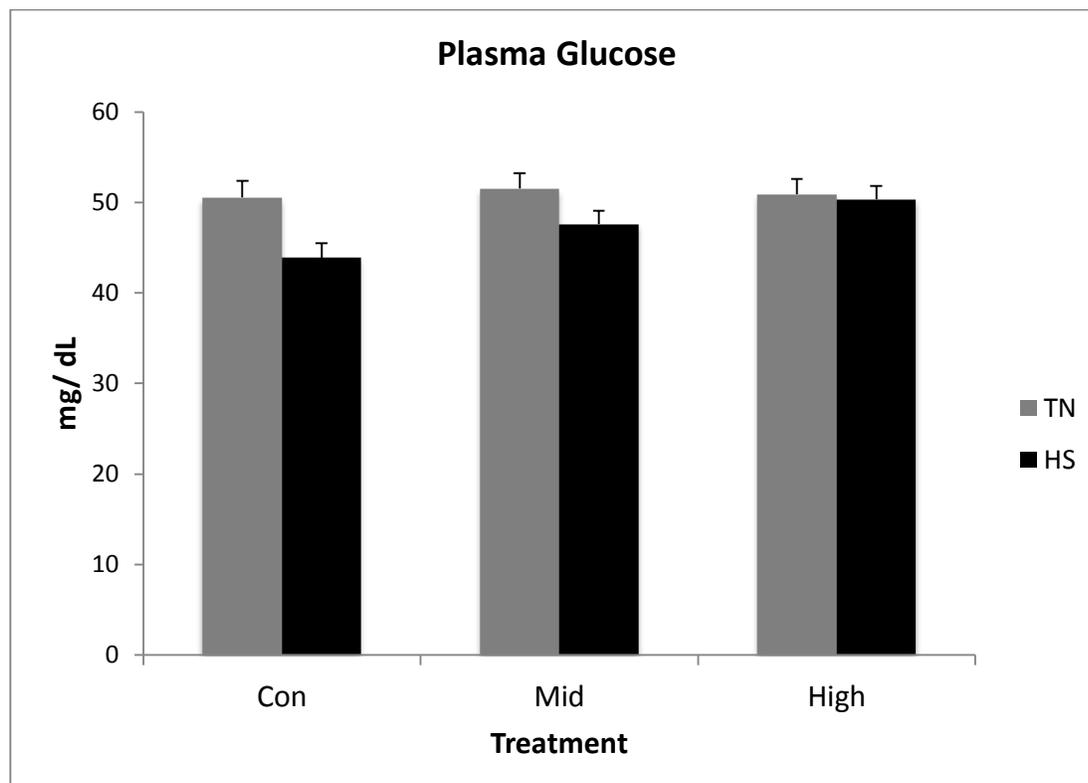


Figure 2.8. Mean plasma glucose with different doses of betaine in TN and HS.

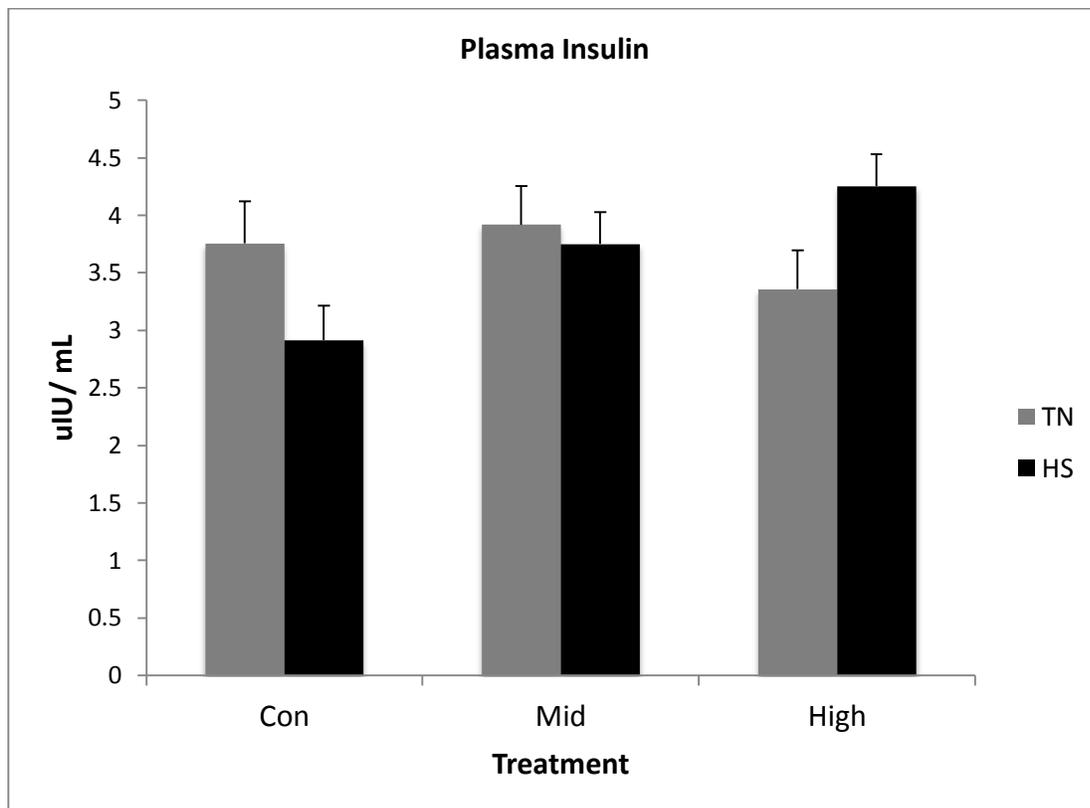


Figure 2.9. Mean plasma insulin with different doses of betaine in TN and HS.

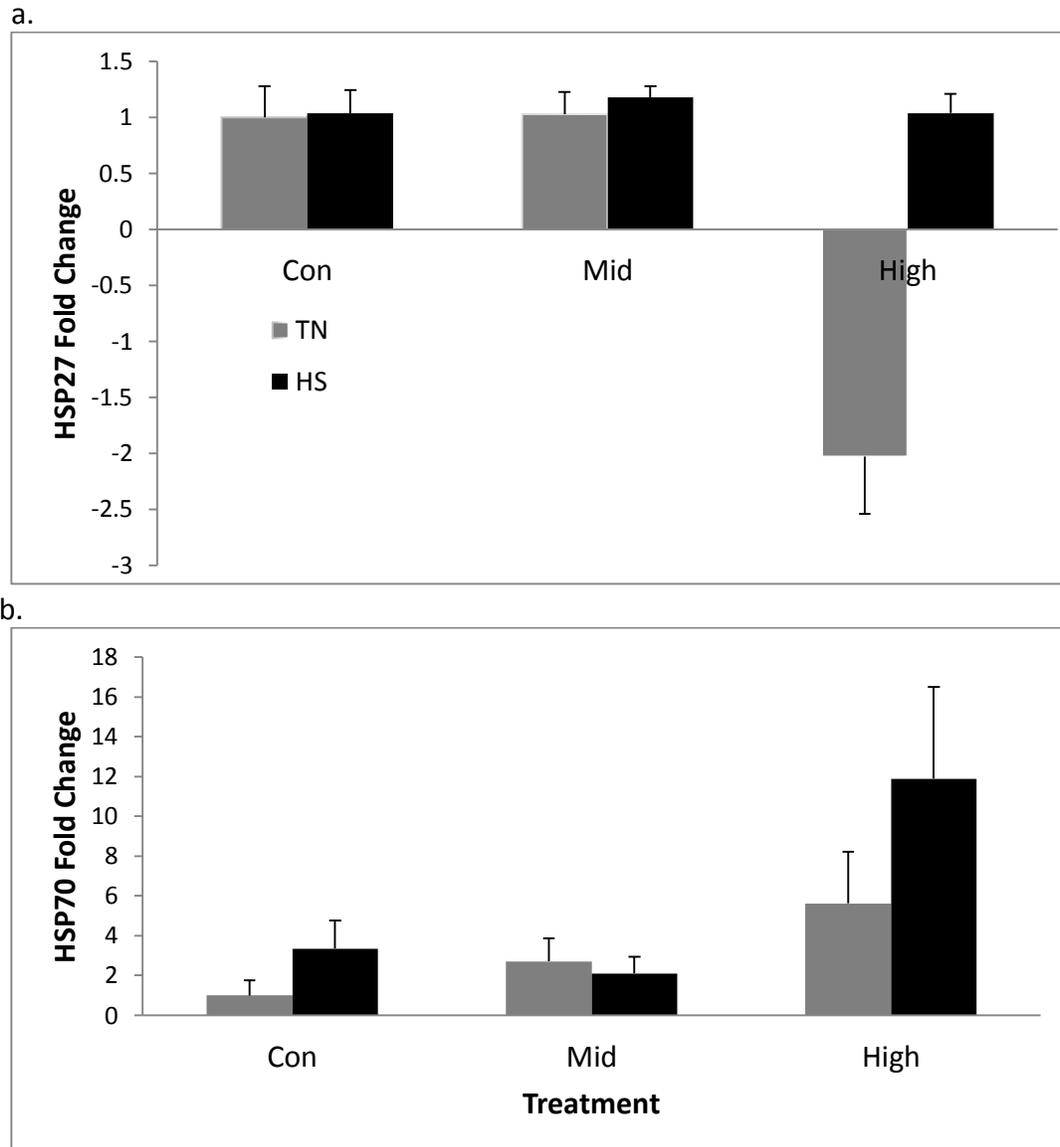


Figure 2.10. Fold change in HSP27 (a) and HSP70 (b) during TN and HS at different levels of betaine. Values are mean gene expression in buffy coat fraction of the blood.

CHAPTER THREE: THE EFFECTS OF CALSPORIN® ON PERFORMANCE, PHYSIOLOGICAL RESPONSES AND CHANGES IN MICROBIAL POPULATIONS IN LACTATING HEAT-STRESSED HOLSTEIN COWS

Abstract

The DFM Calsporin (*Bacillus subtilis* C-3102) was fed to lactating dairy cows under TN or HS conditions to evaluate physiological and production responses of dairy cows to DFM under HS conditions. Twenty-four cows were balanced by parity and DIM and fed 0 g/d or 10 g/d (6×10^9 cfu/d/head). The cows were randomly divided into two groups of twelve, assigned a treatment and exposed to TN or HS conditions. The addition of Calsporin numerically increased MY (1.26 kg, $P = 0.11$) during TN and reduced MY under HS (-2.67 kg, $P < 0.01$) and decreased milk protein ($P = 0.05$). The DFM tended to decrease SCC across periods ($P = 0.07$). There was no advantage to Calsporin in respiration rates and it increased rectal temperature at 1800 h in HS ($P = 0.02$). The expression of HSP27 was decreased with Calsporin with treatment and ($P = 0.03$) in both HS and TN. The microbial count did not change with the exception of the Calsporin strain in treated animals ($P < 0.01$). Calsporin did not reduce the impact of HS, but did numerically increase MY in TN and decrease SCC.

Introduction

Heat stress has tremendous economic impacts on the dairy industry (St-Pierre et al., 2003) by reducing production at the individual level. Despite major advancements in facility management and heat abatement, heat waves and hot and/or humid environments continue to

threaten production in lactating dairy cows. Exposure to HS causes immediate issues and later down-stream implications that can all have devastating effects on the production of a cow.

The rumen and the hindgut gastrointestinal tract (GIT) are also susceptible to HS. The GIT is a lumen through the body and is thus a potential source of external pathogens and potential toxins that are derived from microbes and fermentation. The bulk of immune cells are proximate to the GIT. The mucosa of the rumen and hindgut can be impaired during HS leading to leakage of toxins and microbes into the circulation and impacting immune function and animal performance (Bolton and Pass, 1988).

Direct fed microbials are bacteria and yeast cells (variable strains) that are a normal gut commensals or beneficial types which can be added to the diet of production animals. These microbials reduce harmful bacteria in the large intestine which leads to improved performance of production animals. We evaluated the impact of using a commercially available direct fed microbial, Calsporin, a *Bacillus subtilis* C-3102 strain, (Calsporin®, Calpis Co. USA, Peachtree, GA) in the diet of HS animals. This specific strain is fed to humans, swine, broilers and layers as a stable endospore. The bacteria (variable strains) are a normal gut commensals that can improve performance in production animals and reduce harmful bacteria in the large intestine. A study in humans found that supplementation of the spore increased *Bifidobacterium* species and decreased some *Clostridium* groups of microbes (Hatnaka et al., 2012). The *Bacillus subtilis* group is a Gram-positive, rod-shaped bacterium that was thought to be an obligate aerobe, but research has shown that this bacterium can also have anaerobic growth (Nakano and Zuber, 1998). This specific species is able to utilize oxygen that is toxic in an anaerobic environment like the rumen but also increase specific enzymes that aid in the digestion of proteins (subtilisin

and catalase). Vegetative forms of *Bacillus subtilis* have been shown to enhance mitogenic-induced T cell proliferation, but did not modify lymphokine production (Ciprandi et al., 1986). There are potential benefits that may come from supplemental Calsporin in the diet of a lactating dairy cow. In swine diets fed between birth and 42 d, Calsporin improved feed efficiency resulting in a lower feed intake compared to control animals, with higher daily gains and 42 d weights with this DFM (Medel et al., 2009). Garcia et al. (2007) had similar findings in trials with broilers. They had lower mortality, favorable feed conversions and better growth with Calsporin. *Bacillus subtilis* C-3102 has also been shown to reduce certain pathogenic strains of bacteria such as *E. coli*, *campylobacter* and some *clostridium*s (Bhandari et al., 2008; Maruta et al., 1996). The duration of feeding decreased *campylobacter* populations as the number of days of Calsporin supplementation increased (Maruta et al., 1996).

The ruminant digestive tract requires specific considerations compared to monogastric species. The rumen provides an anaerobic environment with constant temperature and stable pH that favors specific bacterial conditions. Advantages of a DFM may be partially lost with HS. The rumen is buffered to prevent acidosis and alkalosis. *Bacillus subtilis* C-3102 promotes the growth of lactic acid producing bacteria. During HS buffers are lost with panting allowing volatile fatty acids (VFAs) to lower the pH, and lactic acid is 10 times stronger than VFAs (Russell and Hino, 1985).

The hindgut can also suffer under thermal stress. Hindgut fermentation provides 5-10% of dietary energy under normal conditions (Gressley, 2011). Rumination time decreases with exposure to heat (Soriani et al., 2013). Digestibility of diet, specifically starches can decrease with less rumination. Larger amounts of starches in the hindgut can cause rapid microbial

growth, decrease the pH and cause hindgut acidosis (Gressley et al., 2011). DMFs can also protect against pathogenic strains that can infect or cross the mucosa and cause disease with the compromise of hyperthermia. The quantification of pathogenic strains and recovery time can help assess the role of DFMs during HS in lactating dairy cows.

Though there have been many advancements in environmental control with dairy cows, rumen health needs to be considered in both acute and chronic heat exposure to reduce the immediate and post-stress effects on health, production and reproduction in dairy cows. Maintaining rumen, colon and cecal fermentation and protecting the mucosal linings against infection of and passage through of pathogens can reduce the net impact of HS. The decrease in pH leads to the death of Gram-negative bacteria that release endotoxins, jeopardizing tissues and production. The supplemental use of DFMs may reduce immediate and delayed severity of thermal stress.

Our hypothesis is that Calsporin (*Bacillus subtilis* C-3102) supplementation before and during heat stress will improve milk yield by promoting favorable microbial fermentation, improving anaerobic condition (facultative aerobe), and have a faster return to pre heat stressed conditions in lactating Holstein cows.

Materials and Methods

This study was separated into two phases; the on-dairy portion and the controlled environmental chamber exposure at the University of Arizona, William J. Parker Agricultural Research Center (ARC).

The on-dairy portion of the study took place during the summer months on a southwest US dairy in Eloy, AZ (32.7636° N, 111.6000° W). All cows were housed in dry lots having Saudi

barns with cooling, feed line soakers and shade with cooling at the parlor and holding area. Cows were milked 3x and fed fresh feed 2x daily. Fecal samples were taken after the morning milking at 415 h (control) and 500 h (Calsporin). Cows (n = 60) were selected and balanced based on parity (2.7 ± 0.99), previous lactation production at 75 DIM (93.9 ± 15.5) at freshening. The cows were randomly assigned to either control or Calsporin fed pens. At 5 days postpartum cows were moved into one of two group pens (250 cows/pen) fed with or without Calsporin. Calsporin was measured at the recommended dosage for cows at 6×10^9 cfu/day/head, adjusted for the number of cows in the pen and mixed in a fixed mixer into the TMR at each feeding. Milk weights were automatically recorded by RFID and stored in DairyComp 305, (Valley Ag Software, Tulare, CA). The cows were fed Calsporin prior to the start of the ARC portion of the study to measure the effect of HS with preconditioning to the DFM. Weekly milk yields and fecal samples were the only recorded measurement taken from the dairy phase. During the on-dairy trial 5 cows were removed for health related issues (3 controls and 2 Calsporin cows).

Data for weekly milk weights is calculated from daily milk yields and presented as changes in weekly means by treatment and corrected to week 1 production.

Environmental rooms

The ARC portion consisted of two groups of 12 cows (6 control and 6 Calsporin) selected from the 60 cows which completed the on dairy portion of the study. The cows were maintained on the same treatment groups from the dairy. Both environmental rooms housed 6 cows (3 Control, 3 treated). One room was designated HS and the other TN and the rooms switched environments between groups of cows. After cows were shipped from the dairy to

the ARC they were weighed, fitted with a halter and randomly assigned to a room and a tie stall within the room. There were three periods; acclimation (7 days), HS or thermal neutral depending on the room (10 days), and recovery period (3 days; figure 3.1).

The diet was an alfalfa based TMR that was balanced to supply a similar composition as the on-dairy TMR. Grab samples were collected when the feed was freshly mixed. Samples were analyzed at Dairy One, INC, Ithaca NY by wet chemistry. The TMR is shown in table 1. Bypass fat (Maxxer, Chandler AZ) was added to the TMR along with a high producer mineral supplement (Dairy Nutrition Services INC, Chandler, AZ) table 3.1.

Blood sampling:

Blood samples were collected by coccygeal venipuncture. The area was wiped clean with sterile gauze, sprayed with 70% EtOH and wiped clean with sterile gauze. Samples were collected in BD Vacutainer™ brand tubes with a 20 gauge transfer needle. During TN and HS, samples were collected every 4 hours for a 24-hour period (400, 800, 1200, 1600, 2000 and 2400). Samples were collected on day 7 of HS/ TN. Samples for buffy coat and plasma were collected in tubes containing sodium heparin, placed on ice and centrifuged at 1,500 x g for 15 min at 4° C. Samples for serum were collected in blank tubes, stored at 4° C for 12 hours then centrifuged at 1,500 x g for 15 min at 4° C. Plasma and serum were aliquoted into previously labeled microfuge tubes, the plasma was stored at -20° C and the serum was stored at -80° C until analysis. The buffy coat was removed after plasma and placed into microfuge tubes containing Trizol LS® and stored at -80° C. Serum samples were collected in blank Vacutainer tubes and allowed to sit at 4° C for 12 h, then centrifuged at 1,500 x g for 15 min at 4° C. Aliquots were stored at -80°C. Buffy coat samples were thawed and homogenized using the

polytron (Polytron PT 3100, Kinematica, Bohemia, NY). RNA was extracted using the Trizol LS and RNA was cleaned up using the Omega clean-up columns. Clean RNA was quantified using the Nanodrop, treated with DNase and synthesized into cDNA. Gene expression was measured using quantitative PCR.

Physiology and production:

Cows were individually fed 2 times/d at 500 and 1700 h. Refusals were removed and weighed at 445 daily. Individual water consumption was recorded daily at 515 h. Cows were milked between 530 and 630 am, and 1730 and 1830 h daily. Milk was weighed at each milking, samples for milk composition were taken at the am milking and milk was discarded. Samples were individually stored with a preservative (bronopol tablet, D&F Control Systems, San Ramon, California) at 4°C. Aliquots were analyzed by Arizona DHIA (Tempe, Arizona) by infrared for butter fat, protein, somatic cell count (SCC), lactose and solids-not-fat (SNF).

Physiological parameters were taken 3 x/ d (0500, 1400, and 1700 h). Skin temperature was measured at the tail head, shoulder and rump using a Raytek® infrared thermometer, and rectal temperature was measured using a GLA® M700 thermometer (San Luis Obispo, CA). Respiration rates (breaths per minute) were recorded at the same time points as temperatures. Any cow with a rectal temperature over 40.5°C was removed from the environmental chamber and sprayed with cold water for 5 minutes.

Fecal samples were collected from the rectum with a clean glove and placed into a sterile plastic bag. Microbial counts were analyzed by Calpis technicians for number of CFU and data is given in $n \times 10^9$ cfu/d/head.

Statistics

The dairy phase consisted of two pens, one for control cows and one for Calsporin, so the experimental unit was pen. The pre-feeding of Calsporin was to increase levels of Calsporin in the cows before arrival at the ARC. At the ARC, animal was the experimental unit. The study was set up as a 2x2 factorial (control and Calsporin x TN and HS) design with two groups of cows, one following the other. Results were analyzed using the mixed procedure (SAS® Institute Inc., Cary NC) with the LSMEANS and PDIF options.

One cow that could not adapt to the tie stall and the fluctuating environments, became severely hypophagic and was removed from the study and omitted from the results

Results and Discussion

During the on-dairy portion of the study, both groups experienced a reduction in milk yield that was likely due to thermal stress. There was no difference in persistency of lactation between treatments ($P = 0.10$), there was an effect with week ($P = 0.03$), but no treatment x week interaction ($P = 0.92$). The Calsporin fed cows had reduced milk production as did the controls, but the control cows as a group had numerically greater losses in milk yield during weeks 2 to 6 (figure 3.2). This may be a result of improved rumen function with the DFM added to the TMR. Out competing unfavorable microbes could increase nutrient availability compared to the control cows.

During the controlled environment (ARC) trial we did not detect an effect of Calsporin on dry matter intake (figure 3.3). There was no treatment effect ($P = 0.77$) or difference within periods between treatments. There was a period effect that reduced DMI that is typically seen

with HS ($P < 0.01$), but not treatment by period interaction ($P = 0.75$). It appears that both groups experienced reduced intake associated with HS and this DFM did not alter this response.

The advantage seen in on-dairy milk production did not carry over to the ARC portion of the study. There was a numeric advantage of 1.35 kg milk/day with Calsporin supplementation during TN ($P = 0.11$). The advantage was lost and Calsporin cows had lower milk production during HS compared to controls ($P < 0.01$). This is typical of supplements that improve milk yield; during HS they often lose the advantage and have lower milk yields. Both groups increased milk production when exposed to TN conditions and we did not detect a treatment effect in this response. The recovery period may have been too short to detect differences as levels were still climbing, and did not have time to plateau. To determine the effects of recovery, greater cow numbers and a longer period would be required to determine more sensitive changes in milk production.

There was no overall treatment effect on milk yield ($P = 0.22$) but there was an environment effect and an environment by treatment interaction ($P < 0.001$ and 0.0029 respectively). The environment by treatment interaction was the result of greater loss in milk yield in the group fed Calsporin (figure 3.4). Thus HS reduced feed intake and milk yield between environments and milk yield in both groups.

Dietary Calsporin tended to decrease SCC in daily milk samples throughout the study but there was no period differences or treatment by environment interaction ($P = 0.07$, 0.73 and 0.82). The visible difference in figure 3.5 may be due to the high standard errors within the means ($10.68 \times 10,000$). Individual variability between cows is difficult to adjust for in smaller groups of cows and get the needed sensitivity. There tended to be an advantage to feeding

Calsporin to reduce SCC across environments but there was no detectable difference between treatments in TN, HS or recovery ($P = 0.37, 0.13, \text{ and } 0.30$ respectively).

The greatest economic impact of feeding Calsporin was on milk components (table 2). Lactose was higher in Calsporin fed cows during HS ($P = 0.01$). We did not detect an effect of treatment on milk lactose but there was an environment effect and an interaction between environment and treatment ($P = 0.12, 0.01, \text{ and } 0.01$ respectively). The environment by treatment interaction was due to the fact that milk lactose remained higher in cows fed Calsporin during thermal stress. If the rumen flora is fermenting more substrate or this may be a result of lower milk production. Milk protein percent was lower in cows fed Calsporin but the percent protein did not fluctuate between environments with the DFM. Percent milk protein did not change with environment and we did not detect a treatment x environment interaction on milk protein percent. We also did not detect an effect of treatment or a treatment x environment interaction on percent milk fat, Table 3.2. The SCC decreased with treatment in HS ($P = 0.02$) and was numerically lower in TN and recovery ($P = 0.14 \text{ and } 0.19$).

Respiration rate and rectal temperature were different in 500 h respiration rate and 1700 h rectal temperatures, table 3.3. The control cows in the TN environment had higher respiration rates (figure 3.7), but were not above normal physiological rates. The higher rectal temperatures (figure 3.6) were seen in Calsporin fed cows during HS at 1700 h and were high enough to result in an overall treatment effect ($P = 0.02$). If there was more fermentation occurring in the treated cows this could result in a higher core body temperature. Additionally, if Calsporin promotes the growth of lactic acid producing bacteria, lower ruminal pH has been

shown to increase rumen temperature. Thus, measuring pH in the rumen samples may help clarify why Calsporin fed cows had elevated rectal temperatures during HS.

The heat shock protein response was reduced by Calsporin. HSP27 is a small HSP that has important roles protecting cells from thermal stress, inhibiting apoptotic activities and cell proliferation. Calsporin reduced the response in both TN and HS as seen in figure 3.8. Figure 3.9 shows the change in expression throughout a diurnal cycle. It appears in figure 3.9 to peak at 1200 and 2400 h which may be a delayed response to HS and the resulting misfolded proteins that change expression of the HSP27 gene.

The changes in fecal microflora are reported in table 3.4. There are several strains of *Enterobacter* that are pathogenic and not heat tolerant. Feeding Calsporin or HS had no effect on *Enterobacter* population. *Clostridium perfringens* is a normal inhabitant of the lower digestive tract but can become pathogenic when there are disruptions in microbial populations. Additionally, some Gram negative bacterial strains can release an α -toxin that can cause enterotoxaemia in ruminants. This α -toxin can exfoliate large and small intestine epithelial cells and result in intestinal hemorrhages (Morris et al., 2012). There was no treatment effect but there was a day effect ($P=0.03$). Mean populations of *C. perfringens* were numerically lower at the dairy for all groups and increased when cows were housed at the ARC. This may be due to the effects of stress (acclimation to the rooms, travel, changes in diet and handling procedures) and are significantly different in the Calsporin TN group at the dairy and at the TN group at the ARC ($P = 0.01$). Calsporin fed cows had numerically less compared to the control at the dairy.

Calsporin (*Bacillus subtilis* C-3102) levels were significantly higher in supplemented cows. Treatment did not alter Lactobacillus populations, but numerically reduced

Bifidobacterium population ($P = 0.11$). This may be attributed to the non-detectable levels in the Calsporin HS group of cows. Total number of anaerobes was also not changed with the supplementation of Calsporin.

Conclusion

Calsporin fed cows had a numeric advantage in milk yield in TN and lower SCC in the environmental rooms. The milk production was lost with HS.

Supplementing Calsporin did not improve the physiological responses to HS or milk yield. However, at the cellular level, the feeding of Calsporin increased the expression of HSP27. There was a numerical advantage in milk production to feeding Calsporin on the dairy and under TN conditions in the environmental rooms. This was lost during HS, and treated cows experienced a numerically lower milk yield during HS.

With studying microbial populations, more cow numbers would increase sensitivity and help explain changes that occur. The fecal sampling intervals could also be shortened to understand acute changes in population associated with stressors (adding the 1st and 2nd day after shipping and exposure to HS). The addition of a commercial dairy study with sampling under normal condition and HS conditions may eliminate variables (new stress) that can alter microbial populations. The cows were preconditioned yet fecal bacterial populations seemed to change with the transition from the dairy to the ARC.

Figure 3.1. Room environments during the ARC phase of the Calsporin study.

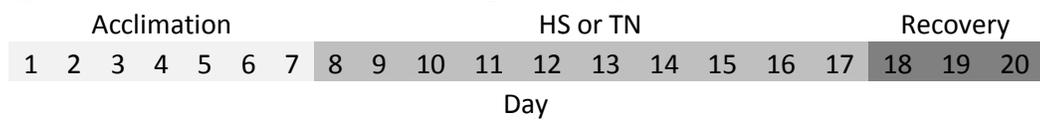


Table 3.1. Ingredients and chemical composition of the diet¹

Item	% of DM
Alfalfa Hay	65.02
Corn (Steam Flaked)	22.12
Whole Cottonseed	7.28
Distillers Grains (dry)	2.58
Supplement RS-1299 ²	2.04
MAXXER ³	0.96
Chemical analysis	
CP, %	18.40
NDF, %	39.30
ADF, %	28.70
Fat, %	4.77
DM, %	53.00
NE _L , Mcal/kg	1.63

¹Diet DM averaged 53% by weight of DM and moisture including added water

²The supplement contained 1.14% fat, 10.42% Ca, 4.49% P, 3.80% Mg, 0.49% S, 0.19% K, 15.83% Na, 7.52% Cl, 2,029.06 mg/kg Zn, 1,991.82 mg/kg of Mn, 974.24 mg/kg of Fe, 583.45 mg/kg of Cu, 67.86 mg/kg of Co, 12.28 mg/kg of Se, 6.81 mg/kg of Mo, 43.68 mg/kg of I, 304.9 IU/g of vitamin A, 30.2 IU/g vitamin D, and 1.0 IU/g of vitamin E

³Calcium salts of palm oil (Tarome Inc., Eloy, AZ)

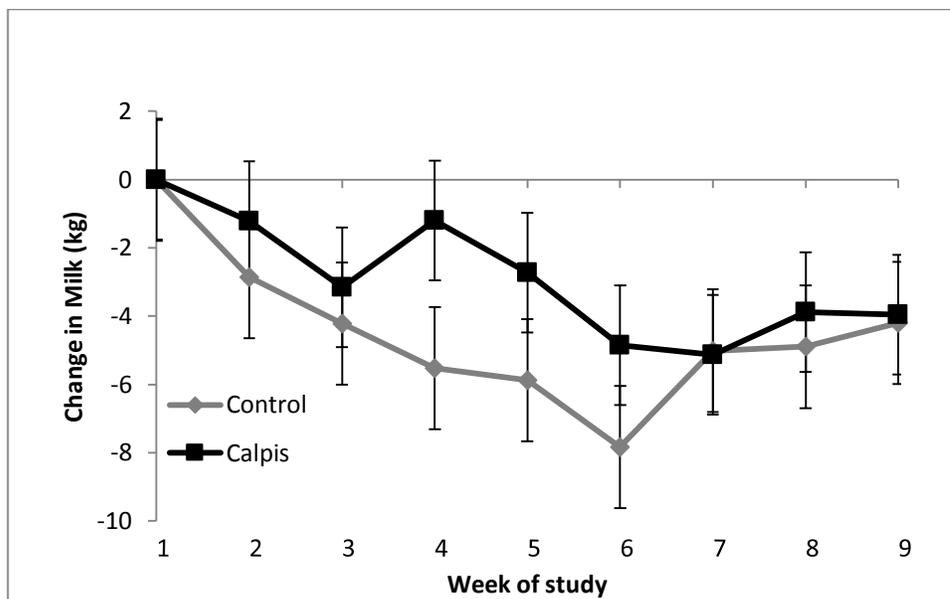


Figure 3.2. Change in on-dairy milk production with treatment of Calsporin by week.

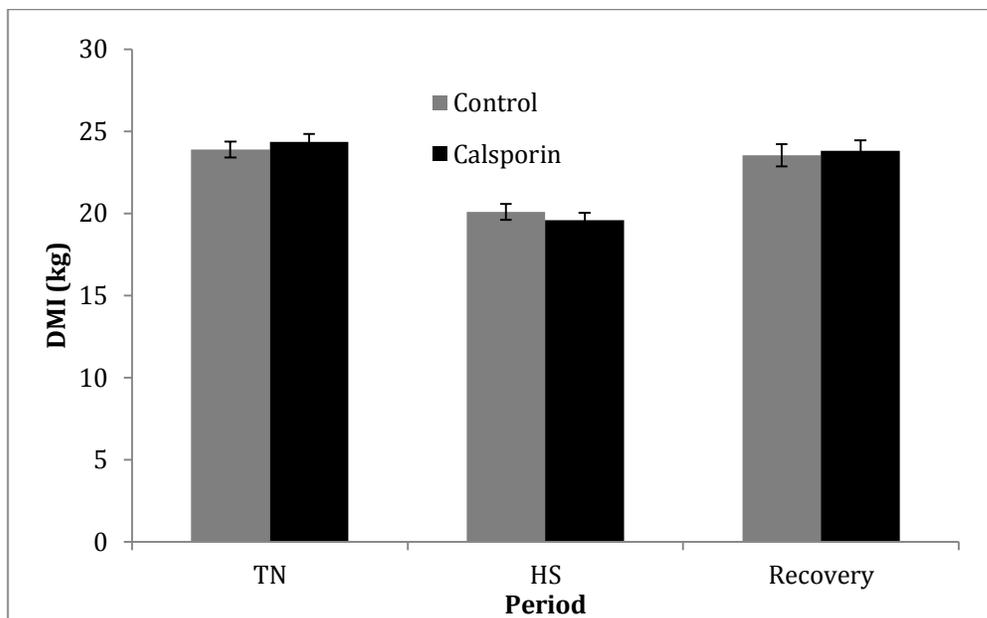


Figure 3.3. Dry matter intake (ARC) in Calsporin fed cows

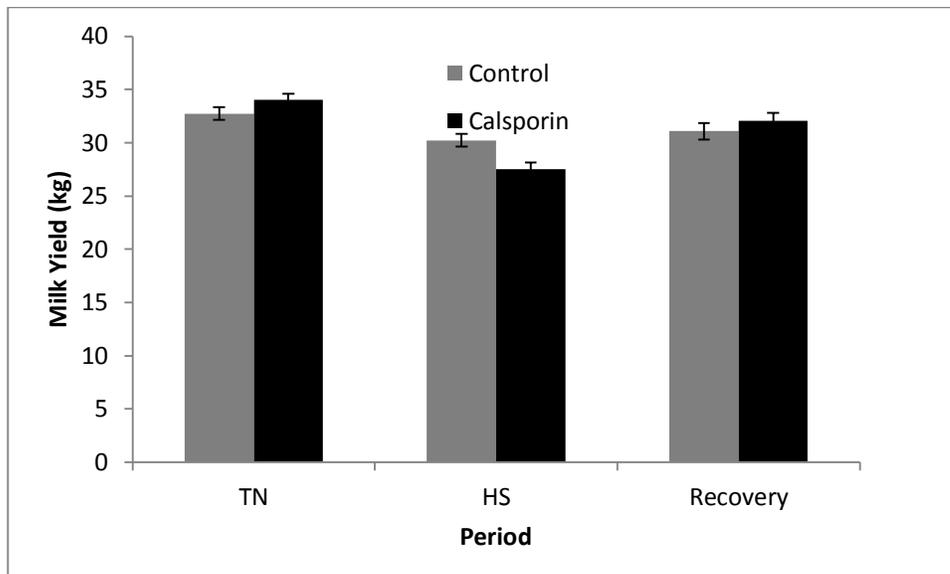


Figure 3.4. Milk yield during ARC portion with supplemental Calsporin during TN and HS.

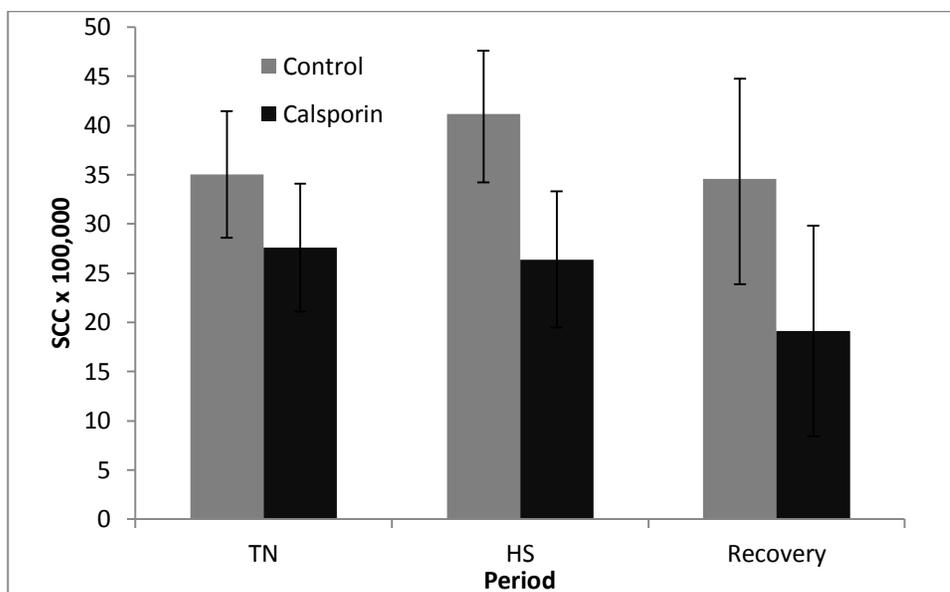


Figure 3.5. Effect of Calsporin, heat stress and thermal neutral conditions on somatic cell count in milk from lactating Holstein cows.

Table 3.2. Effect of Calsporin, heat stress and thermoneutral conditions on milk composition of lactating Holstein cows

Item	Control		Calsporin		SEM	P value
	TN	HS	TN	HS		
Fat (%)	3.94	4.12	3.91	3.95	0.20	0.49
Protein (%)	2.91	2.86	2.80	*2.80	0.05	0.05
Lactose (%)	4.92	4.79	4.87	4.90*	0.04	0.12
SCC (x 10,000)	35.1	41.2	27.6	26.4	10.7	0.07

* $P < 0.05$ between treatments in the same environment and is placed on the higher value

Table 3.3. Effect of Calsporin, heat stress and thermoneutral conditions on respiration rate and rectal temperatures of lactating dairy cows

Item	Control		Calsporin		SEM	P value
	TN	HS	TN	HS		
Respiration rate						
500	30.2	40.9	27.9	38.3	1.11	0.03
1400	39.9	73.2	37.5	74.8	1.95	0.83
1700	43.6*	77.6	39.0	79.1	1.48	0.32
Rectal temperature						
500	38.1	38.3	38.1	38.3	0.04	0.97
1400	38.1	39.1	38.2	39.2	0.06	0.18
1700	38.3	39.5	38.3	39.8*	0.06	0.02

* $P < 0.05$ between treatments in the same environment and is placed on the higher value

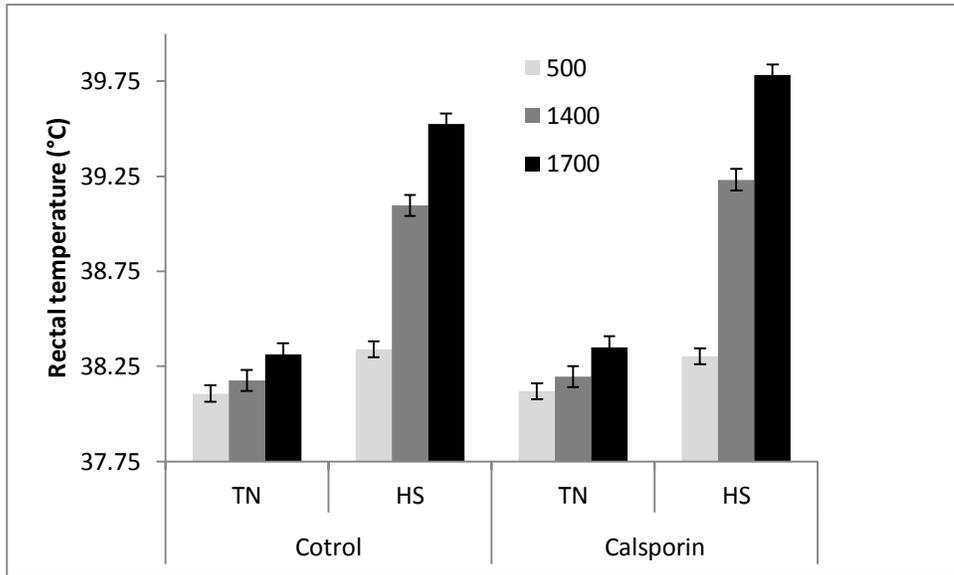


Figure 3.6. Effect of HS and Calsporin on mean rectal temperatures at 0500, 1400 and 1700 hours in lactating dairy cows.

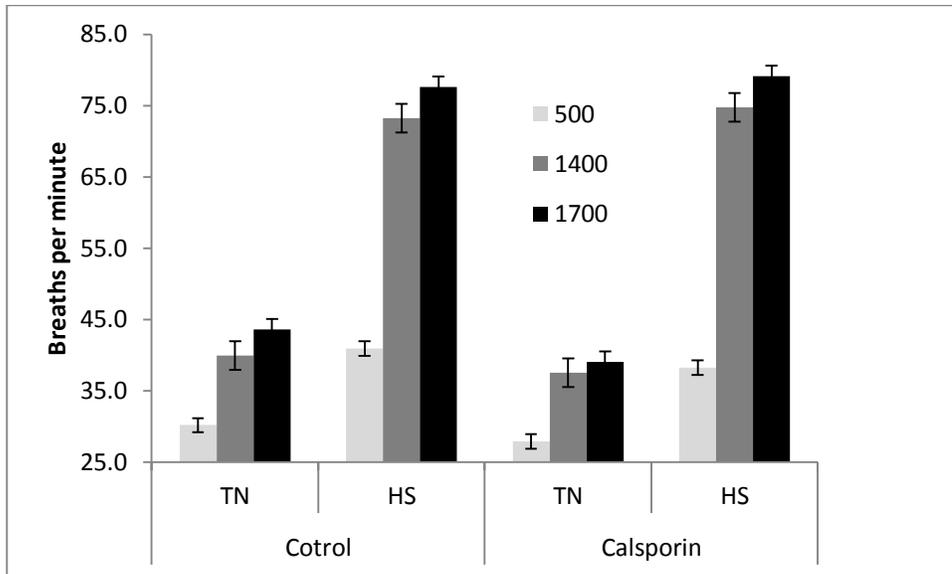


Figure 3.7. Effects of HS and Calsporin on respiration rates at 0500, 1300 and 1700 hours in lactating dairy cows.

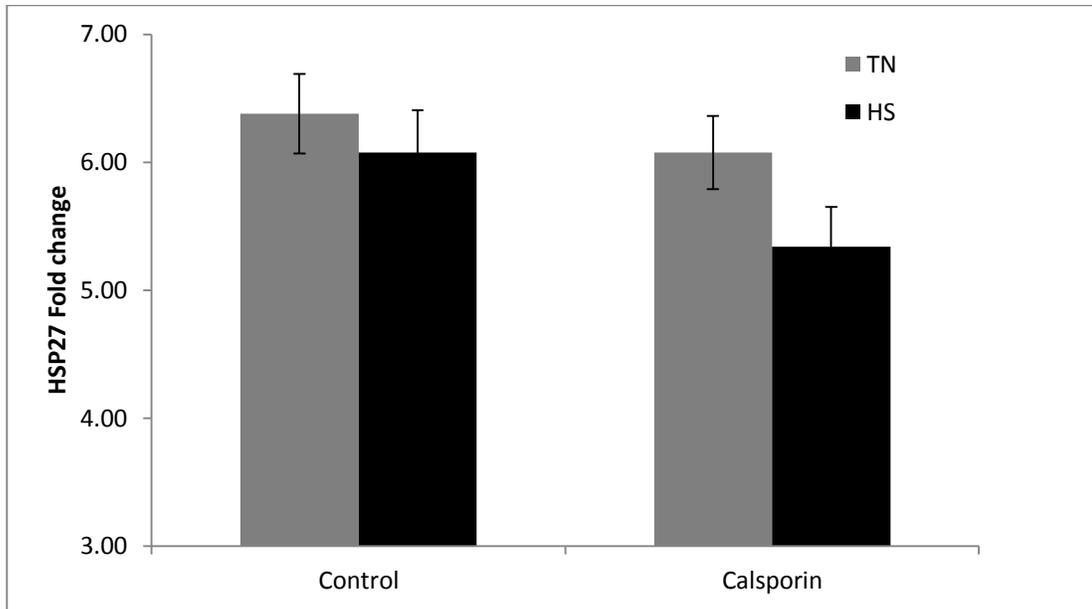


Figure 3.8. Effect of HS and Calsporin on HSP27 gene expression in leukocytes of lactating dairy cows

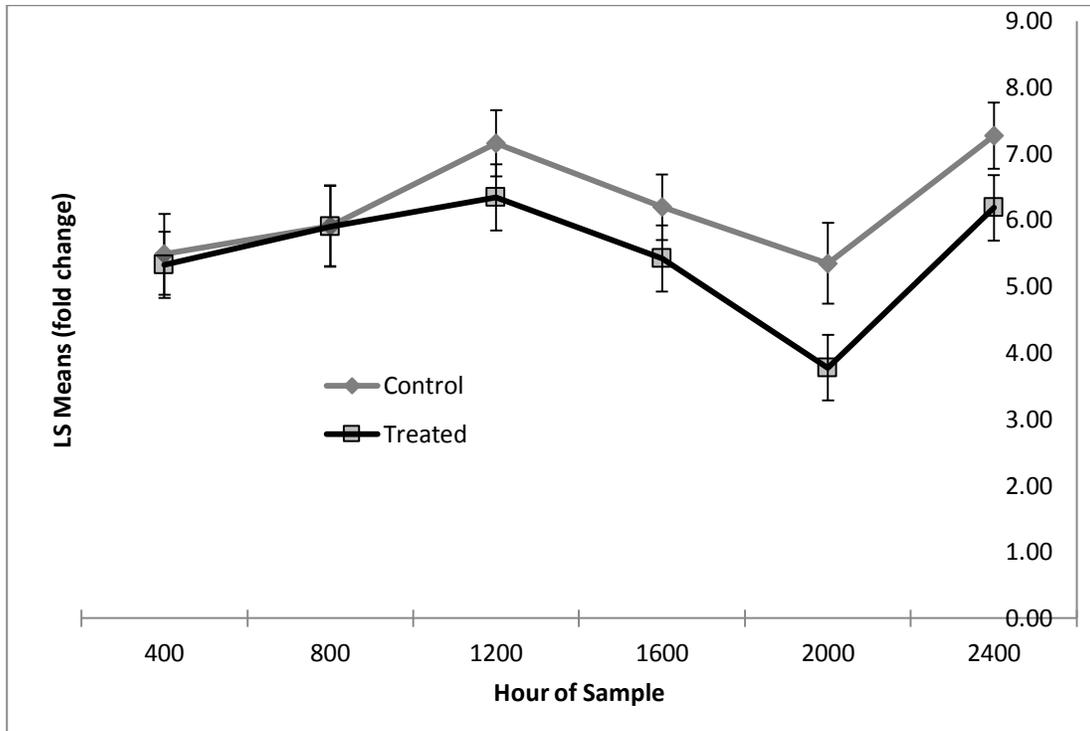


Figure 3.6. Effects of feeding Calsporin to lactating dairy cows on circadian expression of HSP27 in the buffy coat fraction of the blood.

Table 3.4. Effects of feeding Calsporin to lactating dairy cows on selected microbial cell populations while at the dairy and in the environmental chambers

Item	Control TN				Control HS				Calsporin TN				Calsporin HS				SEM	P value
	Dairy	Accl	TN	Rec	Dairy	Acc	HS	Rec	Dairy	Acc	TN	Rec	Dairy	Acc	HS	Rec		
Enterobacter	5.41	5.64	5.13	5.50	6.26	5.50	5.91	5.96	6.39	5.47	5.26	5.20	5.69	5.56	5.25	5.11	0.39	0.34
C. perfringens	3.00	3.73	3.41	2.98	3.00	3.36	3.28	3.18	2.60	3.78	3.53	3.54	2.66	3.39	3.21	3.13	0.48	0.75
Calsporin	4.81	4.60	4.08	2.83	4.72	4.60	3.35	2.58	4.97	5.00	5.04	4.05	4.99	5.46	5.23	4.15	0.17	<.01
Lactobacillus	6.43	5.74	6.06	5.97	6.39	5.48	5.79	5.99	6.21	5.55	5.90	6.02	6.67	5.36	5.60	6.05	0.17	0.77
Bifidobacterium	7.24	3.59	3.78	4.94	7.58	5.21	4.60	ND	6.85	4.57	5.01	5.12	7.40	ND	ND	4.29	0.54	0.11
Total anaerobes	7.84	7.32	7.28	7.02	8.29	6.89	7.59	7.20	7.72	7.08	7.18	7.41	8.03	7.01	7.54	7.27	0.21	0.97
Lactobacillus (%)	5.09	3.00	8.02	8.86	2.82	4.08	1.64	7.74	3.28	3.22	10.03	6.54	4.90	4.30	1.17	7.19	2.47	0.98
Bifidobacterium (%)	26.35	0.02	0.01	0.79	31.38	3.33	0.09	ND	29.63	0.31	0.82	1.79	24.76	ND	ND	0.59	20.13	0.98

Dairy = on-dairy, Acc = acclimation ARC, TN = TN ARC, HS = HS ARC, and Rec = recovery ARC

CHAPTER FOUR: AN EVALUATION OF *OMNIGEN-AF™* IN HEAT-STRESSED LACTATING HOLSTEIN COWS: PHYSIOLOGICAL RESPONSES, PRODUCTION RESPONSES, MILK QUALITY, MEASURES OF IMMUNITY AND HORMONES OF THE ADRENAL AXIS.

Abstract

Holstein cows (n=30) were balanced by DIM, milk production and parity (91 ± 5.9 DIM, 36.2 ± 2.5 kg/d, and 3.1 ± 1.4 respectively) and fed OmniGen-AF (OG; Prince Agri Products, Quincy, IL) at 0 g/d for control or 56 g/d for OG for 52 days on a commercial dairy. At 52 d of lactation cows were randomly selected (n = 12) from both groups (6 OG and 6 CON) and housed in environmentally controlled modules for 21 d at the University of Arizona. The OG was top-dressed 2x/d with molasses as the carrier, and the CON cows received the molasses carrier 2x/d. The treatment was mixed into the top one-third of the TMR. The cows were subjected to 7 days of TN conditions, 10 days of HS, and 4 days of recovery (TN). Feed intake, milk production, and milk composition were measured daily. Rectal temperatures and respiration rates were recorded 3x/d (600, 1400, and 1800 h). Blood samples were taken on days 7 (TN), 8 (HS), 10 (HS), 17 (HS) and 18 (TN) during the ARC segment. During the environmental room phase of the study cows fed OG had higher feed intake than CON during HS (HS) (46.8 kg vs. 42.9 kg, $P < 0.0001$) but there was no differences during TN. Cows fed OG maintained a numerical 1 kg milk yield advantage compared with CON (TN =33.1 vs 33.9, HS = 30.3 vs. 31.4 kg, $P = 0.26$) during HS but not during TN. Cows fed OG had lower milk fat percent (3.8 vs 4.2%, $P = 0.02$) and milk protein percent during HS (CON = 2.98 and OG = 2.86, $P = 0.04$) but there was an increase in protein yield during HS. There was no difference in

3.5% FCM between treatments. Water consumption was lower (12.4 l/d in OG treated cows, $P < 0.01$) than control cows. Respiration rates were lower in treated cows at 1400 h and 1700 h (4.7 and 8.4 less respirations/min, $P = 0.05$, < 0.001 respectively) and rectal temperatures were also lower (0.15° C and 0.25° C lower than CON, $P = 0.05$, < 0.001 respectively) in treated cows. Serum Cortisol levels were highest on first day of HS (day 8). Cows fed OG had lower serum cortisol compared to Con on day 8 (0.4838 ug/dL vs 0.8372 ug/dL, $P = 0.03$) and not different from Con on other days. Serum insulin and plasma glucose levels were not different between groups ($P = 0.8248$ and $P = 0.945$). Cows fed OG maintained lower SCC compared to control ($P < 0.01$) during the recovery period. We did not detect differences between groups in serum calcium ($P = 0.44$), while serum NEFA concentrations ($P = 0.10$) tended to be greater in OG fed cows across the ARC trial including HS. Serum adrenocorticotrophic hormone (ACTH) levels were greater in OG cows ($P < 0.0001$) across all sample days. Feeding OG reduced the HS response including serum cortisol. This suggests that OG treatment may alter adrenal response to ACTH or that hypothalamic negative feedback is altered in OG fed cows. Additional research is needed to determine the cause of reduced serum cortisol and elevated serum ACTH in cows fed OG. Feeding OG reduced physiological responses to HS in lactating dairy cows.

Introduction

A report by Kadzere et al. (2002), estimated that 48% or 4.2 million dairy cows in the United States are subjected to HS on an annual basis, negatively affecting milk yield, reproduction and cow health. Dairy cows begin to physiologically adjust to the

detrimental effects of HS when the ambient temperature exceeds 32.2° C or the Temperature-Humidity Index (THI) reaches 68 (Kadzere et al., 2002 ; Smith et al., 2013). The physiological responses of dairy cows to HS include increased body temperature and elevated respiration rates (Igono et al., 1992). These physiological adaptive measures are followed by declined feed intake, milk yield, milk components and reproductive efficiency (Johnson et al., 1976 and Jordan, 2003). In addition to the negative impact on production, immune function and cow health is also negatively affected by thermal stress associated with elevated of cortisol associated with exposure to heat (Sordillo, 2013 and Christison and Johnson, 1972).

The additive OG is a nutritional supplement for ruminants has been shown to bolster immune function in replacement dairy heifers (Ryman et al., 2013), lactating dairy cows (Wang et al., 2009) and sheep (Wang et al., 2007). In a report by Ortiz-Marty et al., (2012) neutrophils harvested from mice fed OG were more resistant to the effects of cortisol as measured by the adhesion protein L-selectin, a marker indicative of innate immune function. In two separate field studies, multiparous dairy cows fed OG either from dry-off through 30 days into lactation (Holland et al., 2012) or beginning at 120 days in lactation (Holland et al., 2013) and exposed to thermal stress were observed to have higher milk responses than the control cows. These studies prompted a more detailed investigation to evaluate the physiological, immunological and production effects of pre-feeding OG to lactating dairy cows prior to HS. We postulate that feeding OmniGen-AF to lactating dairy cows before and during heat stress will improve the heat

induced immune response, HPA axis activity, reduce infection (SCC) and improve milk yield in thermoneutral and thermal stress.

Materials and methods

Cattle were selected and sorted by days in milk (DIM), production (previous lactation and current lactation), and parity ($n \geq 2$). There were two phases of the study, the on-dairy portion and the environmental chamber portion. The on-dairy portion was conducted at a commercial dairy in Eloy, Arizona. The environmental chamber portion took place at the University of Arizona, William J. Parker Agriculture Research Center (ARC), Tucson, Arizona.

The on-dairy part of the study was needed to elicit an immune response prior to arrival at the ARC. Previous studies have established that a 52 day feeding period of OG supplementation is required to demonstrate differences in markers of immune function (e.g., L-selectin) between OG fed cows and controls (Wang et al., 2004). Following selection, 30 cows (15 controls and 15 OG) were placed into one of two pens that held 252 cows each. The control cows were offered fresh feed twice daily. The OG fed group received the same base TMR as the control, and OG was mixed in at 56 g/ head/ d. Animals were pen fed and pen was confounded with treatment. The dairy utilized for the study (Caballero Dairy, Eloy, Arizona) was a dry lot dairy with Saudi barns which were cooled by Advanced Dairy Systems-Shade Tracker (ADS-ST, Chandler, AZ), an oscillating evaporative cooling system. Cows were milked 3 times daily in a rotary style parlor.

The ARC portion took place in environmentally controlled rooms. The ARC portion of the trial lasted 21 d. There were 7 d of acclimation at TN to allow the cows to settle and adjust to their surroundings. After the acclimation period, the cows were subjected to 10 d of HS. Cows were given a recovery period at TN for 4 d prior to returning to the commercial dairy (figure 4.1).

Of the original 30 cows in the on farm phase, six were selected from each group to provide 12 cows to be used for the environmental room portion of the study. Cows in each group were balanced for pre-trial lactation records, parity and days in milk. After arrival at the William Parker Agricultural Research Complex in Tucson, AZ, the cows were weighed and fitted with halters to accommodate their tie-stalls. The twelve cows were randomly assigned to one of two rooms with 3 control and 3 OG cows per room. Cows were continuously monitored for the first 48 hours following arrival to prevent injury during acclimation to the rooms and the tie-stalls. During the night, cows were observed through remote access cameras.

Feeding and milking occurred twice daily at 500 and 1700 h. Cows were individually fed and milked in their own tie-stalls. Control cows were fed the base TMR (table 4.1) plus ~25 g molasses (as-fed) mixed into the top one-third (2x) and the OG cows received the TMR + molasses + 28 g OG mixed into the top third of each meal. Orts were removed daily at 445 h and weighed. Water consumption was metered and recorded daily before the am feeding. Milk bucket weights were taken and a milk sample was taken from the am milk. Samples were individually stored with a

preservative (bronopol tablet, D&F Control Systems, San Ramon, California) at 4° C. Aliquots were analyzed by Arizona DHIA (Tempe, Arizona) by infrared for butter fat, protein, somatic cell count (SCC), lactose and solids-not-fat (SNF).

Temperature, respiration rate and skin temperatures were taken 3x/d at 0500, 1400 and 1800 h. Rectal temperatures were taken using a GLA® M700 (San Luis Obispo, CA) hi-performance digital thermometer. Respiration rates were visually counted as breaths per minute. Skin temperature was measured at the tail head, shoulder and rump using a Raytek® infrared thermometer. Temperatures and respiration rates were recorded at each time for all cows.

Blood Samples

Blood samples were taken on d 0, 26 and 52 during the on-dairy portion and analyzed for L-selectin to ensure that cows were receiving the OG. During the ARC portion, blood was collected on d 1, 7, 8, 14, 17, and 18. The blood was collected 6 times per day (0400, 0800, 1200, 1600, 2000, and 2400 h) on d 7, 8, 17, and 18. Blood was collected at the time of arrival at the ARC on d 1, and at 2000 h on day 14. Blood samples were collected by venipuncture from the coccygeal vein. The area was wiped with sterile gauze, sprayed with 70% EtOH and wiped clean with sterile gauze. Samples were collected in BD Vacutainer tubes (BD Vacutainer, Franklin Lakes, NJ) with an 18-gauge transfer needle. Samples needed for immune markers were collected in Vacutainer tubes containing sodium citrate. Buffy coat and plasma samples for metabolites, heat shock proteins and hormones assays were collected in Vacutainer

tubes containing sodium heparin. All blood samples were immediately placed on ice after collection. Blood samples for plasma and buffy coat were centrifuged at 1,500 x g for 15 min at 4° C within 15 minutes of collection. Plasma was collected and transferred into aliquots and stored at -20° C until analyzed. The buffy coat layer was then removed and placed in TRIzol LS[®] for RNA extraction. Buffy coat samples were stored at -80° C. Prior to extraction the samples were homogenized. Serum samples were collected in blank Vacutainer tubes and allowed to sit at 4° C for 12 h, then centrifuged at 1,500 x g for 15 min at 4° C. Aliquots were stored at -80° C.

ASSAY

Serum NEFA levels were determined enzymatically through a commercial kit (*Wako NEFA-HR(2)*; Wako Chemicals USA, Richmond, VA). Plasma insulin and cortisol levels were determined using a radioimmunoassay (RIA) and plasma glucose was quantified using a colorimetric assay, both described by Ford et al. (2007). Serum haptoglobin and ACTH levels were determined ELISA kits (*Bovine Haptoglobin*, ELISA Kit MBS564002 and Bovine adrenocorticotrophic hormone kit, San Diego, CA).

L-selectin and regulated on activation, normal T cell expressed and secreted (RANTES) were determined at OmniGen Research Laboratory (Corvallis, OR). Ionized serum calcium was measured using a blood-gas analyzer (ABL 700). Gene expression within the buffy coat fraction of heat shock proteins (HSPs) was quantified using q-real time PCR. Buffy coat samples were homogenized and RNA was extracted using a chloroform extraction. Samples were cleaned using the MicroElute *RNA Clean-Up*

(Omega Bio-tek, Norcross, GA) spin columns, treated with DNase and synthesized into cDNA.

Data were analyzed using the PROC MIXED procedure (version 9.3, SAS Institute, Cary, NC). Cow was the experimental unit (ARC Phase) and data is presented as least square mean with significance declared at $P \leq 0.05$

Results and Discussion

Treatment groups did not differ in milk yield at the start of the on farm phase. CON, 38.6 kg/d, vs OG, 38.6 kg/d). Throughout the 45-day Phase 1, no differences were detected in milk yields between the Control and cows fed OG (36.8 kg/d and 38.3 kg/d), however, numerically the cows fed OG averaged 1.5 kg more milk per day than the Controls while at the dairy (Figure 4.3). Feed intake was not measured at the dairy, animals were pen fed.

Feed intake, milk and milk components during the environmental room component of the study (Phase 2) are shown in Table 4.2. Milk yields recorded during TN and HS were not different ($P > 0.23$) between the CON and OG fed cows, however in HS cows fed OG produced an average of 1.1 kg/day of milk more than the Controls ($P = 0.26$). Milk protein (%) was higher with OG ($P = 0.04$) during HS and milk fat (%) was lower ($P = 0.04$) in TN and HS between the CON and OG cows. The other measured milk components, protein (kg), lactose (%) and fat corrected milk (kg) were not different between treatments. The SCC did not differ until recovery TN ($P = 0.03$) when CON cows averaged 594,000 cells/ml compared to 263,000 cells/ml for the OG cows, figure 4.4.

The CON and OG treated cows both had a spike in SCC during the recovery period, but the OG-AF cows maintained lower SCC levels during the spike.

Dry matter intake increased during TN as the cows adjusted to the tie-stalls and the environmental chambers in both control and OG fed groups (figure 4.5). Feed intakes during Phase 2 did not differ during TN, but were higher in HS ($P < 0.001$) for cows fed OG (24.8 kg DM/d) compared to the Con (22.7 kg DM/d). There was an effect of environment on feed intake in the CON cows ($P = 0.01$) but not in the OG group ($P = 0.81$).

Water consumption differed by treatment ($P \leq 0.001$) and environment ($P = 0.01$) but there was no treatment by environment interaction ($P = 0.13$). The differences were seen during TN and HS, but during recovery there were no differences between groups (figure 4.6), with cows fed OG having lower water intakes during HS.

The data presented for plasma insulin and glucose is the mean level by group and across time points. The figure for day 14 only represents the 2000 h time point. Glucose levels did not differ between treatments ($P = 0.95$) or period ($P = 0.24$) and there was no treatment by period interaction. We did not detect differences in plasma insulin between treatment groups ($P = 0.79$) but there was a period effect ($P = 0.001$) as insulin levels were highest in the recovery period.

Physiological, HPA axis and Immunological Measures

Serum cortisol an important indicator of the physiological status of the cow in HS was measured during Phase 2 at day 7 (TN), days 8, 14 and 17 (HS) and day 18 (REC)

(Figure 4.9). Cortisol levels (ug/dl) were only observed to be different ($P < 0.006$) between the CON and OG fed cows on day 8 (HS) (figure 4.9).

Adrenocorticotrophic hormone (**ACTH**) that is produced by the anterior pituitary gland is important in regulating the stress response via the adrenal axis. Cortisol is a corticosteroid whose secretion from the adrenal gland is regulated by ACTH. Cortisol levels typically increase with the immediate insult of HS. We observed this response in the mean serum cortisol concentrations of the CON cows on day 8 which was the first day of HS. OG-treated cows did not have an increase in serum cortisol levels during d 8 (first day of HS). There was no overall treatment or period effect. OmniGen-AF treated cows had elevated ACTH compared to the controls and an environment effect was also detected ($P = 0.0001$), with higher levels of ACTH throughout sample days, Figure 4.10.

Immune function and responsiveness to stress was monitored by measuring three levels of expression; neutrophil L-selectin mRNA, Interleukin 8 receptor mRNA (IL8R) and upregulated on activation, normal T cells expressed and secreted (RANTES) mRNA. Neutrophil L-selectin mRNA was used as the biomarker during the commercial dairy phase to validate that cows assigned to the OG diet were consuming the appropriate daily amount of the supplement. Over Phase 1 the cows fed OG expressed greater levels of this biomarker than the CON. IL8R, a surface receptor on leukocytes used to detect signals from other immune cells was observed to be expressed at a higher level in the OG fed cows versus CON, particularly during HS (Figure 4.12). Cows fed OG also had higher RANTES levels than CON during HS but lower in REC.

The HSP27 expression in buffy coat was reduced compared to the controls across all treatments ($P < 0.01$; figure 4.13). The OG cows did not have the same degree of stress (RR, RT, MY, DMI, cortisol) and this was also evident in the HSP27 response.

Conclusion

Feeding OG to cows reduced the impact of HS on physiological responses and maintained feed intake during HS. This may be explained by the altered cortisol response. More research focused on the hypothalamic-pituitary-adrenal axis response to OG could define the mechanism behind the favorable changes seen with OG.

The threshold for HS in lactating dairy cows are a $THI > 68$ (Smith et al., 2013), a respiration rates of greater than 60 breaths per minute (BPM) and rectal temperatures above 38.5°C . In this experiment, the OG fed cows were observed to have respiration rates that did not exceed 60 bpm and rectal temperatures that were -0.2 and -0.3°C cooler than control cows during the periods of peak thermal load. This was not true in CON cows that displayed respiration rates of 63.1 and 60.8 bpm and rectal temperatures of 38.7 and 39.1°C during 1400 and 1800 h. The reduced HSP27 expression in buffy coat with OG cows supports the reduced HS responses observed with treatment with production, physiological responses and hormonal measures.

A decline in milk yields and feed intake of heat-stressed lactating cows is normal. In this study, milk production decreased in all cows. However, milk yield of OG fed cows was numerically higher ($+1.1$ kg/cow/day) than CON both on farm and in the environmental rooms. Feed intake was also higher in OG fed cows ($+2.07$ kg DM/cow) throughout both the TN and HS periods. Difference in milk components were observed,

with CON cows maintaining higher milk fat (%) and milk protein (%) in both TN and HS, although component yield was not different. Changes in SCC were consistent with thermal stress, however, the OG fed cows had lower SCC compared to the CON with the greatest difference occurring in the recovery period.

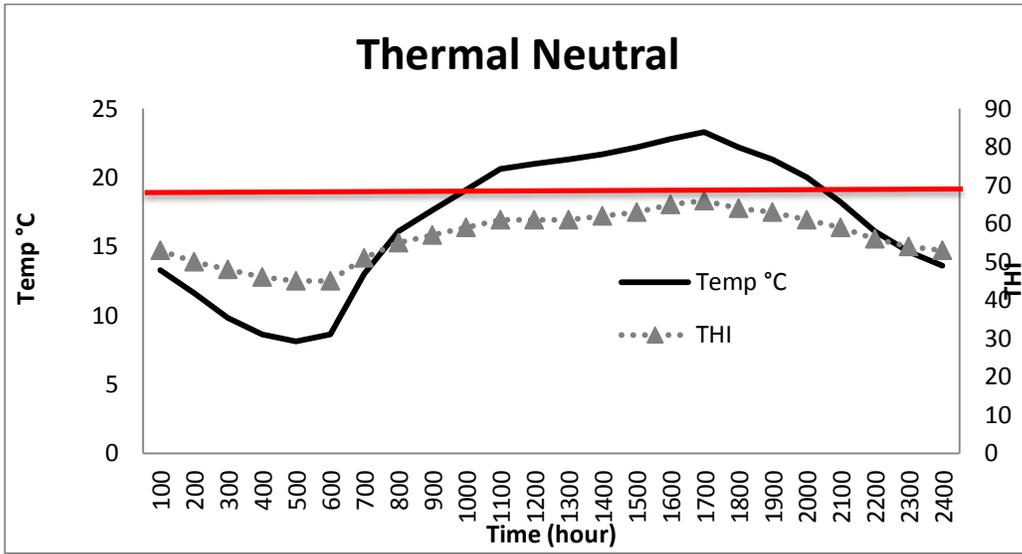
Biomarkers used in the study to monitor immune function were different between CON and OG fed cows. These results were consistent with those reported in previous experiments in which cows supplemented with OG exhibited higher levels of neutrophil L-selectin, IL8R and RANTES during periods of stress (Ortiz-Marty et al., 2012 and Wang et al., 2007).

In conclusion, supplementing lactating dairy cows with OG beginning 45 days prior to and during exposure to moderate thermal stress resulted in a reduction of the typical physiological and production responses associated with HS as measured by feed intake, water intake, rectal temperature, respiration rate, cortisol secretion and selected measures of immune function. Elevated ACTH concentrations in OG fed cows was unexpected and warrants further investigation. Possible mechanisms include altered corticosteroid binding globulin in plasma which would alter free cortisol or direct effects on hypothalamic centers regulating corticotrophin releasing factor secretion.

Environmental Rooms																				
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Thermoneutral							Heat Stress										Thermoneutral			

Figure 4.1. Environmental conditions in the environmental rooms during the 21-day ARC study.

a.



b.

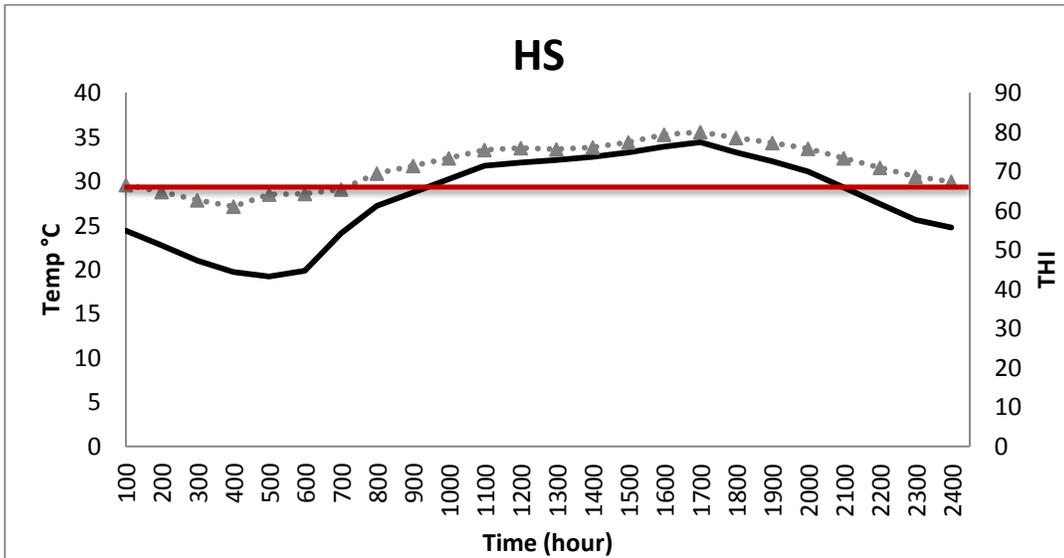


Figure 4.2. Environments in the controlled rooms during the ARC portion of the OmniGen trial. The red line represents the threshold THI for HS.

Table 4.1. Ingredients and chemical composition of the diet¹

Item	% of DM
Alfalfa Hay	65.02
Corn (Steam Flaked)	22.12
Whole Cottonseed	7.28
Distillers Grains (dry)	2.58
Supplement RS-1299 ²	2.04
MAXXER ³	0.96
Chemical analysis	
CP, %	19.75
NDF, %	25.39
ADF, %	19.75
Fat, %	4.74
DM, %	53.00
NE _L , Mcal/kg	1.76

¹Diet DM averaged 53% by weight of DM and moisture including added water

²The supplement contained 1.14% fat, 10.42% Ca, 4.49% P, 3.80% Mg, 0.49% S, 0.19% K, 15.83% Na, 7.52% Cl, 2,029.06 mg/kg Zn, 1,991.82 mg/kg of Mn, 974.24 mg/kg of Fe, 583.45 mg/kg of Cu, 67.86 mg/kg of Co, 12.28 mg/kg of Se, 6.81 mg/kg of Mo, 43.68 mg/kg of I, 304.9 IU/g of vitamin A, 30.2 IU/g vitamin D, and 1.0 IU/g of vitamin E

³Calcium salts of palm oil (Tarome Inc., Eloy, AZ)

Table 4.2. Dry matter intake, milk yield and milk composition of CON and OG fed animals housed under TN or HS conditions in environmentally controlled rooms

Item	Control			OmniGen-AF			SEM	P-value
	TN	HS	Recovery	TN	HS	Recovery		
DMI	24.4	22.7	25.2	24.9	24.8	26.0	0.55	0.01
Milk yield (kg)	33.1	30.3	30.4	33.9	31.4	31.3	1.02	0.23
FCM (kg/ d)	35.0	33.7	33.7	34.7	32.8	32.6	1.45	0.39
Protein (%)	2.95	2.98*	2.86	2.95	2.86	2.79	0.07	0.15
Protein (kg)	0.97	0.89	0.89	1.00	0.92	0.92	0.30	0.13
Lactose (%)	4.87	4.85	4.99	4.89	4.78	4.96	0.08	0.61
SCC (x 10,000)	20.3	23.9	59.4*	19.6	22.9	26.3	9.12	0.03

* = P-value \leq 0.05 and indicates the higher value

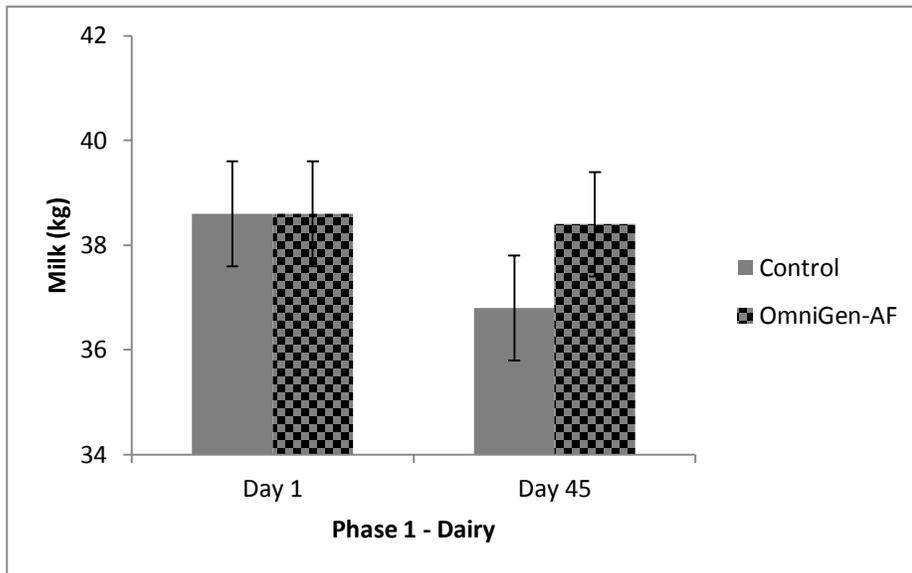


Figure 4.3. Mean milk production at the start of the study and on day 45 at the dairy

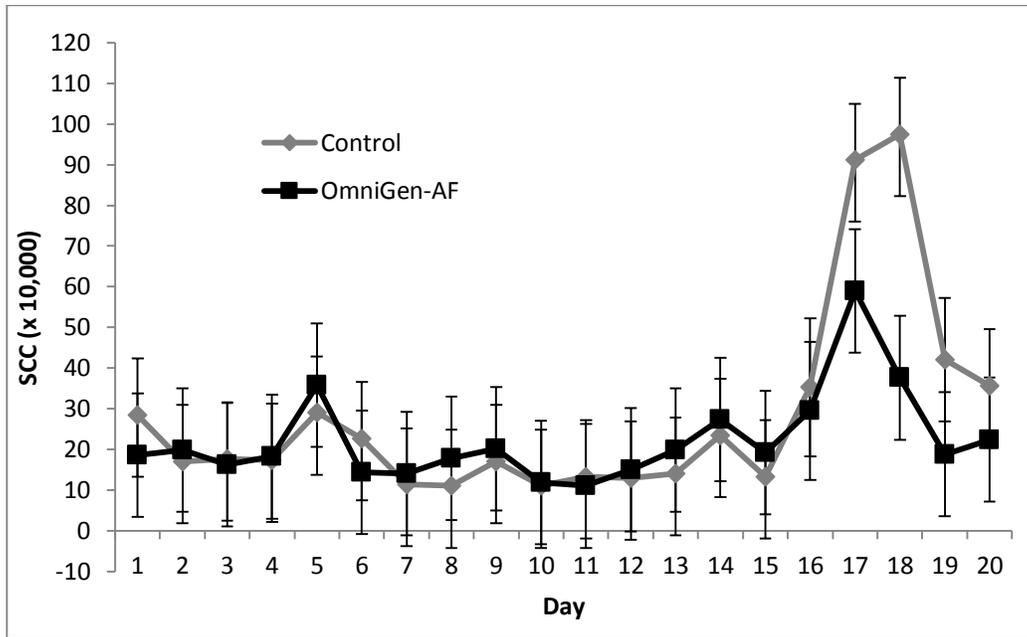


Figure 4.4. Milk Somatic cell count by day in CON and OG Fed cows during TN (d 1-7), HS (8-17) and recovery (18-20) periods.

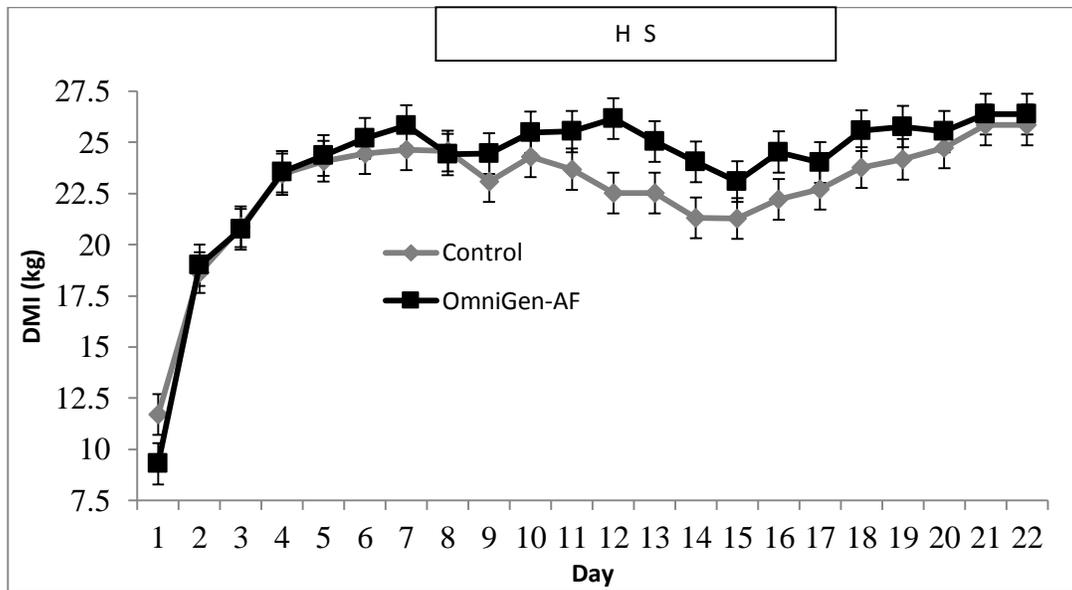


Figure 4.5. Dry matter intake by day during the ARC phase.

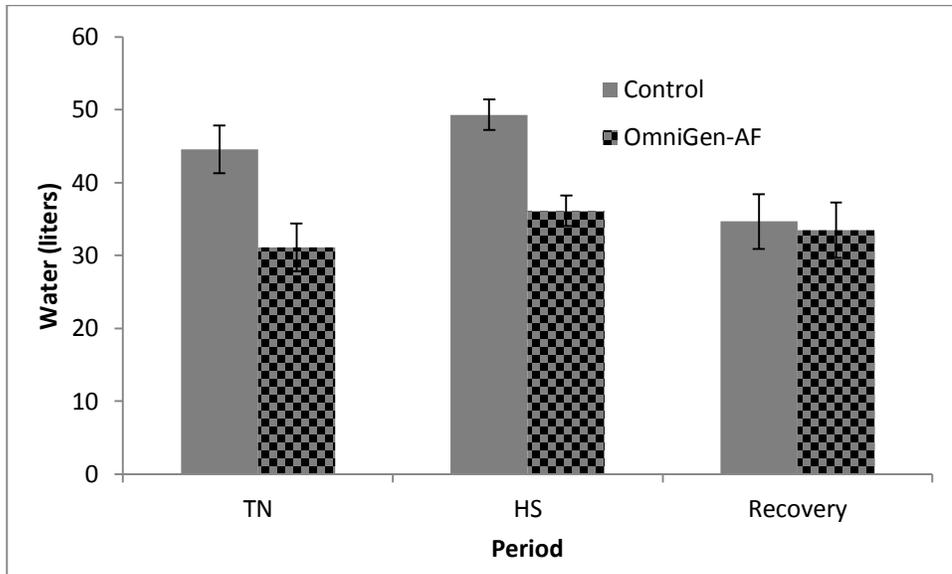


Figure 4.6. Mean daily water consumption in CON and OG fed cows during TN, HS and recovery periods.

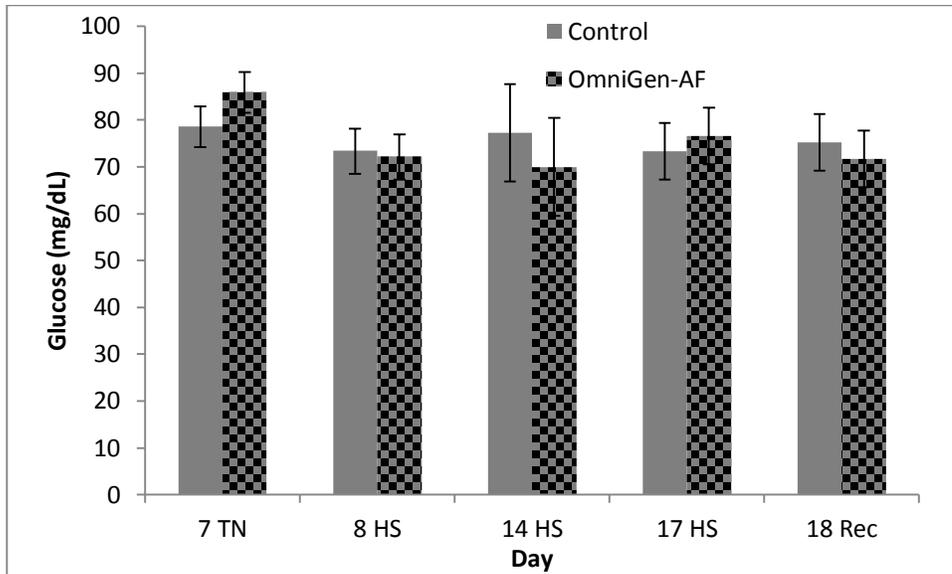


Figure 4.7. Plasma glucose concentrations in CON and OG Fed cows during TN, HS and recovery periods

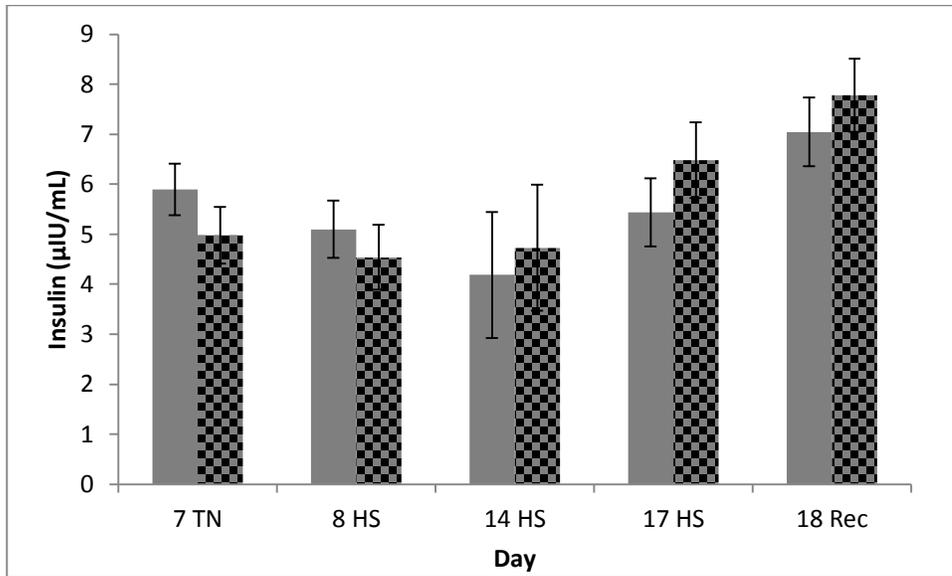


Figure 4.8. Plasma Insulin (ARC) concentrations with OG during TN and HS

Table 4.3. Mean respiration rates and rectal temperatures from cows fed OG and exposed to TN and HS

Item	Control			OmniGen-AF			SEM	P-value
	TN	HS	Recovery	TN	HS	Recovery		
Resp/ min								
600	26.9	31.9	28.3	26.6	30.4	27.9	1.40	0.40
1400	34.3	63.1*	35.3	30.1	58.3	35.5	2.99	0.20
1800	34.9*	60.8*	32.1	29.5	52.4	29.7	2.62	0.01
Rectal Temp (°C)								
600	38.2	38.0	37.9	38.2	38.1	38.1	0.05	0.26
1400	38.0	38.7*	38.0	38.1	38.5	38.1	0.09	0.77
1800	38.2	39.1*	38.2	38.2	38.8	38.3	0.08	0.25

* = P -value ≤ 0.05 and indicates the higher value

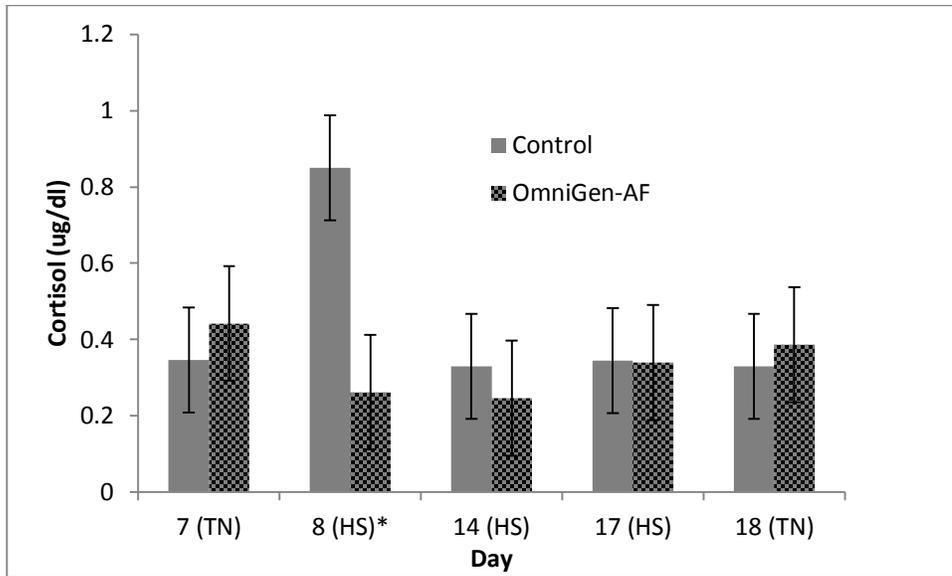


Figure 4.9. Mean serum cortisol in CON and OG fed cows during TN, HS and recovery periods.

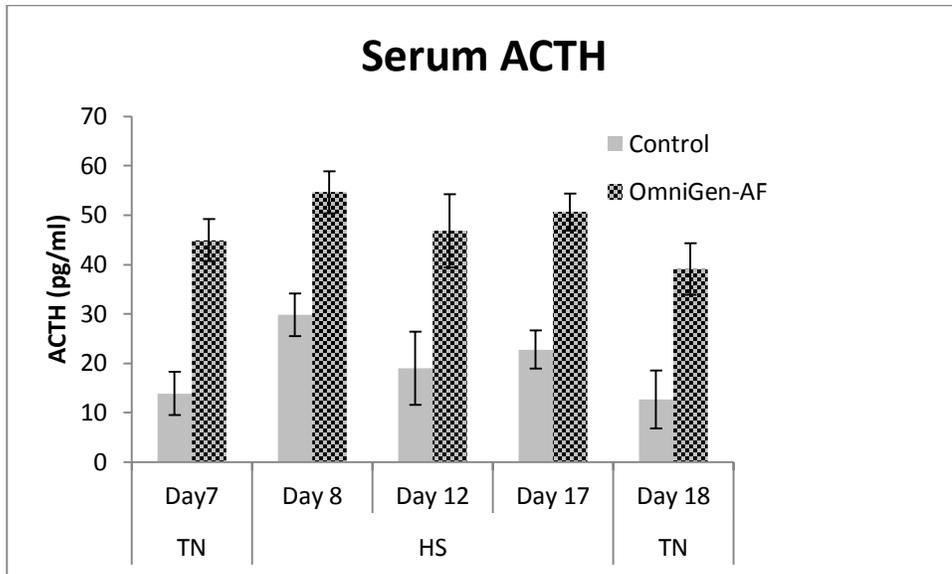


Figure 4.10. Serum ACTH concentrations in CON and OG fed animals during TN, HS and recovery periods.

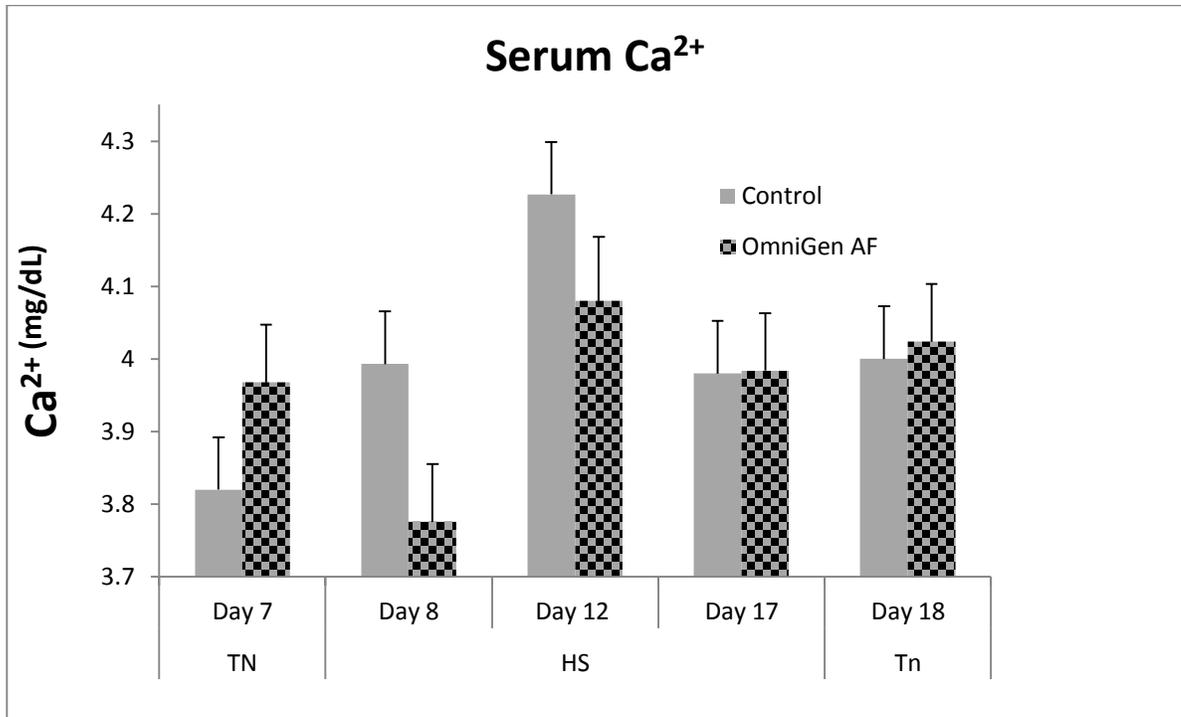


Figure 4.11. Ionized serum calcium in CON and OG fed cows during TN, HS and recovery periods. The figures represent the mean daily values, but day 14 is only at the 2000 h.

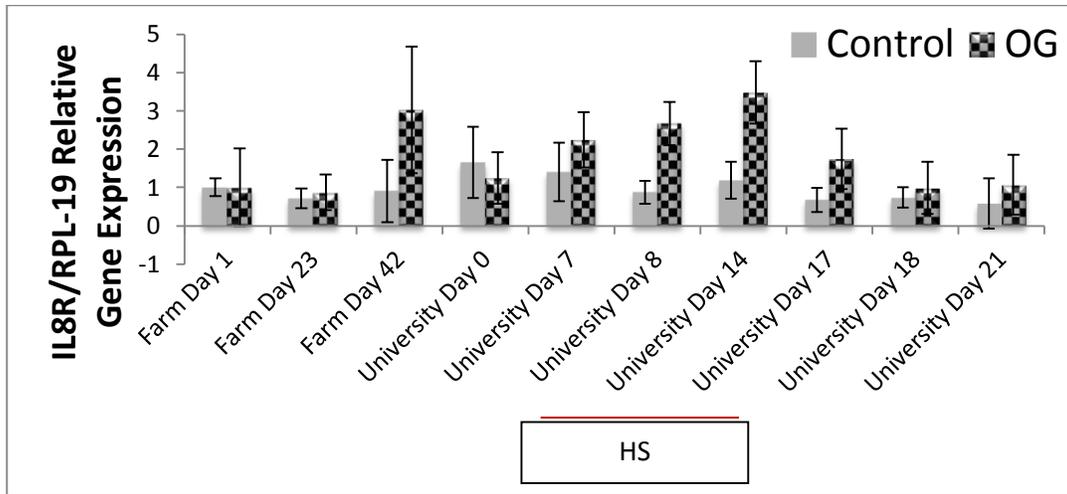


Figure 4.12. IL-8R Gene Expression at dairy and ARC with OmniGen-AF.

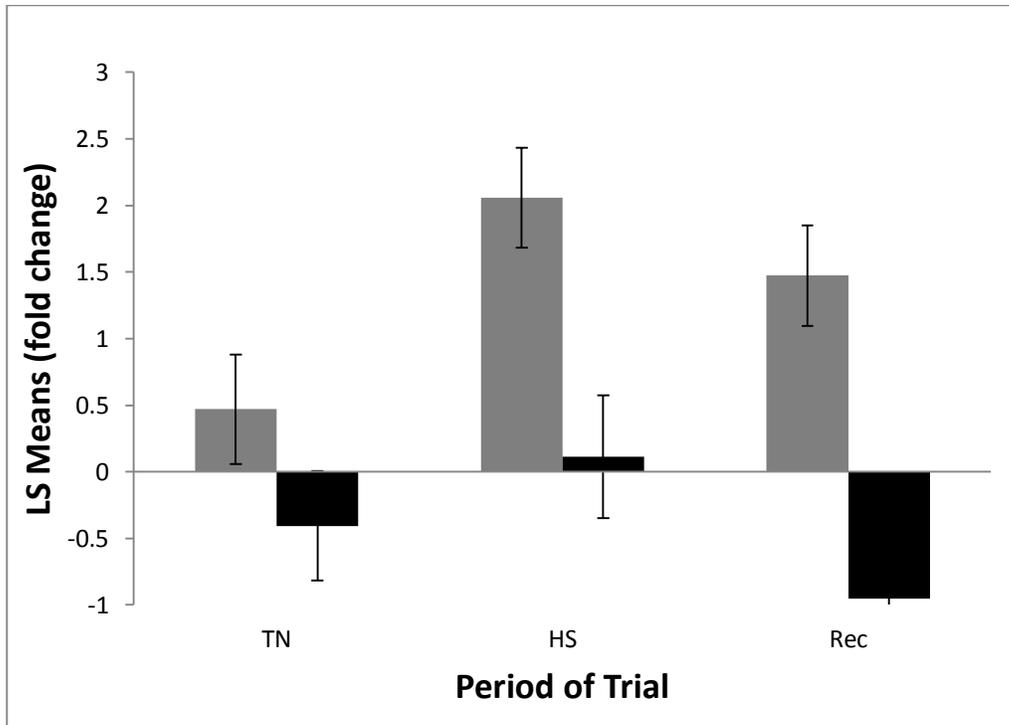


Figure 4.13. The change in expression of HSP27 with treatment of OG by period. The overall treatment effect was $P < 0.01$.

IMPACT OF β -HYDROXYBUTYRATE AND MERCAPTOACETATE ON FEED INTAKE SERUM METABOLITES, AND SERUM INSULIN IN LACTATING HOLSTEIN COWS

Abstract

Heat stressed (HS) dairy cows have elevated concentrations of serum β -hydroxybutyrate (BHB) and a decrease in feed intake. BHB is a ligand for the nicotinic acid receptor that has a higher binding affinity than niacin. Pancreatic β -cells increase insulin production with BHB. Insulin signals satiety and reduces feed intake. The role of BHB in dairy cows with feeding behavior and changes in metabolism was determined by two studies. In the first study a bolus of BHB administered at 1 mmol/kg BW or saline was administered to lactating dairy cows ($n=5$) over a two day period in a switchback design. The second study used the same 5 cows under HS conditions and a bolus of mercaptoacetate (β -MA, 1.55 mmol/kg body weight 0.75) or saline was administered using a similar design. BHB increased skin temperature (time 0.5, 1, 2, 3 and 4° C $r^2 = 0.98$ with serum BHB) and decreased serum NEFA levels ($P < 0.01$). There was no change in mean DMI, glucose or insulin. β -MA decreased feed intake, vaginal temperature, and insulin. There was an increase in serum BHB with the initial dose of β -MA and an initial decrease in serum glucose ($P < 0.0001$) with β -MA. Serum glucose and NEFA had overall responses nearing a trend with β -MA. The infusion of BHB did markedly increase skin temperature but did not alter feed intake in this study despite high plasma levels of BHB. This suggests that there was sufficient BHB in plasma to induce peripheral vasodilation but this level of plasma BHB was insufficient to cause

changes in feed intake. The inhibition of β -oxidation by mercaptoacetate did not decrease serum BHB nor did it increase dry matter intake in HS dairy cows.

Introduction

HS decreases feed intake in lactating dairy cows. It has been proposed that ketones suppress appetite and restricts energy intake in non-ruminants (The decrease in feed intake is greater with increased Temperature Humidity Index (**THI**), and indices that accounts for temperature and humidity and has been used by the dairy industry for five decades (Berry et al., 1964). There is also a decrease in milk yield with increasing THI. The lower milk yield is derived from the decrease in feed intake, and other physiological changes that are not fully defined (Rhodes et al., 2009). When HS cows were compared to a group of pair-fed (fed the same amount as the stressed group) under the same production parameters (DIM, daily milk production and parity), cows under TN conditions, the decreased intake only accounted for about 50% of milk losses (Baumgard et al., 2011). This demonstrated that about 50% of milk losses associated with HS came from physiologic alterations. Additionally, the HS cows had lower NEFA levels compared to the pair-fed even though both groups were in a negative energy balance. Plasma insulin levels were also greater in the HS group though plasma glucose levels were lower (Baumgard et al., 2011).

Figure 5.1 indicated that lactating Holstein cows exposed to HS had increased levels of serum BHB within 1 h of heat exposure and were higher at 10 h after HS.

Serum NEFA levels decreased at the same time points with HS and were significantly lower at 10 h.

It has been shown that BHB reduces feed intake in a monogastric (rat, Langhans et al., 1985). This has been difficult to demonstrate in ruminants. Feeding lactose to ruminants increased the production of butyrate in the rumen and caused elevated levels of plasma BHB. When lactose was fed, dry matter intake increased (DeFrain et al., 2004). The intraperitoneal injection of BHB in pygmy goats decreased serum NEFAs but did not cause a robust decrease in feed intake (Rossi et al., 2000). The addition of 1,3-butanediol, a substrate that can be synthesized into BHB caused a decrease in feed intake (Rossi et al., 2000). This hypophagic effect was seen when plasma BHB concentrations were greater than 0.7mmol/l (Rossi et al., 2000).

Vasodilation increases with heat stress (Aoki et al., 1997). This flushing increases peripheral flow of blood to the skin to reduce the core body temperature. Flushing can be regulated by the GPR109a (niacin receptor). Niacin causes flushing by activating the GPR109a receptor resulting in the release of prostaglandins (Ingersoll et al., 2012). Niacin also causes cutaneous flushing by activating the capsaicin receptor TRPV1 even without heat dependent activation (Ma et al., 2014). BHB acts as a competitive ligand to niacin to bind GPR109a (Taggart et al., 2005). The BHB induced flushing is the likely mechanism between increased vasodilation seen with low-calorie diets in humans (Sasaki et al., 2002). Through the activation of the GPR109a receptors on adipocytes, BHB also negatively regulates ketogenesis by blocking lipolysis (Taggart et al., 2005). This give an evolutionary advantage to elevated levels of BHB seen with HS.

The elevated levels of BHB seen during HS may account for the decrease in feed intake, increased insulin levels, lower NEFA and glucose levels, and contribute to increased vasodilation. We hypothesize that administering a bolus of BHB to lactating dairy cows will decrease feed intake, increase serum insulin, cause elevated skin temperatures and decrease serum NEFA and glucose levels. Additionally we propose that a pharmaceutical bolus of β -MA in HS lactating cows will decrease serum BHB levels and increase feed intake.

Mercaptoacetate

β -Mercaptoacetate (β -MA) is a compound that is classified as a fatty acid oxidative (FAO) inhibitor. These FAO inhibitors can alter feeding behavior in many species including humans (Friedman and Tordoff, 1986; Kahler et al., 1999). β -MA also increases food intake in TN exposed rodents maintained on a high fat diet (Darling and Ritter, 2009; Del Prete et al., 2000) while decreasing serum BHB levels (Kahler et al., 1999). Interestingly, both HS and high fat diet feeding increase serum BHB (Soriani et al., 2013; Murata et al., 2013). BHB is known to suppress phagic drive (Langhans et al., 1985).

Our objective was to use β -MA to limit acetyl CoA availability for BHB synthesis during HS in lactating dairy cows to increase feed intake. Additionally, a second study during TN evaluated the effects of a bolus dose of BHB on feed intake and other intermediates. We hypothesize that Serum β -hydroxybutyrate (BHB) levels seen during

heat stress in dairy cows will decrease serum insulin, inhibit cell proliferation, reduce dry matter intake, and increase vasodilation by receiving a bolus of BHB during thermoneutral conditions. Mercaptoacetate will block β -oxidation, reduce acetyl CoA, and inhibit hepatic ketogenesis. This will reduce BHB during HS and increase feed intake.

Materials and Methods

We conducted a preliminary study on blood collected immediately before, 1, 3, and 10 h after exposure to heat stress. This study was conducted to evaluate the short term effects of heat stress on serum BHB and NEFA levels. Samples were obtained from 6 lactating Holstein cows housed in the environmental chambers at the University of Arizona. At each time point we collected blood through venipuncture of the coccygeal (tail) vein. Blood was collected and immediately ejected into borosilicate glass tubes, placed on ice, and stored at 4°C overnight to allow clotting. We collected serum after centrifuging clotted samples at 1,500Xg for 15 min at 4°C, aliquoted the serum to prevent repeated freeze thaw of samples, and stored all samples at -80°C until analysis. We measured serum BHB and NEFA levels to understand when BHB increases and NEFAs decrease relative to heat stress (figure 5.1).

We performed two cross over studies in late lactation multiparous Holstein cows designed to determine the effect of altering serum BHB on dry matter intake (DMI), serum insulin, glucose, beta-hydroxybutyrate, and non-esterified fatty acids (NEFAs). To control heat exposure cows were housed in tie stalls in rooms that allowed

environmental control of temperature and humidity. We prepared a total mixed ration, balanced for high producing dairy cows, daily and provided it at 120% of the previous day's consumption to ensure ad libitum feed intake (table 5.1). Feed intake, milk yield and water consumption were recorded daily, while respiration rate, rectal and skin temperatures were recorded at 0600, 1400, and 1800 h throughout these studies. Skin temperature was measured by Raytek® (Raytek Corporation, Santa Cruz, CA) infrared thermometer, and rectal temperature were measured using a GLA® M700 (**GLA Agricultural Electronics**, San Luis Obispo, CA). We milked cows at 0600 and 1800h. Cows were fed immediately following milking. These studies were approved by the Institutional Animal Care and Use Committee at the University of Arizona.

Blood Collection and Handling

We placed bilateral jugular catheters 2 days prior to the onset of the study. Patency was maintained by flushing 10 mL heparinized saline (1,000 USP/L) twice daily. Between sampling, catheters were flushed with 10 mL heparinized saline (100 USP/mL). We collected samples by first collecting and discarding 6 ml of fluid to eliminate heparin, then collecting 8 ml of blood. Blood was immediately ejected into borosilicate glass tubes, placed on ice, and stored at 4° C overnight to allow clotting. We collected serum after centrifuging clotted samples at 1,500Xg for 15 min at 4° C, aliquoted the serum to prevent repeated freeze thaw of samples, and stored all samples at -80°C until analysis.

Serum Metabolite and Hormone Analysis

We measured serum glucose (Cat. # G7519, Pointe Scientific Inc., Canton, MI), NEFA (NEFA-HR(2); Wako Diagnostics, Richmond, VA), and BHB (Cat. # 700190, Cayman Chemical, Ann Arbor, MI) concentrations using commercially available colorimetric kits. Serum Insulin concentration was assessed using a bovine insulin ELISA kit (Cat # 10-1201-01, Mercodia Inc., Salem, NC).

β -hydroxybutyrate Bolus Study

To isolate the metabolic effects of elevated BHB from those of heat stress we monitored the phagic and metabolic response to BHB in cows maintained at TN (THI < 67). Cows were maintained in the tie stalls at TN for 6 days prior to study onset. On day 6, we treated the cows treated with either BHB (1 mmol/kg bw 30 ml/100 kg bw) or saline (30 ml/100 kg bw), two days later each cow received the opposing treatment. This crossover design allowed for paired statistical analysis.

Bolus BHB or saline was given immediately following morning milking and feed was offered 15 min after infusion. Feed weight was taken at 60, 120, 240, 480, 720 and 1440 minutes after feed provision. We measured skin temperature at the same time points to understand the effect of BHB on peripheral vasodilatation, and collected blood samples at -30, 0, 15, 30, 60, 120, 180, 240, 360, and 480 minutes relative BHB injection. Vaginal temperature data loggers (Product; Company, City, State) recorded core body temperature every 15 minutes throughout the study.

β -Mercaptoacetate

A second study was designed to understand the metabolic effects of pharmacologically inhibiting β -oxidation and ketogenesis in the heat stressed cow. To induce heat stress, we set conditions to a THI = 80 for 19 h, and 4 h of a THI < 68 (figure 5.2). The first 6 d of HS allowed the cows to acclimate and feed intake to plateau. On day 6, we gave the cows a bolus dose of β -MA ($1.55 \text{ mMol/kg}^{0.75}$ in 14 ml/100 kg bw) or saline (14 ml/100 kg bw) 2 h after feeding. Two days later cows were given the opposing treatment and data was collected identically. This crossover design allowed for paired analyses. To calculate dry matter intake, we recorded feed weight daily on all days and at 60, 120, 240, and 480 minutes after bolus on study days.

We measured skin temperatures at the same time points to assess the degree of peripheral vasodilatation and potential heat loss. We collected blood at -0.5, 0, 0.25, 0.5, 1, 2, 3, 4, 6, and 8 minutes relative β -MA injection. We used vaginal temperature data loggers to record core body temperature every 15 minutes.

Statistical Analysis

The studies to assess the response to BHB under thermoneutral conditions and β -MA in heat stress conditions were analyzed separately using paired comparison, repeated measures mixed ANOVA models in SAS (SAS Institute, Cary, NC). The model included main effects of treatment, time and their interaction. To ensure there was no effect of the order of treatment we tested the effect of day and all interactions. There was never a significant effect of day or any significant effect of the interactions with day. Thus, day and all interactions were removed from all models. Comparisons between

least squares means were assessed using the pdiff function and correction for multiple comparisons was performed using the Bonferroni adjustment. The actual mean \pm SEM is presented in all figures made in Graphpad PRISM® version 5.04 for Windows (GraphPad Software, San Diego, CA).

Results

Response to short-term heat stress

Within one hour of heat exposure (THI > 68) cows had elevated serum BHB that remained elevated out to 10 hours of heat exposure (Figure 5.1A). Since BHB is known to suppress adipose tissue lipolysis through signaling at the G α i coupled GPR109a, we evaluated serum NEFAs. Heat stress decreased serum NEFAs significantly by 10 hours (Figure 5.1B).

β -hydroxybutyrate Bolus

Administration of a BHB bolus increased serum BHB ($P < 0.001$) at 0.25, 0.5, and 1h post infusion (Figure 5.4). Coincident with the highest levels of serum BHB, BHB treated cows had lower serum NEFA concentrations 15 minutes after infusion than saline treated cows. Likewise, serum glucose was lower in BHB treated than in saline treated cows at 30 minutes after infusion. Additionally, serum glucose was decreased relative to time 0 at both 15 and 30 minutes following infusion in BHB treated cows, but not in saline treated cows. Although serum insulin did not significantly vary by treatment, the provision of feed at 15 minutes following may have muted the possibility

for a difference between treatments. The glucose:insulin ratio did not change with treatment.

BHB did not affect vaginal or skin temperatures (figure 5.5). Moreover, BHB did not alter dry matter intake (figure 5.6).

B-Mercaptoacetate

β -MA is known to inhibit β -oxidation by limiting activity of long chain acyl-CoA dehydrogenase, which limits acetyl CoA the substrate for ketogenesis. Moreover, β -MA inhibits activity of β -OH butyrate dehydrogenase I which catalyzes the synthesis of acetoacetate to BHB. Thus, we expected that β -MA would decrease serum BHB. Surprisingly, β -MA treatment increased serum BHB concentrations 30 m after treatment initiation (figure 5.7). Although β -MA increases serum NEFA in rodents, we show here that β -MA did not affect an increase in serum NEFA concentrations (figure 5.7). This apparent difference may result from the anti-lipolytic effects of BHB. β -MA decreased serum insulin concentrations relative to saline from 1 to 6 h post β -MA bolus (figure 5.7). This effect of β -MA on serum insulin elucidates the importance of β -oxidation in the secretion of insulin in the cow. β -MA increased serum glucose concentrations, presumably by decreasing pancreatic insulin release (figure 5.7). However, serum glucose increased prior to a measurable decrease in serum insulin. These changes in serum glucose and insulin resulted in a robust increase in the glucose insulin ratio at 1 and 2 hours following β -MA infusion.

β -MA reduced vaginal temperature from 30 m to 6 h post β -MA bolus (figure 5.8). Although rectal temperatures were not recorded continuously, when measured at identical time points (0, .25, .5, and 1 h), vaginal and rectal temperature were highly correlated ($P < 0.001$; $R^2 = 0.61$; figure 5.9). Shaved skin temperature was not affected by treatment. β -MA decreased dry matter intake at 6, 10, and 24 h following treatment ($P < 0.0001$; figure 5.10).

Discussion

Heat stress has a tremendous impact on the dairy industry with annual losses estimated to cost \$900 million (St. Pierre et al., 2003). Heat stress depresses food intake and reduces milk yield in lactating dairy cows. Although reducing the environmental heat load can effectively limit heat exposure, the costs of heat abatement and the associated water use associated prevent complete limit full application to eliminate heat stress (Armstrong et al., 2003; Burgous et al., 2007; Anderson et al., 2013). Alternatively, we propose to dampen the losses associated with heat stress through pharmacological inhibition of the physiological changes that decrease milk production and feed intake.

Approximately, 50% of the decrease in milk yield is attributed to the reduced dry matter intake and the remaining 50% of milk losses are physiologically induced (Rhoads et al., 2007; Baumgard et al., 2011). Heat stress reduces feed intake, milk yield, serum NEFA and glucose concentrations, while increasing vasodilation and serum insulin concentrations in lactating dairy cows (Rhoads et al., 2009; Di Costanzo et al., 1997).

Vasodilation increases with heat stress (Aoki et al., 1997). This flushing increases peripheral flow of blood to the skin to reduce the core body temperature. Flushing can be regulated by the GPR109a (niacin receptor). Niacin causes flushing by activating the GPR109a receptor resulting in the release of prostaglandins (Ingersoll et al., 2012). Niacin also causes cutaneous flushing by activating the capsaicin receptor TRPV1 even without heat dependent activation (Ma et al., 2014). BHB acts as a competitive ligand to niacin to bind GPR109a (Taggart et al., 2005). The BHB induced flushing is the likely mechanism between increased vasodilation seen with low-calorie diets in humans (Sasaki et al., 2002). Through the activation of the GPR109a receptors on adipocytes, BHB also negatively regulates ketogenesis by blocking lipolysis (Taggart et al., 2005). This give an evolutionary advantage to elevated levels of BHB seen with HS.

As increased serum BHB induces many similar effects as HS. Elevated levels of BHB can decrease feed intake (Rossi et al., 2000), mammary cell proliferation (Kennedy et al., 2002), and adipose tissue lipolysis and the resulting serum NEFA concentration (Taggart et al., 2005). Similar to HS, BHB also increases insulin release from pancreatic β -cells (Itoh et al., 1998; Biden and Taylor, 1983) and decreases serum glucose concentrations. The decrease in glucose may result from the elevated serum insulin (figure 5.4). Like Nardone et al. (1997), we have shown that HS (figure 5.1) increases BHB levels in cows. This rise remained elevated out to 10 h of heat stress. In other studies plasma BHB levels were positively increasing levels of THI and this is consistent with the increase we saw over 10 h as THI increased (Soriani et al., 2013). In other studies we have seen that heat stress increases serum BHB concentrations 4 days after

initiation of HS. Surprisingly, in the studies reported here we don't see any heat stress effect on serum BHB at 6 days after HS initiation. This may result from increased BHB clearance by heat adapted tissues in the heat stressed cow. In fact, our data with β -MA may suggest that BHB clearance increases with heat stress. The BHB levels seen on or after d 6 of the β -MA study done under HS are lower than those seen in other studies the first 4 days of hyperthermia. Lower clearance of BHB could increase circulating levels, inducing vasodilation and short term acclimation to HS.

To recapitulate the heat stress response in thermoneutral cows, we gave a BHB bolus that increased BHB, decreased serum NEFA and decreased serum glucose, as is observed during heat stress. Early research showed that the infusion of propionate and butyrate into the rumen increased plasma insulin levels four fold, but BHB had no effect on plasma insulin (Horino et al., 1968). Despite, reports that BHB bolus doses increase insulin release independent of glucose in non-ruminants (Hermansen, 1982; Biden and Taylor, 1983; Malaisse et al., 1990) and from isolated islets (Grant et al., 1980; MacDonald et al., 2011), we observed no significant effect on serum insulin in response to a bolus dose of BHB. This incongruent result may result from study design. The primary objective was to understand the relationship between BHB and feed intake. As such, we conducted the study at thermoneutral and fed the cows after they received the treatment. This timing of feed provision may have masked the insulin response that is typically measured while the subject is fasting. Additionally, during heat stress lactic acid production increases with panting and anaerobic respiration (Lorenzo et al., 2011). Both lactate and BHB are similarly transported into β -cells (Muller et al., 2002) and both

induce insulin release (Ishihara et al., 1999). In fact, their co-application can result in 10 to 20-fold increase in insulin secretion *in vitro* (McDonald et al., 2008). Thus, during heat stress, the increase in BHB and lactate may work additively to affect a rise in serum insulin concentrations. Future measurements of lactate with heat stress and the relationship between lactate and BHB should be evaluated. This may explain why the BHB bolus did not alter serum insulin during TN.

BHB and reduces feed intake in rats (Le Foll et al., 2014). However, in goats BHB alone was unable to decrease feed intake. Yet, when infused with both BHB and 1,3-butanediol feed intake decreases in goats (Rossi et al., 2000) indicating that other intermediates may influence feeding behaviors seen in HS, but not replicated in our TN study. This comparison, suggests that ruminants may be less sensitive to the hypophagic effects of ketones. Given the ruminants are continuously ketogenic they may have adapted a greater tolerance. Alternatively, the rapid clearance of BHB is opposite to the sustained levels of BHB seen in rodents may have prevented the hypophagic effect of BHB, while explaining how 1,3 butanediol can encourage a hypophagic response. Our findings are in agreement with previous findings in dairy cows where DMI was not altered by BHB (Zarrin et al., 2013).

In an attempt to limit heat stress induced BHB production, we infused β -MA into heat stress cows to limit substrate availability for ketogenesis. Surprisingly, despite limiting BHB production β -MA increased serum BHB. β -MA inhibits the enzyme fatty acid CoA dehydrogenase, the enzyme responsible for the first step in fatty acid oxidation

(Li et al., 2013). Inhibition of fatty acid oxidation should also block the activation of ketogenic enzymes including acetoacetyl-CoA thiolase, β -hydroxybutyrate dehydrogenase and 3-oxoacid-CoA transferase (Hagopian et al., 2012). β -MA also inhibits palmitoylcarnitine (an ester involved in the metabolism of fatty acids) supported respiration (Sabbagh et al., 1985). The inhibition of palmitoylcarnitine increased the output of lactate and increased the ratio of BHB to acetoacetate under gluconeogenic conditions in rat hepatocytes (Pryor et al., 1987). There are likely other effects of β -MA on cellular energetics that are not accounted for.

β -MA inhibits long chain acyl CoA dehydrogenase in the first step of β -oxidation (Li et al., 2013) and inhibits BDH1 by reducing acetyl CoA. Acetyl CoA is required for ketogenesis and fatty acid oxidation increases ketogenic enzymes (Hagopian et al., 2012). We expected to have a decrease in BHB but β -MA treated cows had elevated BHB 30 m post infusion. Perhaps there was an effect on BDH2 and BHB clearance. Serum NEFA levels were unchanged in our ruminant model, yet in monogastrics NEFAs increase with β -MA in fasted animals and BHB decreased 7-fold (Bernard et al., 2002). This may occur due to the clearance of BHB, elevated BHB decreases NEFA concentrations (Taggart et al., 2005) and in our study BHB was elevated. Insulin levels decreased with β -MA. During FAO there is a rise in acyl-CoA esters that correlates inversely to insulin release and this could explain the decreased insulin seen with β -MA in our study (Prentki et al., 1992). In our study the reduced serum insulin levels may explain why the NEFA levels were unchanged. We expect that the increased glucose is a result of decreased serum insulin, not a direct effect of β -MA. In fact, by inhibiting β -

oxidation, we would expect β -MA to increase glucose clearance and decrease serum glucose if it didn't affect a decrease in serum insulin.

Although this study with β -MA didn't induce the expected decrease in serum BHB concentration, which would have allowed us to ascertain the effect of BHB in the phenotypes common to heat stress, it does provide insight into the direct effect of core body temperature on phagic drive. β -MA decreased vaginal temperatures of HS cows. At times when both rectal and vaginal temperatures were taken, they were highly correlated ($R^2 = 0.61$; $P = 0.0004$). β -MA decreases metabolic rate and body temperature by inhibition of β -oxidation (Westman and Geiser, 2004). Although, β -MA lowered core body temperature there was no concurrent increase feed intake. In fact, β -MA decreased feed intake.

Herein, we have shown that BHB recapitulates many of the phenotypes common to heat stress in the dairy cow. Moreover, we have shown that pharmacologically reducing core body temperature will not necessarily affect an increase in feed intake. Future studies to ascertain the role of BHB in heat stress induced hypophagia should aim to inhibit β -OH butyrate dehydrogenase 1 or activate β -OH butyrate dehydrogenase II to inhibit BHB synthesis or activate BHB clearance and degradation, respectively.

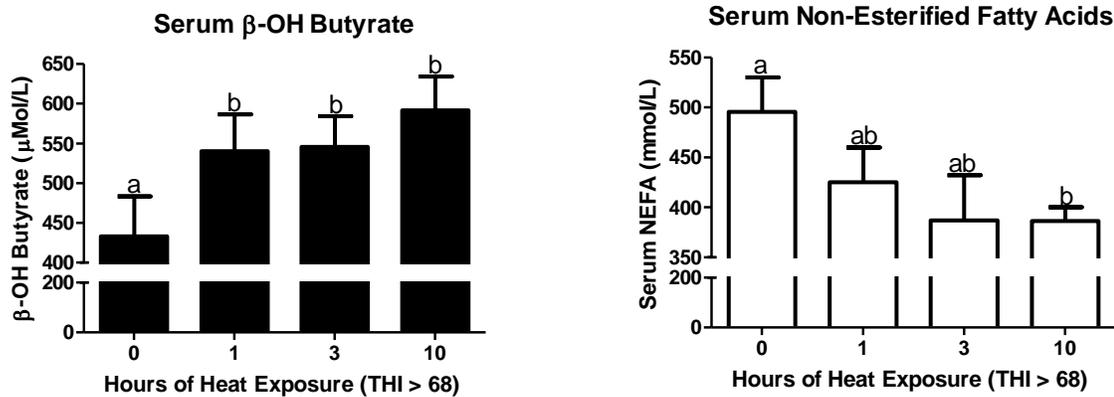


Figure 5.1. Serum β -OH Butyrate and non-esterified fatty acid concentrations 1, 3, and 10 hours following exposure to temperature humidity index (THI) > 68. The THI at time 0 = 67.6, 1 = 73.7, 3 = 80.7, and 10 = 84. ^{a,b} Bars that do not share a common letter differ significantly ($P < 0.05$).

Figure 5.2. Treatment schedule of β -mercaptoacetate and β -hydroxybutyrate bolus studies with times of treatment, sampling, feeding and milking.

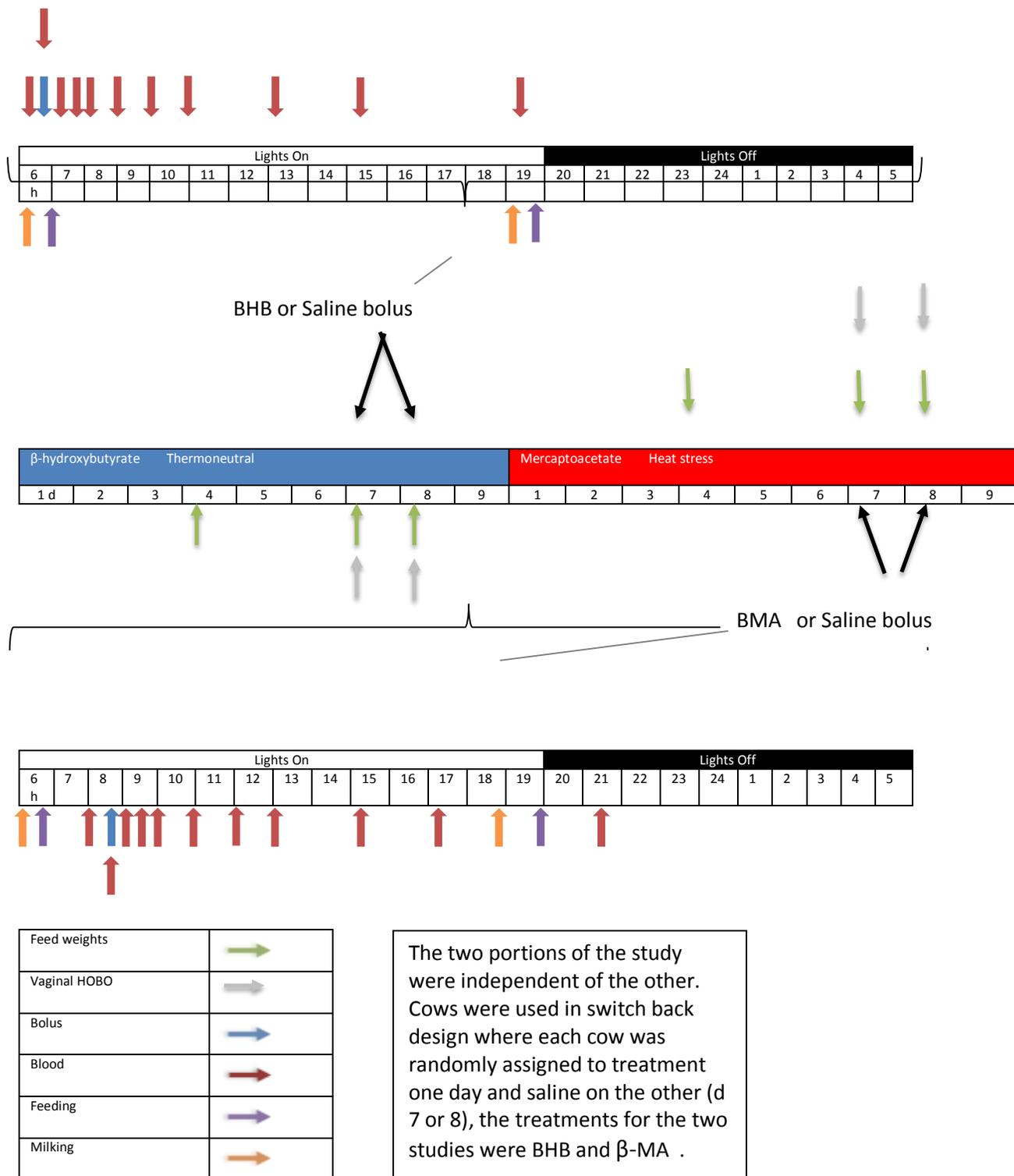


Table 5.1. Ingredients and chemical composition of the diet¹

Item	% of DM
Alfalfa Hay	65.02
Corn (Steam Flaked)	22.12
Whole Cottonseed	7.28
Distillers Grains (dry)	2.58
Supplement RS-1299 ²	2.04
MAXXER ³	0.96
Chemical analysis	
CP, %	17.00
NDF, %	26.43
ADF, %	20.24
Fat, %	4.99
DM, %	53.00
NE _L , Mcal/kg	1.76

¹Diet DM averaged 53% by weight of DM and moisture including added water

²The supplement contained 1.14% fat, 10.42% Ca, 4.49% P, 3.80% Mg, 0.49% S, 0.19% K, 15.83% Na, 7.52% Cl, 2,029.06 mg/kg Zn, 1,991.82 mg/kg of Mn, 974.24 mg/kg of Fe, 583.45 mg/kg of Cu, 67.86 mg/kg of Co, 12.28 mg/kg of Se, 6.81 mg/kg of Mo, 43.68 mg/kg of I, 304.9 IU/g of vitamin A, 30.2 IU/g vitamin D, and 1.0 IU/g of vitamin E

³Calcium salts of palm oil (Tarome Inc., Eloy, AZ)

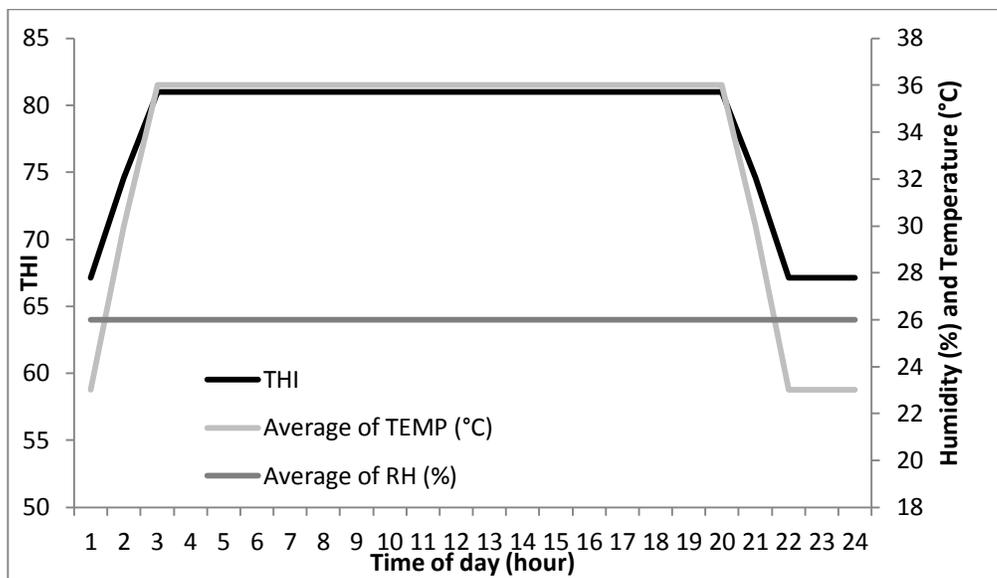


Figure 5.3. HS conditions during the β -mercaptoacetate portion of the study.

Table 5.2. The change in production and physiological responses with β -mercaptoacetate and β -hydroxybutyrate

Item	TN				HS			
	Saline	BHB	SEM	<i>P</i> value	Saline	BMA	SEM	<i>P</i> value
Milk (kg)	21.2	20.8	2.76	0.89	15.5	14.05	2.76	0.62
Feed (kg)	21.45	23	1.35	0.44	16.3	12.9	13.47	0.09
Water (l)*	99.1	96.1	53.15	0.96	179.55	182.2	75.16	0.97
Skin Temp (°C)								
Shoulder	32.8	33	0.40	0.71	37.1	37.7	0.57	0.29
Rump	32.9	32.1	0.49	0.21	37.2	38.1	0.69	0.18
Tailhead	32.7	39.9	0.43	0.74	37.4	37.8	0.61	0.48
Rectal temp (°C)	38.1	38.2	0.14	0.51	39.7	39.8	0.19	0.73
Respirations/min	35.2	34.4	6.51	0.93	90.8	93.6	9.21	0.76

* There were two cows that played with their water and regularly splashed it out.

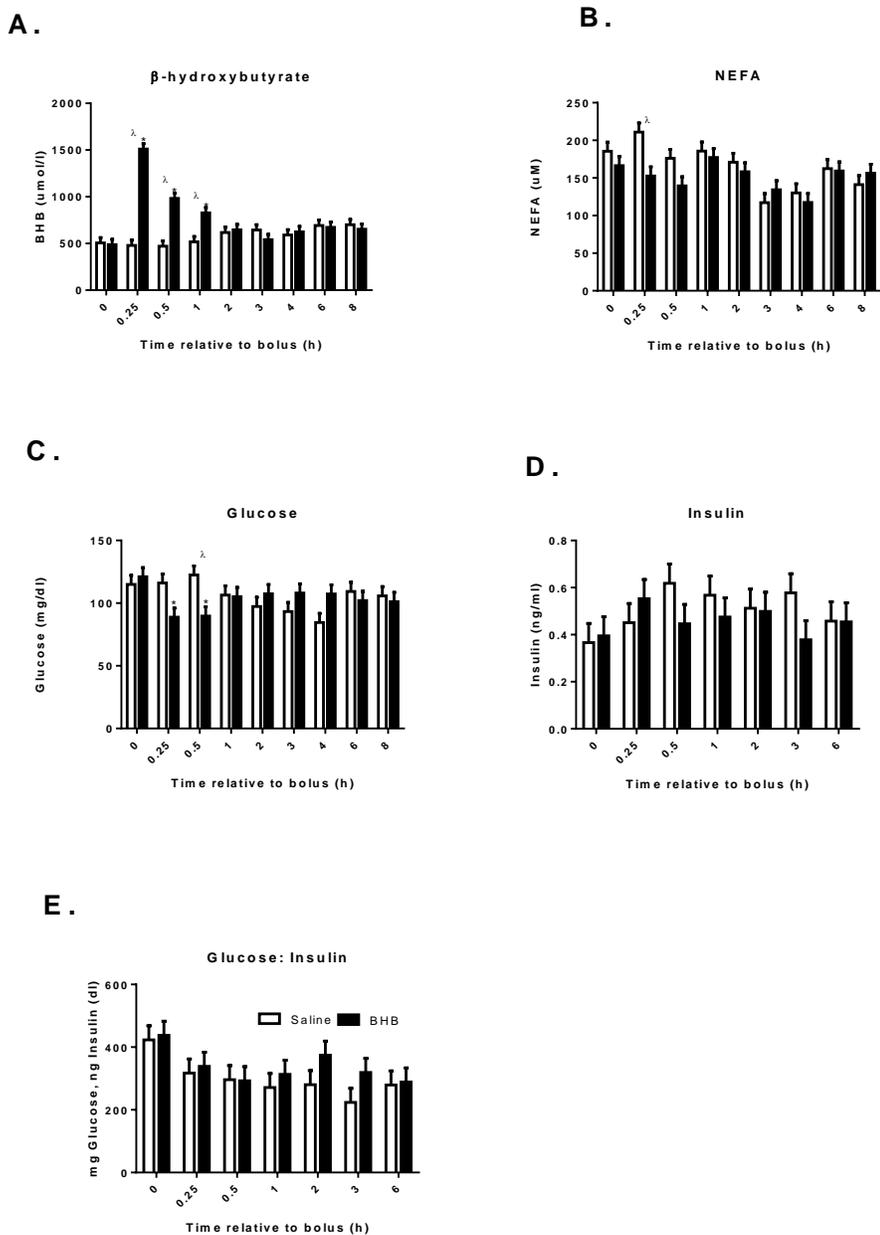
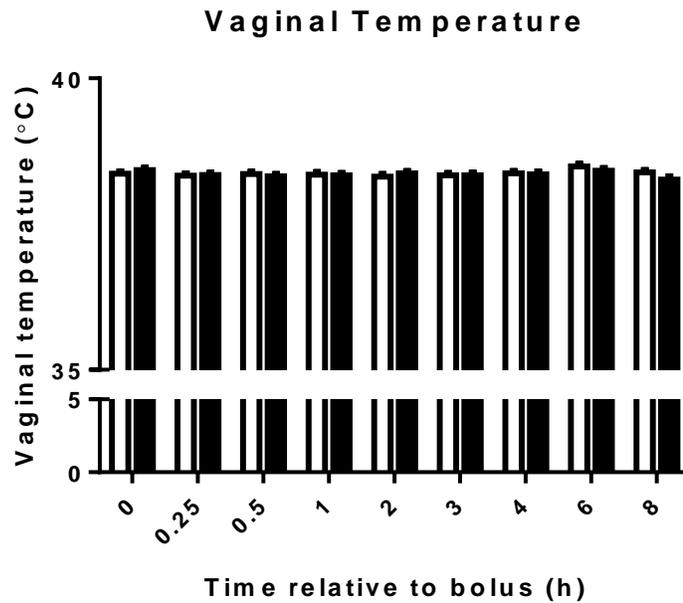


Figure 5.4. The change from baseline (time 0) in; (a) serum BHB, (b) serum glucose, (c) serum NEFA, and (d) serum insulin and (e) glucose : insulin in cows treated with a bolus of β -hydroxybutyrate. * Bars that have an asterisk differ significantly from time 0, λ bars within a time point that share a lambda symbol are significantly different.

A.



B.

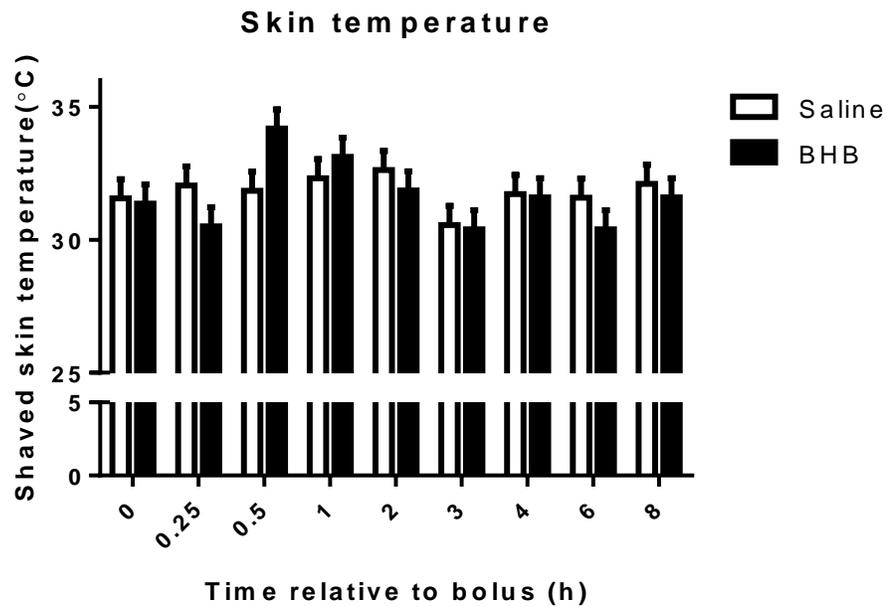


Figure 5.5. The change in temperature from baseline (0 h) by treatment. Skin temperature (a) and vaginal temperature (b). * Bars that have an asterisk differ significantly from time 0, λ bars within a time point that share a lambda symbol are significantly different.

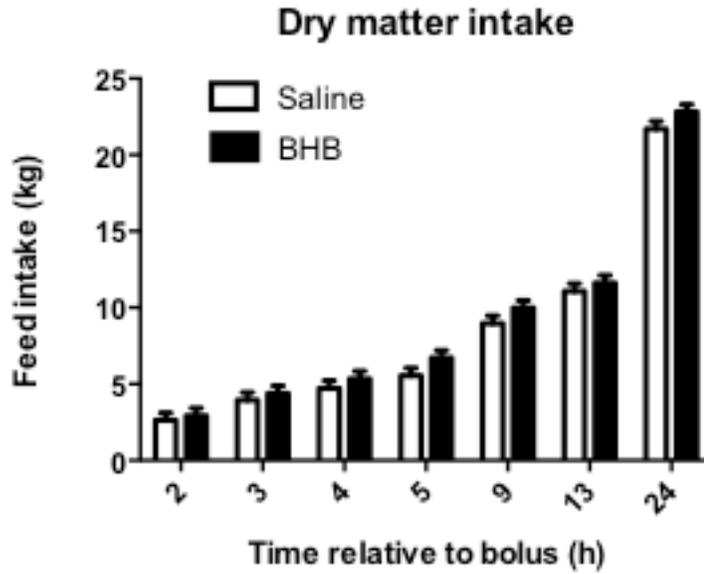


Figure 5.6. The mean change in dry matter intake (a) between measurements, and the cumulative total in DMI (b) by time point in BHB treated cows. * Bars that have an asterix differ significantly from time 0, λ bars within a time point that share a lambda symbol are significantly different.

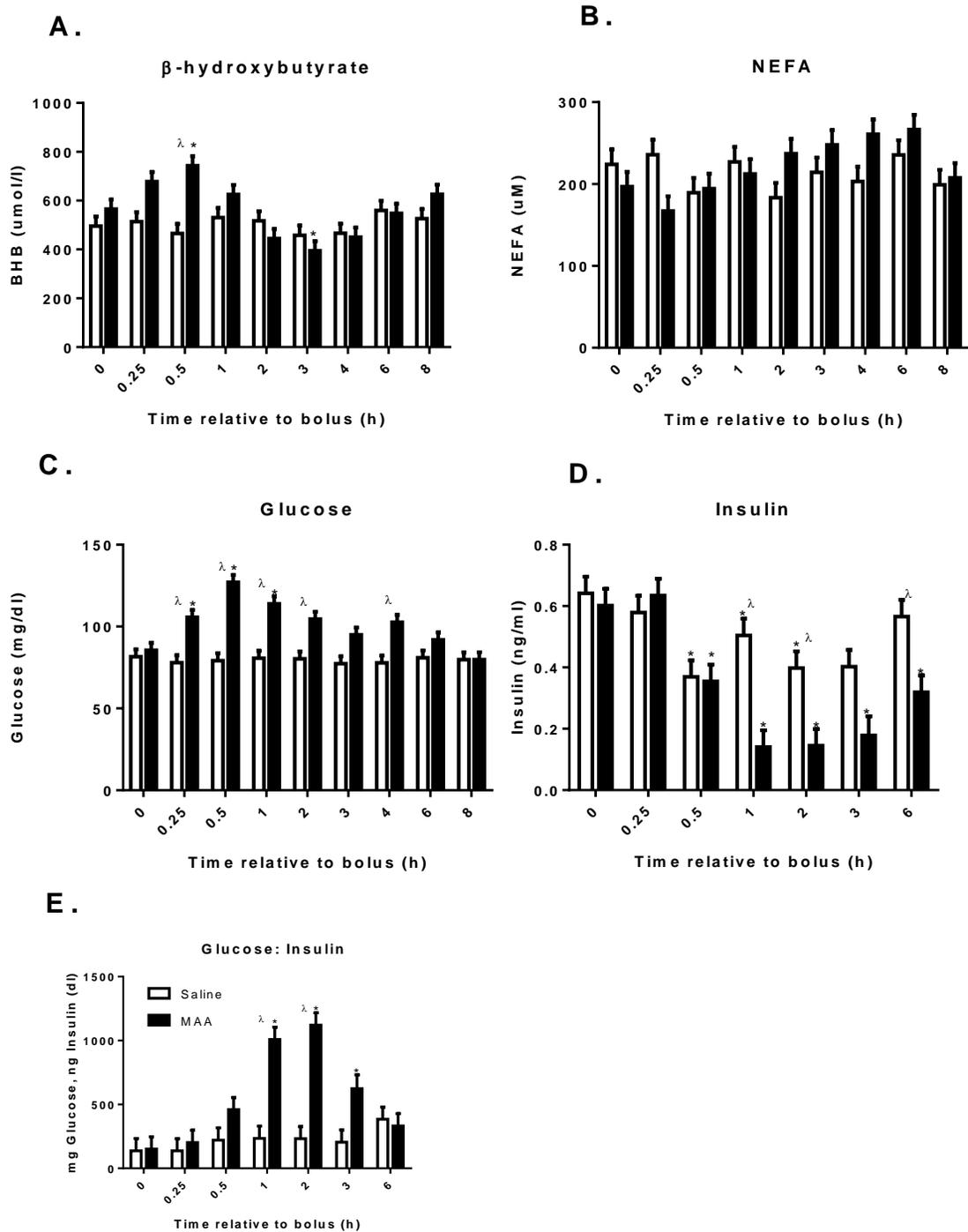


Figure 5.7. The change from baseline (time 0) in; (a) serum BHB, (b) serum glucose, (c) serum NEFA, and (d) serum insulin and (e) glucose : insulin in cows treated with a bolus of β -mercaptoacetate. * Bars that have an asterix differ significantly from time 0, λ bars within a time point that share a lambda symbol are significantly different.

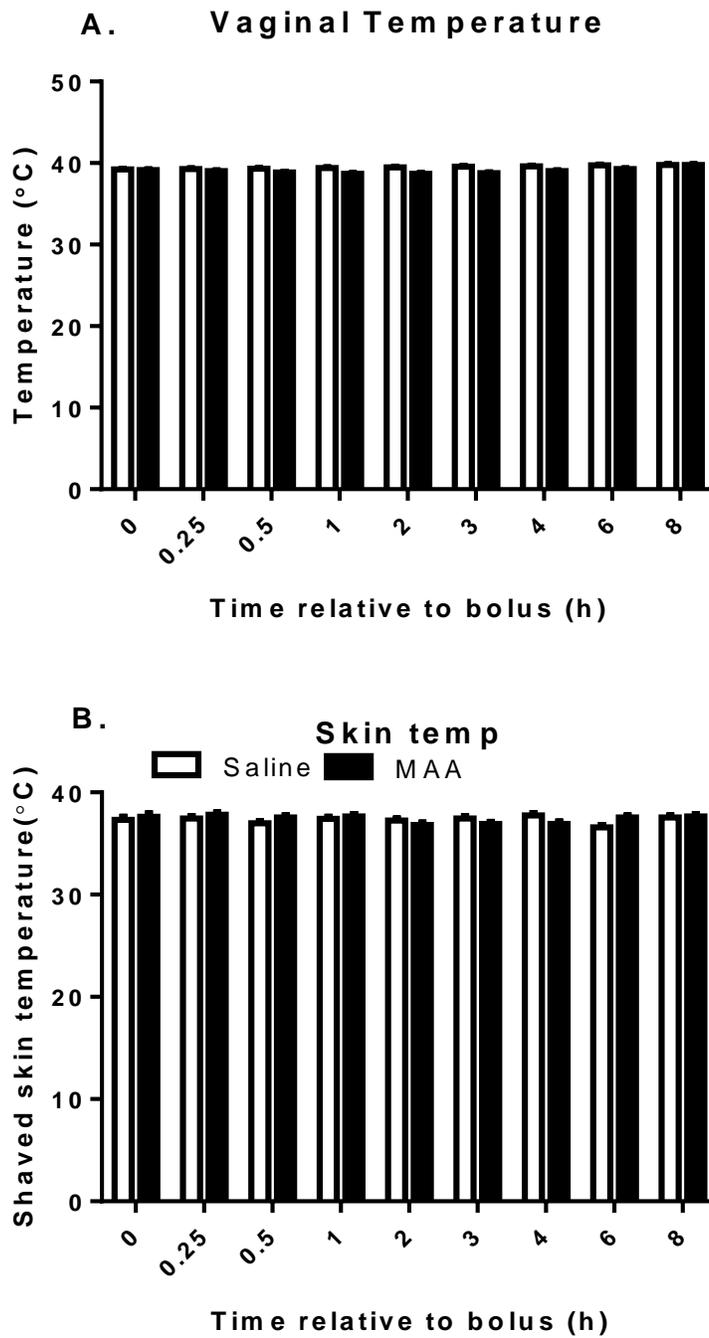


Figure 5.8. Changes in mean (a) skin temperature and (b) vaginal temperature with BMA. * Bars that have an asterisk differ significantly from time 0, λ bars within a time point that share a lambda symbol are significantly different.

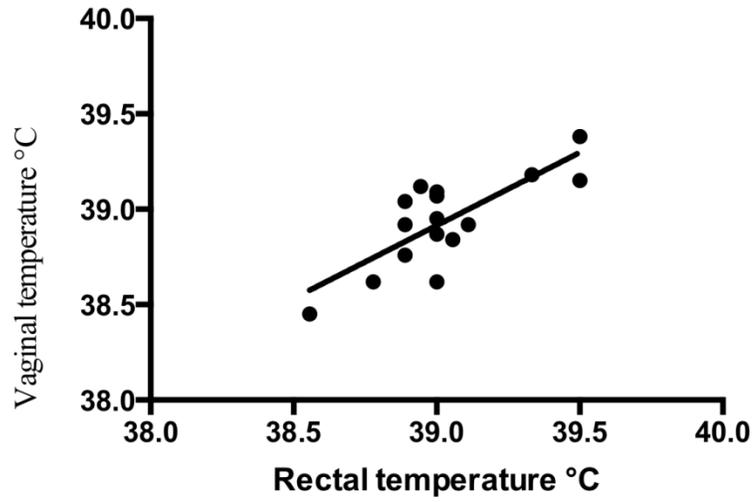


Figure 5.9. The correlation of vaginal and rectal temperatures in β -mercaptoacetate treated cows at times 0, 15m, 30m, and 1h relative to bolus. Saline cows are not represented in this figure as there was no change in vaginal temperatures. $R^2 = 0.61$, $P = 0.0004$

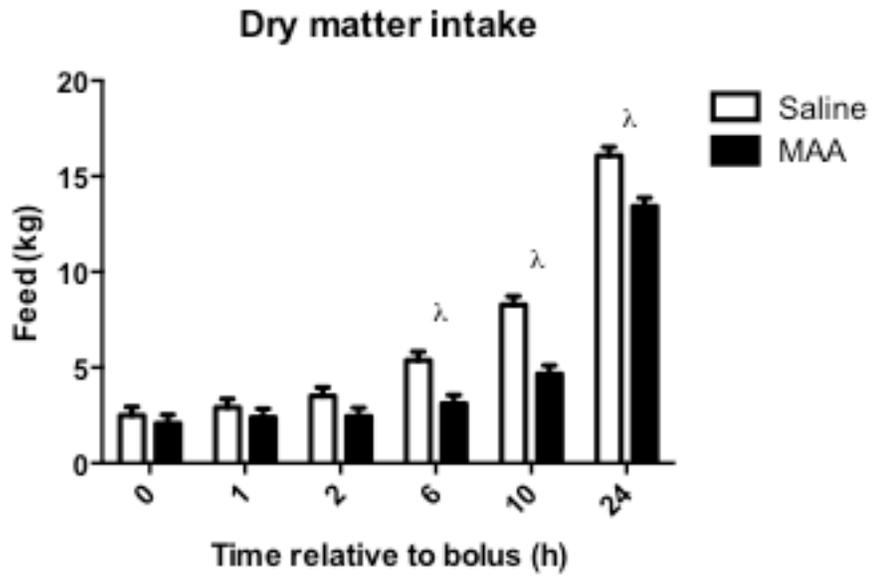


Figure 5.10. Cumulative dry matter intake by hour with saline or BMA treatment. λ Bars within a time point that share a lambda symbol are significantly different

SUMMARY AND CONCLUSION

The use of each dietary supplement provided some advantage to production in dairy cows but some of those were not carried into HS and some were intensified. Milk production responses were all increased (numerically or significantly) during TN, but the advantages were lost in HS. We used BHB to understand the mechanisms behind these losses and found that BHB did not control feed intake, insulin or serum metabolites at the bolus levels. The role of BHB in ruminants is likely different than those seen in monogastrics.

The use of BET to ameliorate HS in lactating dairy cows did not work. Betaine increased CBT and did not improve DMI or MY during HS. The most promising results include increased MY during TN and plasma glucose during HS. Betaine increases VFA production and though the milk (kg) advantage was lost with HS, but the increased level of plasma glucose may be an indication that the rumen microbes may be protected by BET, and are functioning. Betaine was not able to overcome the physiological costs associated with HS at the doses fed. Future research could include a higher dose of BET as the HI cows had lower HS CBT compared to MID, higher TN MY and HS plasma glucose. It needs to be determined where the BET is being utilized, if it stays in the GIT or if it makes it to the host cells.

Feeding Calsporin did not reduce the impact of HS. There was a numerical TN MY advantage, but that was lost during HS. Calsporin did decrease SCC throughout the study. The physiological response to HS was less favorable in Calsporin cows compared

to controls. This is likely due to a higher level of production. Future implications should include more cow numbers to better understand the change in microbial populations. Calsporin supplementation decreased the expression of HSP27. Calsporin may be a beneficial supplement to dairy production systems.

The impact of HS was reduced using OG in lactating dairy cows. The OG numerically increased TN and HS MY, reduced SCC, demonstrated a lower cortisol response to HS, maintained HS DMI, and reduced physiological HS responses (RR and RT). Feeding OG likely improved the immune response and hormonal response to HS. Future research should help define the mechanism of immunity and the hormones of the adrenal axis in HS OG fed cows.

The increased levels of BHB seen during HS in lactating dairy cows are not the likely culprit for the reduced HS DMI. This is not definitive, we treated cows with a bolus of BHB, yet a long term infusion of BHB would better mimic the BHB HS response. Ruminants are likely more tolerant of ketones due to their reliance on VFAs and gluconeogenesis. MAA did not decrease BHB, but had demonstrated increased levels of circulating serum BHB. This may indicate BHB is being synthesized by rumen epithelial cells, or that the uptake of BHB is compromised. MAA did reduce vaginal temperatures, but this did not improve DMI, RR or metabolite concentrations. The relationship between lactate and BHB during HS could be helpful in understanding the true impact of BHB during HS.

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