Evaluating the impact of heat stress and altered glycemic state on plasma γ-Aminobutyric Acid (GABA) in lactating Holstein cows

Alicia Gest Arneson

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Michelle L. Rhoads, Chair
Robert P. Rhoads
Susan L. Campbell

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Evaluating the impact of heat stress and altered glycemic state on plasma γ-Aminobutyric Acid (GABA) in lactating Holstein cows

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ABSTRACT

Heat stress (HS) induces hyperinsulinemia and hypoglycemia in lactating dairy cows. We hypothesized that γ-aminobutyric acid (GABA) participates in the regulation of this altered glycemic state as it is produced by the pancreatic beta cells and has a stimulatory effect on pancreatic secretion of insulin. Multiparous lactating Holstein cows (n=6; 63.33±2.35 DIM, 3.17±0.40 lactations) were placed in environmentally controlled rooms for four experimental periods: 1) thermoneutral (TN; d 1-5; 18±4°C), 2) TN + hyperinsulinemic-hypoglycemic clamp (HHC; d 6-10), 3) heat stress (HS; d 16-20; 33±4°C), and 4) HS + euglycemic clamp (EC; d 21-25). Cows were milked twice daily, and blood samples were collected once daily via coccygeal venipuncture into heparinized evacuated tubes. Plasma GABA concentrations were determined using a competitive ELISA. The data were analyzed in two ways. The first analysis included data from all treatment periods and yielded no period-based differences in plasma GABA concentrations. In this analysis, plasma GABA was lowly correlated to plasma insulin concentrations (r = -0.29, P<0.01). The second excluded data from HHC and EC periods so that GABA concentrations during TN were directly compared to concentrations during HS. In this analysis, plasma GABA concentrations tended to be higher in TN than HS (16.31±2.14 vs 13.80±2.15 ng/ml, respectively, P = 0.06). Milk production was moderately correlated with plasma GABA (r=0.42, P<0.01) and the average plasma GABA during TN and HS was moderately correlated to baseline glucose levels for those periods (r=-0.57, P=0.05). Furthermore, the percent change in plasma GABA was strongly correlated with the percent change in plasma glucose from TN to HS (r=−0.95, P<0.01). Plasma GABA was again lowly correlated to plasma insulin concentrations (r = -0.35, P = 0.01). While these analyses are not indicative of causality, the results suggest that GABA is involved in the regulation of the altered glycemic state observed during HS. More research is needed to determine its precise role in heat-stressed lactating dairy cattle.
Evaluating the impact of heat stress and altered glycemic state on plasma γ-Aminobutyric Acid (GABA) in lactating Holstein cows

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GENERAL AUDIENCE ABSTRACT

Heat stress causes large annual financial losses for the dairy industry and presents potential welfare issues for dairy cows when they can no longer cope appropriately with their environment. As climate change continues, the intensity and duration of heat stress experienced by dairy cows will increase which will cause the effects of heat stress on the dairy industry, as well as on the wellbeing of dairy cows, to become more significant. For this reason, it is important to understand the physiological processes underlying a cow’s adaptive response to heat stress to improve future farm management in a changing climate. It is well documented that heat stressed dairy cows experience an increase in plasma insulin concentrations, as well as a concurrent decrease in plasma glucose concentrations. It is not well understood how or why these changes occur, but γ-aminobutyric acid (GABA) is known to be secreted from the same cells that secrete insulin and to have effects on the secretion of insulin, as well as on concentrations of glucose in the blood through effects on pancreatic glucagon secretion. This work began the process of determining the physiological mechanism behind these changed concentrations by determining how plasma GABA changes during heat stress in lactating dairy cows and how those changes are related to other physiological changes observed during heat stress. It was determined that plasma GABA tends to decline during heat stress and is significantly related to milk production, as well as blood glucose concentrations. While these results cannot be taken without more research to imply cause, they do support the idea that GABA plays a role in coordinating the altered glycemic state observed in heat stressed dairy cattle and would be an interesting research target in the future.
DEDICATION

This work is dedicated to the twenty-three incredible bovine Hokies who participated in our experiment – especially to Tallulah, whose kindness is beyond my comprehension.

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LIST OF ABBREVIATIONS

3-MH = 3-Methlyhistidine
BBB = Blood Brain Barrier
BHBA = β-Hydroxybutyric Acid
CCC = Chloride-Cation Cotransporter
CL = Corpus Luteum
DMI = Dry Matter Intake
E = Epinephrine
EC = Euglycemic Clamp
ELISA = Enzyme Linked Immunosorbent Assay
FSH = Follicle Stimulating Hormone
GABA = γ-Aminobutyric Acid
GC = Granulosa Cell
HHC = Hyperinsulinemic – Hypoglycemic Clamp
HS = Heat Stress
IR = Insulin Receptor
IVF = In Vitro Fertilization
KCC = Potassium Chloride Cotransporter
LDCV = Large Dense Core Vesicles
LH = Luteinizing Hormone
LPA = Lysophosphatidylcholine
MCFA = Medium Chain Fatty Acid
NE = Norepinephrine
NEBAL = Negative Energy Balance
NEFA = Non-esterified Fatty Acids
NKCC = Sodium-Potassium-Chloride Cotransporter
PCOS = Polycystic Ovarian Syndrome
PUN = Plasma Urea Nitrogen
SCC = Somatic Cell Count
SCFA = Small Chain Fatty Acid
SCS = Somatic Cell Score
SLMV = Synaptic-Like Microvesicles
TAG = Triacylglycerol
TCA = Tricarboxylic Acid
THI = Temperature-Humidity Index
TN = Thermoneutral
WAT = White Adipose Tissue
INTRODUCTION

Heat stress occurs when an organism’s thermoregulatory mechanisms cannot dissipate heat sufficiently to maintain homeostasis. For the livestock industry, this translates to major production losses during the summer months, especially in regions with hot climates. St-Pierre and co-workers (2003) reported $2 billion in annual losses for US livestock industries. Of those, about $1 billion were to the dairy industry specifically. These losses result from a decline in milk production, impairment of reproductive parameters, and increased culling during times of high ambient temperatures (St-Pierre et al., 2003). These losses are liable to continue to increase with climate change. A production loss of between 0.5 and 2% is expected by 2030, using 2010 as a base year (Key and Sneeringer, 2014). This will result in up to $269 million in costs to be shared by producers and consumers (Key and Sneeringer, 2014). Part of this cost will be due to the need for stronger heat abatement systems to mitigate the effects of heat stress, such as air conditioning (Gunn et al., 2019; Fournel et al., 2017). Continued genetic selection for increased milk production will add yet another strain to the system. Milk production has more than doubled over the last 50 years (ERS, 2020). Because milk production is responsible for approximately 53% of produced heat energy in a lactating dairy cow (Coppock, 1985), it is reasonable to think that continued increased selection for milk production at the current rate will require that cows are able to dissipate heat far more efficiently. Failure to do so will greatly increase the negative impact that heat stress has on the industry’s productivity.

In addition to the economic costs associated with heat stress, it is perhaps more important to consider the detrimental effects on animal welfare. Animal welfare is compromised any time an animal cannot cope well with its environment (Silanikove, 2000). These situations can fall under one of three categories (Fraser et al., 1997). In the first case, an animal’s adaptations no longer serve a meaningful purpose in its environment. This case brings about a negative affective state and frustration. In the second case, an animal’s environment presents a challenge for which the animal has no adaptation. This case negatively affects health and biological parameters. In the final case, an animal has some adaptations to cope with its environment, but they are
insufficient. This will affect both biological and affective functioning. It is in this category that heat in the environment beyond what an animal can effectively dissipate falls (Fraser et al., 1997). Similar to the economic costs to the dairy industry, the negative impacts on animal welfare caused by heat stress will continue to be exacerbated as climate change and genetic selection for milk production continue. It is important that the scientific community seek to understand the physiological mechanisms underlying adaptations to heat stress that result in diminished production parameters. From this understanding, better management and selective practices can be designed and implemented to limit the detriment to animal welfare, as well as the economic losses associated with heat stress in the dairy industry.
**Defining Heat Stress**

Physiological stress can be defined as any situation in which an animal’s environment imposes too intense a demand for its regulatory mechanisms to properly compensate (Koolhaas et al., 2011). Therefore, heat stress occurs when the environment does not allow for an animal to properly cool itself. Most apparently this pertains to ambient temperature, however humidity is also an important factor because high humidity conditions limit the capacity of an animal to undergo evaporative heat dissipation (Kibler et al., 1964). The range of ambient climatic conditions that allows an animal to maintain thermal balance without negatively impacting production metrics is considered the thermoneutral zone (Berman et al., 1985). The upper and lower temperatures that define that range are considered critical temperatures, beyond which an animal’s productivity and homeostatic ability will be hindered (Berman et al., 1985). As aforementioned, humidity also plays an important role in determining whether the ambient conditions are conducive to an animal’s wellbeing and production ability. Because of that, temperature-humidity index (THI) is considered to be a more effective way to measure heat stress conditions (Kibler, 1964). Many equations exist to calculate THI, however all of them incorporate terms that include some combination of dry bulb temperature, wet bulb temperature, and relative humidity (Dikmen and Hansen, 2009).

The THI at which dairy cattle begin to experience heat stress is most often determined by analyzing the impact of increasing THI on milk production parameters, including both milk yield and composition. Heat stress threshold THI values as low as 60 and as high as 80 have been recorded, with the most frequently documented value being around 72 (Bernabucci et al., 2014; Gorniak et al., 2014; Brugemann et al., 2012; Boonkum et al., 2011; Bohmanova et al., 2007). The multitude of available equations has created a lack of consistency across studies and is likely partially to blame for the variation seen. A study by Brugemann and co-workers (2012) found that using two different equations produced two different THI thresholds for heat stress. The use of differing metrics among these studies to determine the onset of heat stress also likely contributed to the wide range of THIs recorded.
In addition to unintentional sources of variability, several controllable factors can influence the THI at which dairy cows exhibit production loss from heat stress. Multiparous cows appear to be more severely affected by heat stress in terms of milk production than primiparous cows in terms of milk production, with a more pronounced effect seen with each increasing parity (Bernabucci et al., 2014; Boonkum et al., 2011; Novak et al., 2009). Stage of lactation also seems to have an effect, though studies are split on whether early or late-stage cows are more adversely impacted (Lambertz et al., 2014; Novak et al., 2009; Broucek et al., 2007). More research is needed to determine how stage of lactation is related to production loss due to heat stress.

The location in which a study is performed has a profound effect on the THI corresponding to the onset of heat stress. Bohmanova and co-workers (2007) found that the THI threshold in Arizona is two index points higher than in Georgia. The authors attribute that difference to a difference in humidity in the two regions. Studies performed in the Czech Republic and Germany noted thresholds substantially lower than ones performed in the US, while a study done in Thailand registered a substantially higher threshold than the ones from the US (Gorniak et al., 2014; Brugemann et al., 2012; Boonkum et al., 2011; Bohmanova et al., 2007). Some of the geographic effect observed may be linked to differences in genetic background of the cows from each location (Boonkum et al., 2011).

**Heat Stress Effects on Energy Intake and Expenditure**

Numerous studies have demonstrated that dry matter intake (DMI) is reduced in dairy cattle by heat stress (Gorniak et al., 2014; NRC, 2001; Itoh et al., 1998; Abilay et al., 1975). Reduced DMI is even used as a basis for pair feeding studies to separate the direct effects of heat stress from those indirectly caused by this reduction in DMI (Cowley et al., 2015; Wheelock et al., 2010; Rhoads et al., 2009). Unfortunately, heat stress also results in increased energy requirements if a cow is to fully dissipate sufficient heat to maintain homeostasis (NRC, 2001). This combination causes heat stressed cattle to generally exist in a state of negative energy balance (NEBAL) (Wheelock et al., 2010).

An animal existing in NEBAL will mobilize body tissue reserves to meet energy demands, resulting in observable weight loss as seen in a study performed by Rhoads et
al. (2009). White adipose tissue (WAT) is the major energy reserve tissue, serving as a reservoir for triacylglycerol (TAG) to be hydrolyzed in the absence of sufficient nutrients (Ahmadian et al., 2009). Therefore, it is expected that in times of NEBAL, WAT will be mobilized and the resulting metabolites will be present in higher concentrations in the bloodstream. Non-esterified fatty acids (NEFA) are commonly used as a metric for this, as they are the primary energy molecule formed during the process of lipolysis (Ahmadian et al., 2009). Bauman and co-workers (1988) established a significant correlation between NEBAL and circulating NEFA in lactating dairy cows.

Unanticipatedly, while pair fed dairy cows do have an increased concentration of NEFA in their plasma, heat stressed cows do not exhibit this same increase (Soriani et al., 2013; Wheelock et al., 2010; Rhoads et al., 2009; Abeni et al., 2007). Some studies have shown that levels of beta-Hydroxybutyric Acid (BHBA), a ketone body formed from the metabolism of NEFA in the liver (Emery et al., 1992), increase slightly during heat stress (Soriani et al., 2015; Abeni et al., 2007). This would suggest that NEFAs are used as a fuel source during heat stress, which seems counter to the observation that NEFAs themselves are not also found in increasing amounts in the plasma. Other studies have found no change in levels of BHBA in the plasma during heat stress (Lamp et al., 2015; Cowley et al., 2015). Therefore, more research is needed to elucidate what may be causing this discrepancy in findings.

Interestingly, it has also been shown that plasma urea nitrogen (PUN) (Wheelock et al., 2010; Shwartz et al., 2009), as well as 3-methylhistidine (3-MH) and creatinine, levels are increased in dairy cows during periods of heat stress (Soriani et al., 2013; Lamp et al., 2015; Kamiya et al., 2006; Schneider et al., 1988). Plasma urea nitrogen is elevated in dairy cattle most often by either inefficient incorporation of ammonia into ruminal microbial protein or from the hepatic deamination of amino acids from muscular proteins for use in gluconeogenesis (Wheelock et al., 2010). However, the concurrent increases in plasma 3-MH and creatinine indicate that the increase in PUN during heat stress is likely due to the breakdown of skeletal muscle for use as an alternative energy source during NEBAL (Harris and Milne, 1980; Salazar, 2014). These findings in conjunction with the finding that plasma NEFA concentration is not elevated by heat stress imply that dairy cows are preferentially mobilizing skeletal muscle over WAT in
order to neutralize the state of NEBAL that exists during heat stress. It is not yet understood how or why this adaptation developed. Therefore, studies relating to hypothesized mechanisms would be of great value.

**Heat Stress Impact on Glucocorticoids and Catecholamines**

Cortisol has long been used as a metric hormone to evaluate animal welfare under varying circumstances (Greenwood and Shutt, 1992). Catecholamines, such as epinephrine (E) and norepinephrine (NE) are also sometimes used due to their role in adrenergic activation of the sympathetic nervous system. These “stress” hormones also impact metabolism. For instance, all three are known to encourage lipolysis and therefore are also considered thermogenic (Scotney et al., 2017; Jessen, 1980; Ankermann et al., 1972). Therefore, it is important to understand what impact heat stress may have on these hormones.

Regarding cortisol, the consensus for dairy cattle is that plasma levels are elevated during the first 48 hours of heat stress, then make a return to baseline levels, and ultimately are depressed over the long term (Ronchi et al., 2001; Abilay et al., 1975; Alvarez and Johnson, 1973; Christison and Johnson, 1972). This overall depression was also observed in milk concentrations of cortisol (Correa-Calderone et al., 2004). One study performed by Wise and co-workers (1988) is often cited in papers mentioning an overall increase in plasma cortisol levels during heat stress. However, biological metrics recorded in this study reveal that the cooled cows were also under significant heat stress. Additionally, insufficient sampling was performed to be reflective of the more intricate changes in cortisol observed by the other studies. Because of the dynamic nature and ultimate depression of plasma cortisol during heat stress, it may not be prudent to use it as an indicator of dairy cow welfare under high ambient temperature conditions.

Elucidating catecholamine concentrations during heat stress has proven more problematic. Alvarez and Johnson (1973) detected an overall increase in these hormones over the acute, as well as the chronic, period of heat stress. Katti and co-workers (1991) recorded a similar pattern to cortisol levels, with both NE and E showing an acute increase and then returning to baseline levels after 48 hours. Lamp and co-workers (2015) found that the stage of production may play a role in heat stress-induced changes to
plasma catecholamines. Post-partum (early lactation) cows showed an increase in only E, but not NE while cows just before parturition showed the opposite. It is well documented in dairy cattle that catecholamines acting through beta-adrenergic receptors stimulate lipolysis (Contreras et al., 2017). Considering the lack of increase in NEFA observed during heat stress, it would be counterintuitive that peripheral catecholamine concentrations should rise. However, in humans, catecholamines can also act as lipolytic inhibitors via alpha-adrenergic receptors (Morigny et al., 2016). Therefore, adrenergic receptor gene expression research on these mechanisms under heat stress conditions in dairy cattle may be useful to understand the varying results of these studies.

**Heat Stress Impact on Milk Production**

The most tangible economic loss in dairy production resulting from heat stress is the significant decrease in milk yield. In controlled environment studies, heat stress has been shown to cause as much as a 40% decrease in milk yield (Rhoads et al., 2009). Some studies have shown more modest declines around 30% (Wheelock et al., 2010), but all demonstrate a decrease in milk yield that would prove economically significant when scaled to the farm, and further to the industry level. Because of its strong association with heat stress, diminished milk yield is often used as a parameter to ensure cows in heat stress trials are sufficiently stressed (Cowley et al., 2015) and it is used as a primary parameter to assess Temperature-Humidity Index (THI) thresholds for heat stress (Gorniak et al., 2014; Brugemann et al., 2012; Bohmanova et al., 2007). Though it was initially accepted that the detriment to milk yield was nearly entirely attributable to a decrease in dry matter intake (DMI) seen in heat stressed cows (West, 1994), more recent studies utilizing pair feeding have shown that a reduction in DMI is only responsible for around 50% of the lost milk yield (Rhoads et al., 2009). Rhoads and co-workers (2010) were able to establish that this was not due to an uncoupling of the IGF-GH axis, however research of mechanisms involved in the repartitioning of nutrients during heat stress is still lacking.

Milk components are also altered by heat stress. Milk protein content is reduced by heat stress (Cowley et al., 2015; Wheelock et al., 2010; Rhoads et al., 2009). Not only that but, like milk yield, it is reduced above and beyond the effect of decreased DMI.
This was demonstrated by Cowley and co-workers (2015) and Rhoads and co-workers (2009) via pair feeding trials. There is some debate here because Wheelock and co-workers (2010) did not see an effect above restricted DMI. This suggests that there may be other factors involved that may serve as confounding variables.

Milk lactose content appears to decrease during heat stress (Shwartz et al., 2009), but pair feeding trials link that change to decreased DMI (Wheelock et al., 2010; Rhoads et al., 2009). The reductions shown by those studies are modest, ranging from 3% (Rhoads et al., 2009) to 5% (Shwartz et al., 2009). Data from Cowley and co-workers (2015) also show a numerical decrease in milk lactose content; however, the study was unable to demonstrate statistical significance. The Cowley study’s experimental design was fairly similar to those of the studies reporting decreased milk lactose content, so the results seen by Cowley and co-workers (2015) may be due to differing statistical procedures or because, by design, the heat stress presented was only enough to cause a 4 to 5 liter decrease in milk yield (Cowley et al., 2015) while the heat stress in the other studies was assessed based on biological parameters, such as rectal temperature and respiration rate (Rhoads et al., 2009; Wheelock et al., 2010).

Heat stress induced changes in milk fat content are somewhat debated. Most climate-controlled studies do not note any significant change in milk fat content (Rhoads et al., 2009; Wheelock et al., 2010; Ma et al., 2019; Cowley et al., 2015). However, many of the farm-based studies assessing THI thresholds for deterioration of production parameters use milk fat as one of the threshold-determining factors (Bernabucci et al., 2014; Bohmanova et al., 2007; Gorniak et al., 2014). One study of this kind conducted by Brugemann and co-workers (2012) in Germany did not show any change in milk fat content. These results suggest that the changes in milk fat content during the summer months observed on farm may be the result of other seasonal variables, such as changing forage composition. However, it may be useful to study further whether the duration of heat stress has any impact on milk fat content because farm-based studies take into account heat stress over the course of several months whereas climate-controlled studies typically only sustain heat stress conditions for 7 to 9 days. One definitive study by Liu and co-workers (2017) shows that the composition of milk fat is subject to change under heat stress conditions. Both the triacylglycerol (TAG) and polar lipid profiles changed
considerably in response to acute heat stress. Triacylglycerol species composed primarily of short and medium chain fatty acids were reduced. Because SCFAs and MCFAs in milk are predominantly produced de novo in the mammary gland, this reduction may be an indicator of diminished anabolic activity (Liu et al., 2017). Liu and co-workers (2017) also determined that the polar lipid class lysophosphatidylcholine (LPC) may be useful as a biomarker for heat stress because of the stark change in its relative abundance.

The number of somatic cells in milk has long been used as a marker for mastitis or other inflammatory processes and is tightly regulated by government agencies to ensure milk quality and safety. The impact of heat stress on somatic cell shedding is still not well understood. A few studies have noted an increase in the somatic cell score (SCS) in response to heat stress (Lambertz et al., 2014; Brugemann et al., 2012). Cowley and co-workers (2015) noted no difference, however this study used somatic cell count (SCC) as an inflammatory index, rather than SCS. Table 1 (Norman et al., 2001) below shows how SCC and SCS are related to one another. Because SCS is based on threshold values, a small change in SCS can represent either an exceptionally large or only a slight change in SCC. This discrepancy makes evaluation of somatic cells contained in milk rather complicated and reduces uniformity across studies. Therefore, more research is needed to determine what effect heat stress has on somatic cell values.

<table>
<thead>
<tr>
<th>SCS (cells/ml)</th>
<th>SCC (cells/ml)</th>
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<tbody>
<tr>
<td>0</td>
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<tr>
<td>1</td>
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<td>9</td>
<td>6,400,000</td>
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Figure 1: Equivalent values of SCS and SCC from Norman and co-workers (2001).

**Heat Stress Impact on Glycemic Balance**

In both heat stressed and pair fed dairy cattle, plasma glucose concentrations are reduced from their original values (Wheelock et al., 2010; Rhoads, et al., 2009). Soriani et al. (2013) noted that blood glucose was negatively correlated with the daily maximum
THI which confirms this observation. Cowley and co-workers (2015) did not observe a change in blood glucose levels during heat stress or pair feeding, however this may be due to the use of a specific amount of milk production loss as a heat stress indicator, rather than biological metrics such as respiration rate and rectal temperature. This method of determining heat stress limits the severity of the heat stress to mild, which may limit the impact the heat stress has on physiological measurements. Because hypoglycemia is not observed in pair fed cattle, it is likely that this effect can be attributed to the reduced DMI seen in heat stressed animals.

In relation to that, heat stressed dairy cows exhibit a state of hyperinsulinemia (Itoh et al., 1998). This effect is not observed in pair fed cows (Wheelock et al., 2010; Rhoads et al., 2009). This discrepancy between pair fed and heat stressed cows suggests an underlying difference in metabolic dynamics that is unique to heat stress. Insulin is considered the primary physiological inhibitor of lipolysis (De Koster et al., 2018). Accordingly, the lack of increase in plasma NEFA levels despite NEBAL discussed previously may be at least partially attributable to this rise in plasma insulin.

Reproductive Impacts of Heat Stress

Reproductive performance of dairy cows is known to be hindered during the summer months as a result of heat stress (Ray et al., 1992). This is most directly observable on farms in the form of depressed conception rates. Du Preez and co-workers (1991) showed that conception rate is negatively correlated with increasing THI. Concordantly, conception rate declines considerably above a THI of 73 (Schuller et al., 2014). First insemination conception rate was 40% lower for heat stressed than for cooled cows and conception rates for subsequent inseminations were also significantly lower for heat stressed cows (Wolfenson et al., 1988). Pregnancy rates at days 90, 120, and 150 post insemination were also notably lower for heat stressed than for cooled cows (Wolfenson et al., 1988). Some studies have shown that embryo transfer, especially from donor cows rather than IVF, may be a useful strategy to improve conception rates during the summer months (Putney et al., 1989; Drost et al., 1999). Pregnancy rates at day 21 post insemination were improved by 30% when embryo transfer was used compared to
artificial insemination (Putney et al., 1989), and the improved pregnancy rates have also been observed at day 42 (Drost et al., 1999).

Additional challenges are imposed by changes to estrus behavior during periods of heat stress. Mounting behavior during estrus is markedly decreased by hot weather (Gangwar et al., 1965; Gwazdauskas et al., 1983; Pennington et al., 1985). Because mounting behavior is often used as a means to detect heat, a reduction in this behavior may make heat detection more difficult during the summer months. The duration of estrus is also reduced by heat stress (Gangwar et al., 1965; Wolfenson et al., 1988), which further increases the chance that heat detection will be unsuccessful. Pennington and co-workers (1985) observed a lengthened period of estrus during hot weather as compared to cold weather, however this study offered no control comparison to cows under thermoneutral conditions.

Heat stress has some effect on the estrous cycle and some of the major events therein. Gangwar and co-workers (1965) found that heat stress lengthened the estrous cycle on average and reduced uniformity in estrous cycle length within the experimental group. Lengthened estrous cycles would imply longer anestrus periods within the cycles (Gangwar et al., 1965). However, more recent studies by Roth and co-workers (2000) and Trout and co-workers (1998) found no significant change in estrous cycle length induced by heat stress. It should be noted that in the study performed by Trout and co-workers (1998), heat stress was only applied for days 11-21. Therefore, the results of this study may not be adequately representative of other forms of acute heat stress or of chronic heat stress. This discrepancy may require further studies performed both on farm and in climatic chambers to determine under which conditions, if any, heat stress affects the length of the estrous cycle. A study by Wilson and co-workers (1998) noted that heat stress increases the incidence of dairy cows exhibiting more than two follicular waves per cycle. Roth and co-workers (2000) did not conclude the same. More studies are necessary to verify either conclusion.

The effect of heat stress on follicular development is somewhat unclear. Wolfenson and co-workers (1995) and Wilson and co-workers (1998) both observed an increase in the rate of growth for the second wave dominant follicle that slowed in later development, which resulted in a final dominant follicle no different or even smaller than
a comparable follicle in non-heat stressed cows. Roth and co-workers (2000) did not observe this change in the rate of development but did note an increased medium follicle cohort size. This is representative of reduced follicular dominance during the second wave which was also observed by Wilson and co-workers (1998). Studies are still largely divided on the specific effects of heat stress on the quantities of small, medium, and large follicles during each follicular wave, therefore more studies documenting the entire span of multiple estrous cycles under climatic control would be beneficial to improve the general understanding of the estrous cycle during heat stress.

Ovulation rate is decreased by heat stress conditions (Lopez-Gatius et al., 2005). This could be related to the longer luteal phase induced by heat stress documented by Wilson and co-workers (1998). The presence of a longer luteal phase in that study was attributed to delayed luteolysis, determined by a prolonged serum progesterone concentration above 1 ng/mL. On day 21 of the estrous cycle, only 18% of heat stressed cows had undergone functional luteolysis, whereas 82% of cows under thermoneutral conditions had (Wilson et al., 1998). While luteolysis is delayed, the size of the corpus luteum (CL) as measured by ultrasound is unaffected by heat stress (Howell et al., 1994; Wolfenson et al., 1995; Roth et al., 2000). CL weight measured post slaughter in beef cows was found to be reduced by heat stress (Biggers et al., 1987), which may suggest a reduced luteal tissue density.

The hypothesis that luteal tissue density is reduced by heat stress has mixed support from studies documenting plasma progesterone levels during heat stress. Some studies document a reduction in plasma progesterone levels during heat stress, especially during the late luteal phase (Howell et al., 1994; Trout et al., 1998; Ronchi et al., 2001). Other studies, however, note no effect (Wolfenson et al., 1995) or even an overall increase during the first heat exposed cycle (Abilay et al., 1975). Bridges and co-workers (2005) recorded an increase in progesterone production by follicular cells in vitro when exposed to elevated temperatures. Higher progesterone levels in the follicular fluid can be indicative of increased follicular atresia (Spicer et al., 1987).

Consensus on circulating estrogen concentrations is equally divided. Roth and co-workers (2000) saw no change in circulating estrogen levels during the luteal phase but did see a reduction during the follicular phase. Ronchi and co-workers (2001) did not see
any effect on estradiol-17β by heat stress or pair feeding. Wilson and co-workers (1998) observed decreased concentrations during the proestrus rise in estrogen during heat stress. Estrogen concentrations in the follicular fluid appear to decrease during heat stress (Wolfenson et al., 1997; Bridges et al., 2005).

There is unfortunately no more unity in opinion on circulating gonadotropin and inhibin concentrations. Regarding follicle stimulating hormone (FSH), one study documented that the peak amplitude was higher during heat stress and that the transient increase in FSH that occurred before the second follicular wave was sustained four days longer (Roth et al., 2000). Another study stated that chronic heat stress instead induced a reduction in circulating FSH, however this observation extended only to cows in a “low estrogen” grouping (Gilad et al., 1993). Ronchi and co-workers (2001) did not observe any significant changes in circulating FSH, however the author notes that heat stress may simply not have been severe enough or of long enough duration to cause changes to circulating gonadotropins. Luteinizing hormone (LH) appears to be unaffected by heat stress, though studies specifically documenting changes in LH concentrations are limited and typically only document one cycle due to the high sampling frequency required to fully document LH pulsatility. This tendency creates a need for more studies that document the effects of heat stress on LH over an extended period with multiple cycles’ samples included. Changes in inhibin concentrations are poorly documented. Wolfenson and co-workers (1995) did not report any significant changes in concentration during heat stress, however Roth and co-workers (2000) observed increased plasma inhibin concentrations for cooled cows versus cows kept under direct solar radiation.

Oocyte quality and competence is adversely affected by heat stress conditions (Rocha et al., 1998; Al-Katanani et al., 2002; Ferreira et al., 2011). Oocytes collected from Holstein cows in Texas and Louisiana during the hot season were morphologically abnormal 35% more often than during the cool season (Rocha et al., 1998). This difference was not sustained in Brahman cows, which implies a positive effect of genetic heat tolerance on oocyte quality (Rocha et al., 1998). The increased incidence of morphological abnormalities may help explain the reduced conception rates during artificial insemination. However even when morphologically normal oocytes are selected and fertilized in vitro, the competence of those oocytes appears hindered. The blastocyst
rate for the resulting embryos is reduced significantly (Al-Katanani et al., 2002; Ferreira et al, 2011). Al-Katani and co-workers (2002) also found that cooling the cows for 42 days prior to oocyte collection did not improve blastocyst rates. The author notes that this could be the result of insufficient cooling or of outside seasonal effects, however it could also be indicative of a delayed effect of heat stress on oocyte quality. Roth and co-workers (2000) documented a delayed effect of heat stress on follicular development; therefore, it is highly possible that the delayed effects of heat stress extend to the oocyte itself as well.

Embryo development is impacted at least to some degree as well. With only 7 days of exposure to heat stress post insemination, Holstein heifers showed a 30% reduction in morphologically viable embryos (Putney et al., 1988). Additionally, morphologically normal embryos collected from heat stressed cows proved to be more heat intolerant in vitro than ones from cows under thermoneutral conditions (Edwards et al., 2009). The blastocyst rate for compact morulae from heat stressed cows that were exposed to elevated temperatures in vitro was significantly decreased when compared to that for compact morulae from thermoneutral cows (Edwards et al., 2009). Decreased embryo quality may be explicative of the increased pregnancy loss observed during heat stress (Wolfenson et al., 1988).

**Effect of Insulin on Reproductive Function**

The negative effects of heat stress on fertility are evident, but the underlying mechanisms involved are less clear. Hyperinsulinemia associated with increased insulin resistance has been implicated in the etiology of polycystic ovarian disease in humans (Park et al., 2001). Therefore, it is worth considering what the heat stress-induced state of hyperinsulinemia observed in dairy cattle may contribute to the effects of heat stress on fertility.

Insulin receptors exhibiting tyrosine kinase activity are widely distributed within both the stromal and follicular tissues of the human ovary (Poretsky et al., 1995). Insulin receptor (IR) mRNA expression is also increased significantly in cultured bovine granulosa cells (GCs) of dominant ovulatory follicles as compared to other types of follicles and adding FSH to the culture medium increased the expression of IR mRNA
(Shimizu et al., 2008). This is in concordance with the finding by Landau and co-workers (2000) that insulin concentrations within dominant follicles are higher than in subordinate ones. This evidence supports the hypothesis that insulin plays a role in the control of follicular dynamics and could impact fertility directly.

Additionally, insulin treatment of cultured bovine granulosa cells (GCs) increased progesterone production by the GCs (Spicer et al., 1993). As aforementioned, increased levels of progesterone in follicular fluid serve as an indicator of atresia (Spicer et al., 1987). However, in an in vivo study, Simpson and co-workers (1994) reported that follicular fluid progesterone concentrations were reduced by long-acting bovine insulin treatment for five days. To add more complexity to this matter, Patel and Shah (2018) reported that hyperinsulinemia in rats induced by a high fat diet did not cause any change in plasma progesterone levels. Duleba and co-workers (1993) also observed no change in media progesterone concentration in cultured rat granulosa cells and theca-interstitial tissue. This discrepancy may be due to species differences, as well as varying levels of hyperinsulinemia relative to baseline insulin levels, as the Spicer study used levels of insulin treatment many times higher than those observed physiologically.

Hyperandrogenism in females with PCOS is well documented and has been linked to the state of hyperinsulinemia often observed in those females (Baptiste et al., 2010). Additionally, androgens are antagonistic to follicular development (Zeleznik et al., 1979). Treatment with insulin sensitizers of rats fed a high fat diet to induce hyperinsulinemia reduced hyperandrogenism as measured by plasma testosterone levels (Patel and Shah, 2018). Holstein cows placed on a hyperinsulinemic euglycemic clamp showed elevated circulating testosterone levels as well, however after 30 hours on the clamp the ratio of testosterone to estrogen had significantly decreased (Butler et al., 2004). This may indicate that desensitization or internalization of insulin receptors occurred in response to prolonged exposure to hyperinsulinemic conditions. Duleba and co-workers (1993) documented the potentiation of the luteinizing hormone-dependent production of testosterone by insulin in cultured rat theca-interstitial tissue. This potentiation was not altered by metformin, an insulin sensitizing drug, indicating that insulin may act directly on this system rather than via its glucose metabolism modulatory ability.
Insulin may also play a role in follicular development dynamics. In a study of beef dairy cross cows over two estrous cycles, cows with diet-induced hyperinsulinemia had fewer total follicles develop (Adamiak et al., 2005). However, in 14-day postpartum dairy cows followed through 80 days postpartum, circulating insulin concentration was positively correlated with the number of follicles able to be aspirated (Matoba et al., 2012). This difference is probably due to the state of hypoinsulinemia seen in postpartum dairy cows (Matoba et al., 2012). The cows in the study were at varying degrees of hypoinsulinemia, so it may be drawn that both hypo- and hyperinsulinemia may have negative impacts for reproductive success. Simpson and co-workers (1994) did not see any difference in the number of follicles when Angus cows were treated with long-acting insulin. That may be due to breed differences or to differences in the insulin treatment used.

The findings of Adamiak and co-workers (2005) and Matoba and co-workers (2012) are consistent with those from an applied feeding study. Garnsworthy and co-workers (2009) found that feeding postpartum dairy cows a diet that increased plasma insulin until cyclicity resumed and then switching to a diet that reduces plasma insulin resulted in the best first service conception rate and overall pregnancy rate. Additionally, it was found that in multiparous Holstein cows, plasma insulin levels varied twice as much during the follicular phase of the estrous cycles as compared to the luteal phase (Landau et al., 2000). It has been demonstrated in Angus cows that hyperinsulinemia resulted in larger dominant follicle diameter (Simpson et al., 1994). This was also seen in rats fed a high fat diet (Patel and Shah, 2018). When the rats were treated with insulin sensitizers, average dominant follicle diameter and wall thickness was decreased. These results provide more support for the hypothesis that insulin plays a role in the control of follicular dynamics.

There is also some evidence that insulin has some impact on oocyte quality. Blastocyst rates of embryos produced from IVF of slaughterhouse bovine oocytes treated with pharmacological doses of insulin in the media were lower when compared to control embryos (Laskowski et al., 2017). Contrarily this study also documented an upregulation of the TEF-3, SOX2, and GSC genes which are all positively associated with oocyte quality. Embryos produced from oocytes collected from hyperinsulinemic dairy cows via
transvaginal follicle aspiration also had reduced blastocyst rates compared to oocytes from euinsulinemic cows (Adamiak et al., 2005). Oliviera and co-workers (2016) did not note any physical differences in oocyte quality, but blastocyst rates were not recorded in that experiment.

While insulin produced locally is likely the primary source of brain insulin (Molnar et al., 2014), there is much evidence that plasma insulin crosses the blood brain barrier (BBB) and provides a source of insulin to the central nervous system (Margolis and Altszuler, 1967; Schwartz et al., 1991; Miller et al., 1994; Tanaka et al., 1999). Insulin and its receptors are widely distributed in the brain, however one of the locations they are most concentrated is the hypothalamus (Havrankova et al., 1978; Baskin et al., 1983). Their localization in the hypothalamus warrants a discussion of the potential impact circulating levels of insulin have on GnRH secretion and subsequent gonadotropin release. Studies of this nature are lacking in the bovine model, however two studies performed in rams showed that infusion of insulin at physiological levels into the third cerebral ventricle or into the lateral ventricle resulted in increased luteinizing hormone pulse frequency (Miller et al., 1995; Tanaka et al., 1999). While it may be suspected that this difference could also result from insulin action on the pituitary gland, Havrankova and co-workers (1978) found that insulin receptors are not expressed in high levels in the pituitary gland, so any effects seen on plasma LH or FSH can be attributed to changes in GnRH secretion. In mice, insulin receptor expression was observed in a GnRH expressing cell line (Kim et al., 2005)

**Participation of GABA in the Regulation of Insulin Secretion**

γ-Aminobutyric acid (GABA) is considered the primary inhibitory neurotransmitter in the central nervous system; however, it is also present in various peripheral tissues and its roles in those tissues have only recently been investigated. γ-Aminobutyric acid has three known receptor types. The type A and C receptors are ionotropic chloride channels, while the type B receptor is a G-protein coupled receptor coupled to calcium and potassium channels via second messengers (Bormann, 2000).

Γ-Aminobutyric acid is released from normal mammalian beta cells in the pancreatic islets of Langerhans (Braun et al., 2010; Adeghate and Ponery, 2002;
Smismans et al., 1997). Its release is independent of that of insulin (Braun et al., 2004a; Smismans et al., 1997), however the machinery involved in its release is somewhat debated. It is thought to be released from synaptic-like microvesicles (SLMVs) within the beta cells (Braun et al., 2004a; Thomas-Reetz et al., 1997), though a more recent study by Braun and co-workers (2010) suggests that it may actually be stored along with insulin in the large dense core vesicles (LDCVs) as well. That study found that GABA is present in some, but not all, LDCVs. The presence of GABA in only some of the LDCVs may play a role in increasing vesicular recruitment in the presence of higher concentrations of glucose (Braun et al., 2010).

The effects of GABA released from beta cells are thought to be exerted in a paracrine and autocrine manner on neighboring islet cells specifically in the presence of glucose, though studies are split on whether those effects are excitatory or inhibitory. Some studies have indicated that perfusion of GABA into isolated mammalian pancreatic islet cells cause an excitatory effect and increase the release of insulin from those cells (Bansal et al., 2011; Braun et al., 2010). These studies suggest that these effects are mediated via GABA type A receptors. Other, older studies have documented an inhibitory effect of GABA on islet cell insulin secretion (Braun et al., 2004b; Gu et al., 1993). Several studies saw mixed effects of GABA depending on the experimental conditions. Dong and co-workers (2006) show that GABA has a depolarizing effect on glucose-induced insulin secretion when glucose concentrations are low and a hyperpolarizing effect at higher glucose concentrations. Perfusion of canine pancreata with GABA inhibited insulin release, however so did perfusion with a GABA type A receptor antagonist, bicuculline, which are conflicting results to some extent (Kawai and Unger, 1983). In cultured human islet cells from type 2 diabetic donors, a GABA type B receptor antagonist increased insulin release. However, there was a downregulation of several GABA type A receptor subunits when compared to normal donor islet cells, suggesting that a downregulation of this receptor may be linked to the reduction in insulin production ability seen in type 2 diabetes (Taneera et al., 2012).

These differing results may be explained by the differing mechanisms by which the GABA type A and type B receptors function. The steady state concentration of chloride ions in beta cells is higher than predicted by the Nernst equation, suggesting that
there must be some form of transport of chloride ions against their concentration gradient (Eberhardson et al., 2000). Eberhardson and co-workers (2000) also observed that glucose stimulates transport of chloride ions across the plasma membrane. Maintenance or disruption of this high intracellular chloride concentration is an important factor in determining how glucose will affect beta cell membrane polarization (Best et al., 2005).

In the central nervous system, differential expression and/or activation of cation chloride cotransporters (CCCs) determine the effect GABA has when activating its A type receptor (Blaesse et al., 2009). The sodium potassium chloride cotransporters (NKCCs) act to import chloride ions into the cells, while the potassium chloride cotransporters (KCCs) act as chloride extruders (Arroyo et al., 2013). High expression of the sodium potassium chloride cotransporter, NKCC1, as well as some expression of the potassium chloride cotransporter, KCC2, has been observed in human islet cells (Taneera et al., 2012). Several other isoforms of KCC have also been identified in rat islet cells (Davies et al., 2004). Kursan and co-workers (2017) confirmed the expression of KCC2 in islet cells, but also documented that NKCC1 and KCC2 are co-localized in islet cells, which suggests that they may work together to regulate chloride ion concentrations similarly to neuronal mechanisms. This study also noted that inhibition of KCC2 resulted in an increased insulin response to glucose by islet cells. The opposite was also observed. Stimulation of KCC2 caused a decreased insulin response to glucose (Kursan et al., 2017). Knockout mouse studies found that mice with a lack or deficiency of NKCC1 had lower fasting plasma insulin concentrations (Alshahrani et al., 2015; Alshahrani et al., 2012). However, these studies also documented that knockout mice had better glucose tolerance. This seemingly contrary finding led to the discovery that in the absence or deficiency of NKCC1, another NKCC isoform, NKCC2, is compensatorily expressed in islet cells (Alshahrani et al., 2015).

The only studies mentioned above that considered culture medium concentration of chloride ions were those performed by Bansal and co-workers in 2011 and by Dong and co-workers in 2006. Taken together, these findings support the hypothesis that GABA acts in an excitatory manner to stimulate insulin release from beta cells in the presence of insulin only if intracellular chloride concentration remains high. This effect is mediated via GABA type A receptor ion channel opening. In the absence of high
intracellular levels of chloride, inhibitory effects may be mediated via the type A or type B receptor. More research is needed to elucidate the full mechanism involved.

Another explanation for the controversy regarding the effect of GABA on beta cell insulin release is that GABA production and release is inhibited by sustained elevation of glucose levels (Pizarro-Delgado et al., 2010; Smismans et al., 1997). This effect is likely mediated by increased GABA metabolism caused by increased GABA transaminase activity mediated by increased glucose metabolite diversion from the TCA cycle into the GABA shunt. (Pizarro-Delgado et al., 2010; Wang et al., 2006). Therefore, the glucose concentration and duration used to stimulate insulin production may impact GABA metabolism and alter outcomes.

![Figure 2: “The GABA Shunt” (Kleppner and Tobin, 2002)](image)

**Action of GABA in Reproductive Tissues**

$\gamma$-Aminobutyric acid and its type A receptor are present in mammalian female reproductive tissues as well. Ovarian tissue homogenates showed relatively high concentrations of GABA compared to other peripheral tissues (Erdo et al., 1982; del Rio and Caballero, 1980). It was also observed that ovarian tissue can convert glutamic acid into GABA, suggesting that the ovary has at least some ability to directly synthesize GABA (del Rio and Caballero, 1982). Additionally, GABA was found in rat fallopian tube tissue homogenate in concentrations higher than those found in brain tissue homogenate (Erdo et al., 1982). $\gamma$-Aminobutyric acid type A receptors have also been identified in ovarian tissue homogenate (Erdo and Laszlo, 1984). Superfusion of GABA onto the ovarian surface in pseudopregnant rats increased ovarian blood flow, increased the rate of estradiol release, and decreased progesterone secretion (Erdo et al., 1985).
Additionally, GABA inhibited norepinephrine (NE) release from follicle strips taken from bovine ovaries electrically stimulated to release NE (Kannisto et al., 1986). This inhibition was found to be mediated by the GABA B type receptor (Kannisto et al., 1987).

Binding sites for GABA in the ovary are mostly associated with granulosa cells (GCs) (Schaeffer and Hsueh, 1982). Inhibition of GABA A type receptors with muscimol decreased the expression of bone morphogenetic proteins 2 and 6 in GCs (Danzy et al., 2011). Bone morphogenetic proteins play an important role in the early phases of follicular growth (Yoshino et al., 2011). That study also documented decreased expression of the luteinizing hormone receptor in GCs with inhibited GABA A receptor activity (Danzy et al., 2011). Peluso and Pappalardo (1998) found that using bicuculline to inhibit GABA A type receptors inhibited progesterone’s ability to prevent insulin-dependent mitosis, as well as apoptosis, of GCs. These findings suggest a role for GABA and its receptors in mediating granulosa cell growth and follicular development, but more research is needed to confirm that role.

γ-Aminobutyric acid has also been implicated in alleviating the pathophysiology associated with polycystic ovarian syndrome (PCOS). In a human study, patients with PCOS exhibited significantly lower serum GABA levels than controls (Radwan et al., 2019). Treating rats with induced PCOS with GABA daily for 16 days reduced plasma testosterone levels and increased circulating estradiol levels (Ullah et al., 2017). It also restored normal cycle length, decreased the number of cystic follicles, and decreased insulin resistance typical of PCOS patients (Ullah et al., 2017).

Conclusions

Heat stress is an impactful phenomenon for both economics and animal welfare in the dairy industry. As the dairy industry tends toward the production of more milk from fewer cows and the Earth’s climate tends toward increased temperature and humidity, it will become ever more important to understand the effects of heat stress and how to mitigate them. Additionally, studying some of the physiological mechanisms in place to allow dairy cows to acclimate to warmer seasons may shed light on how similar mechanisms in human beings impact our own physiology. In particular, the altered glycemic state observed in heat stressed dairy cows is similar to that observed in
individuals with prediabetes, impaired fasting glucose, and hyperinsulinemic forms of PCOS (Dale et al., 1992; Li et al., 2009). γ-Aminobutyric acid (GABA) has fairly recently been shown to have effects on secretion of insulin and glucagon from the endocrine pancreas and therefore has become a target of interest in understanding altered glycemic states. This work will begin the process of elucidating its role in the states of hyperinsulinemia and hypoglycemia that occur in heat stressed lactating dairy cows in order to determine whether GABA could be a target for improved pharmacological or management strategies to mitigate the effects of heat stress in these animals.
CHARACTERIZATION OF THE EFFICACY AND CONSISTANCY OF THE ENVIRONMENTAL FACILITY

Introduction

Several different indices have been used to determine whether lactating dairy cows are experiencing heat stress. Many studies have utilized a certain amount of decline in milk production as one metric to determine at which temperature heat stress is induced (Brugemann et al., 2012; Bernabucci et al., 2014; Gorniak et al., 2014). Several others utilize increased rectal temperatures and respiration rates to verify that cows under heat stress treatment were experiencing sufficient heat stress to invoke a response (Rhoads et al., 2009; Wheelock et al., 2010). It is well documented that heat stress produces a decline in dry matter intake in dairy cows and other species (Gorniak et al., 2014; NRC, 2001; Itoh et al., 1998; Abilay et al., 1975), therefore reduced feed intake is also often used as a metric to verify heat stress.

Because the climate-controlled experiment described in the next chapter was performed in a facility that was not designed to house adult, lactating dairy cows and had never been utilized for that purpose, it was necessary to ensure that the cows that underwent treatment truly experienced heat stress. When considering the experimental conditions, two factors became clear targets for this investigation: 1) the environment to which each cow was exposed, and 2) the physiological response of the cows. Four tie stalls in two climatic chambers were used to house the four cows during each replicate. Because each tie stall may be subject to differing conditions (i.e. room differences in temperature and humidity and one tie stall is always closer to a door), it was necessary to verify that tie stall did not significantly impact the THI experienced by each cow or the physiological parameters mentioned above. Conversely, because the goal of the trial was to investigate heat stress, it was important to ensure that the THI and above-mentioned metrics did change in the expected direction when heat stress was applied. Therefore, an analysis of the effects of both tie stall location and period on THI, rectal temperature, respiration rate, milk production, and feed intake was conducted to ensure consistent and proper application of heat stress treatment during the experiment.
The objective of this study was to investigate the effect of the microenvironment (individual tie stalls) and experimental period on the daily THI experienced by each cow, as well as on physiological parameters used to indicate sufficient heat stress treatment. Specifically, this analysis was completed to ensure that the THI during heat stress periods was significantly higher than during thermoneutral periods and induced a significant change in the expected parameters. Additionally, this analysis was necessary to ensure that the tie stall location did not represent a confounding factor by significantly impacting the environmental conditions experienced by each cow nor resulting in a significant difference in physiological parameters compared to the other tie stall locations.

Materials and Methods

IACUC Statement

All husbandry and experimental procedures performed during this experiment were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Data Collection

For replicates 3, 4, 5, and 6, EL-USB-2 Data Logger continuous temperature and relative humidity sensors (Lascar Electronics, Erie, PA 16505) were affixed to the wall in front of each tie stall at approximately cow head height. The sensors recorded temperature and humidity information every 15 minutes for the duration of the experiment. From this data, the average temperature and humidity values were calculated and input into the equation below from Vitali and co-workers (2009), adapted from Kelly and Bond (1971) for temperature measurements in degrees Celsius:

$$\text{THI} = (1.8 \times AT + 32) - (0.55 - 0.55 \times RH) \times [(1.8 \times AT + 32) - 58],$$

to produce THI values for each cow’s microenvironment throughout the experiment. Each tie stall was assigned a number for coding within the data set. A visualization of how the stalls were numbered is shown below in Figure 3.
Rectal temperatures and respiration rates were recorded at 0800, 1230, and 2000 each day. The average values of these measurements were used for the analyses described in this work. Feed intake was measured by performing feed weighback once every 24 hours at 1230 each day. Fresh feed was offered to the cows in measured increments at 0030 and 1230. Daily milk production was determined by weighing milk collected at 0030 and 1230 daily and summatting the two measurements.

Statistical Analysis

PROC MIXED was used to analyze the effect of period and tie stall location on average daily THI, rectal temperature, respiration rate, milk production, and feed intake (SAS 9.4). Each parameter was analyzed as a response variable separately. The model used for all the analyses included period and tie stall location as fixed effects and cow as a random effect. The repeated statement was also used with day as the repeated time point and cow nested within period as the subject. Cow was nested within subject for the repeated command because each cow served as its own control and was used in all four treatment periods. The covariance structure was set to first-order heterogeneous autoregressive. This covariance structure was determined as the best for use in these models using AICc and BIC as the selection criteria. The degrees of freedom were approximated using the Kenward Roger approach. The full code can be found in
Multiple pairwise comparisons were made among periods using Tukey’s method. Statistical significance was declared at P<0.05.

In addition to the above analyses, a descriptive and visual analysis of hourly temperature changes by period was performed using hourly data recorded by the EL-USB-2 Data Logger continuous temperature and relative humidity sensors (Lascar Electronics, Erie, PA 16505). The least squares means and standard error for hourly temperature by period in degrees Celsius, relative humidity as a percent, and temperature humidity index (THI) were calculated using the lsmeans function within the package emmeans in RStudio version 1.4.1103 (Russel V. Length, 2021). Each period was analyzed separately, and a separate formula was used for each environmental metric with THI, temperature, or relative humidity as the response variable and the hour of the day as the explanatory variable. No random effects were included in this analysis. The min, max, mean, and sd functions in base R were used to determine the minimum, maximum, mean, and standard deviation for each metric by period. The data set produced from the least squares means calculations was used for these determinations.

Because the temperature was set to lower at night during the heat stress periods to mimic a summer day, a comparison of rectal temperatures and respiration rates from the heat stress periods to those from the thermoneutral period was performed to ensure that the lower temperatures did not allow for significant alleviation of heat stress. Data points from the HHC period were excluded due to the elevation in rectal temperatures observed during the HHC period as compared to the TN period (figure 5). It was confirmed that neither rectal temperature nor respiration rate differed significantly between the HS and EC periods, which is why the two periods were both used to represent the heat stress condition. This confirmation analysis was conducted using PROC MIXED with rectal temperature or respiration rate as the response variable and time of day, period, and the interaction of the two as the explanatory variables (SAS 9.4). Cow was included as a random effect and the repeated statement was also used with hour of the day as the repeated measure and cow nested within date as the subject. A first-order autoregressive covariance structure was used, and the Kenward-Roger method was used to calculate the denominator degrees of freedom. Multiple pairwise comparisons were made among
periods using Tukey’s method. Statistical significance was declared at $P<0.05$. The full code can be found in Appendix C.

For the follow up analyses comparing the three time point measurements to the daily thermoneutral measurements, PROC MIXED was again used with rectal temperature or respiration rate as the response variable and time of day (or thermoneutral) as the explanatory variable. Because only one thermoneutral timepoint was available for comparison to all three heat stress timepoints, the repeated statement could not be utilized. To compensate for this, intercept and date were included as random variables with cow as the subject. Multiple pairwise comparisons were made among periods using Tukey’s method. Statistical significance was declared at $P<0.05$. The full code can be found in Appendix C.

Results

The analysis of location yielded no significant differences among the tie stall locations for any parameter tested. The analysis of period exhibited significant differences for all parameters tested. The results of Tukey's pairwise comparison analyses are summarized in the boxplots below. For all boxplots, different capital letters beneath the period groupings indicate that the LS means of those groups are significantly different. No significant differences in THI were found within any tie stall location within the periods (Figures 4-8).

Table 1: P-Values from the SAS PROC MIXED Analyses of Period and Location Effects

<table>
<thead>
<tr>
<th></th>
<th>THI</th>
<th>Rectal Temperature</th>
<th>Respiration Rate</th>
<th>Milk Production</th>
<th>Feed Intake</th>
</tr>
</thead>
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<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.0253</td>
</tr>
<tr>
<td>Location</td>
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<td>0.9715</td>
<td>0.8339</td>
<td>0.5865</td>
<td>0.8364</td>
</tr>
</tbody>
</table>
Figure 4: Boxplot showing the distribution of Temperature-Humidity Index (THI) measurements for each period and location within that period. Cows were distributed into four different tie stall locations (figure 3): 1, 2, 3, and 4. The THIs for each location are shown by period (TN = Thermoneutral, HHC = TN + Hyperinsulinemic-Hypoglycemic clamp, HS = Heat Stress, EC = HS + Euglycemic clamp).
Figure 5: Boxplot showing distribution of average daily rectal temperature measurements by location for each period. Cows were distributed into four different tie stall locations (figure 3): 1, 2, 3, and 4. The THIs for each location are shown by period (TN = Thermoneutral, HHC = TN + Hyperinsulinemic-Hypoglycemic clamp, HS = Heat Stress, EC = HS + Euglycemic clamp).

Figure 6: Boxplot showing distribution of average daily respiration rate measurements by location for each period. Cows were distributed into four different tie stall locations (figure 3): 1, 2, 3, and 4.
The THIs for each location are shown by period (TN = Thermoneutral, HHC = TN + Hyperinsulinemic-Hypoglycemic clamp, HS = Heat Stress, EC = HS + Euglycemic clamp).

Figure 7: Boxplot showing distribution of total daily milk production in kgs by location for each period. Cows were distributed into four different tie stall locations (figure 3): 1, 2, 3, and 4. The THIs for each location are shown by period (TN = Thermoneutral, HHC = TN + Hyperinsulinemic-Hypoglycemic clamp, HS = Heat Stress, EC = HS + Euglycemic clamp).
Figure 8: Boxplot showing distribution of total daily feed intake in kilograms (kgs) by location for each period. Cows were distributed into four different tie stall locations (figure 3): 1, 2, 3, and 4. The THIs for each location are shown by period (TN = Thermoneutral, HHC = TN + Hyperinsulinemic-Hypoglycemic clamp, HS = Heat Stress, EC = HS + Euglycemic clamp).

The hourly environmental analysis yielded the results seen in figure 9. As expected, the temperature, humidity, and THI fluctuated minimally during the thermoneutral (TN) and hyperinsulinemic-hypoglycemic (HHC) period, which were both conducted under THI conditions that would be considered thermoneutral for lactating dairy cows. Using a THI of 72 to represent the threshold value for heat stress conditions (Bernabucci et al., 2014), it is clear from both the visual and descriptive analysis (table 2) that the environment during these periods was sufficiently controlled to produce thermoneutral conditions throughout. During the heat stress (HS) and euglycemic (EC) periods, the room temperatures were set to mimic those typical of a summer day, with the temperature reaching a peak in the mid afternoon and reaching a minimum in the middle of the night. This pattern can easily be observed in figure 9. Temperature and THI follow this expected pattern and relative humidity follows the opposite pattern. The minimum and maximum THIs for these periods shown in table 2 also reflect this.
Figure 9: Time course plot of Temperature (°C), Relative Humidity (%), and Temperature-Humidity Index (THI). Points represent least squares means of each metric over the entire period for each hour of the day. Replicates 3, 4, 5, and 6 were included in this analysis. Error bars represent the mean ± standard error.

<table>
<thead>
<tr>
<th></th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN</td>
<td>Temperature (°C)</td>
<td>19.5</td>
<td>20.1</td>
<td>19.7</td>
</tr>
<tr>
<td></td>
<td>Relative Humidity (%)</td>
<td>49.8</td>
<td>53.8</td>
<td>51.6</td>
</tr>
<tr>
<td></td>
<td>THI</td>
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<td>65.5</td>
<td>65.0</td>
</tr>
<tr>
<td>HHC</td>
<td>Temperature (°C)</td>
<td>19.5</td>
<td>19.9</td>
<td>19.7</td>
</tr>
<tr>
<td></td>
<td>Relative Humidity (%)</td>
<td>48.7</td>
<td>51.4</td>
<td>49.9</td>
</tr>
<tr>
<td></td>
<td>THI</td>
<td>64.6</td>
<td>65.2</td>
<td>64.9</td>
</tr>
<tr>
<td>HS</td>
<td>Temperature (°C)</td>
<td>23.5</td>
<td>31.6</td>
<td>27.5</td>
</tr>
<tr>
<td></td>
<td>Relative Humidity (%)</td>
<td>28.1</td>
<td>40.8</td>
<td>34.8</td>
</tr>
<tr>
<td></td>
<td>THI</td>
<td>69.0</td>
<td>76.7</td>
<td>72.9</td>
</tr>
<tr>
<td>EC</td>
<td>Temperature (°C)</td>
<td>22.6</td>
<td>31.7</td>
<td>27.1</td>
</tr>
</tbody>
</table>
Table 2: Minimum, maximum, mean, and standard deviation for each environmental metric by period. Least squares means derived from hourly data from replicates 3, 4, 5, and 6 were used to determine the daily minimum, maximum, and mean temperature, relative humidity, and temperature-humidity index (THI), as well as the standard deviation for each metric.

<table>
<thead>
<tr>
<th>Metric</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>Standard Deviation</th>
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<tr>
<td>Relative Humidity (%)</td>
<td>24.6</td>
<td>40.0</td>
<td>32.2</td>
<td>5.32</td>
</tr>
<tr>
<td>THI</td>
<td>67.8</td>
<td>76.2</td>
<td>72.1</td>
<td>3.11</td>
</tr>
</tbody>
</table>

Depending on the heat stress THI threshold used, it could be argued that the THI sometimes dropped below this threshold for a few hours and therefore it was necessary to verify that heat stress was not alleviated by this temperature drop by using rectal temperatures and respiration rates as practical metrics. The overall comparison of rectal temperatures shows that the thermoneutral rectal temperatures were significantly lower than any of the rectal temperature measurements from the HS and EC periods (figure 10). This effect is not as obvious for some individual cows (figure 11).

Figure 10: Comparison of all daily heat stress (heat stress and euglycemic periods) rectal temperatures (°C) to all thermoneutral (TN; thermoneutral period only) rectal temperature measurements. Rectal temperatures were measured three times daily during heat stress periods and only once daily during the thermoneutral period. The red boxes and associated points represent all rectal temperatures measured during those periods for replicates 3, 4, 5, and 6 for each designated time point. The blue box represents all rectal temperature measurements from the thermoneutral period.
period only. These measurements were all taken at 1230 daily. Different letters represent significantly different categories using $p < 0.05$ as the threshold for significance.

![Comparison of Heat Stress Rectal Temperatures to Thermoneutral Rectal Temperatures](image)

Figure 11: Comparison of all daily heat stress (heat stress and euglycemic periods) rectal temperatures ($^\circ$C) to all thermoneutral (TN; thermoneutral period only) rectal temperature measurements for each cow included in replicates 3, 4, 5, and 6. Cow ID is designated at the top of each panel. Rectal temperatures were measured three times daily during heat stress periods and only once daily during the thermoneutral period. The red boxes and associated points represent all rectal temperatures measured during those periods for replicates 3, 4, 5, and 6 for each designated time point. The blue box represents all rectal temperature measurements from the thermoneutral period only. These measurements were all taken at 1230 daily.

The respiration rate analyses yielded similar results. The thermoneutral respiration rates were significantly lower than those from the heat stress periods (figure 12). This difference is much more apparent than the difference in rectal temperatures. Additionally, it appears that respiration rates were elevated during heat stress for all individual cows. This is likely because increased respiration rate serves to maintain body temperature at a stable level and is therefore more apparently sensitive to heat stress than rectal temperature.
Figure 12: Comparison of respiration rates during the heat stress periods to respiration rates during the thermoneutral period (TN). Respiration rates were measured three times daily during heat stress periods and only once daily during the thermoneutral period. The red boxes and associated points represent all respiration rates measured during those periods for replicates 3, 4, 5, and 6 for each designated time point. The blue box represents all respiration rate measurements from the thermoneutral period only. These measurements were all taken at 1230 daily. Different letters represent significantly different categories using p < 0.05 as the threshold for significance.
Figure 13: Comparison of respiration rates during the heat stress periods to respiration rates during the thermoneutral period (TN) by cow. Cow ID is designated at the top of each panel. Respiration rates were measured three times daily during heat stress periods and only once daily during the thermoneutral period. The red boxes and associated points represent all respiration rates measured during those periods for replicates 3, 4, 5, and 6 for each designated time point. The blue box represents all respiration rate measurements from the thermoneutral period only. These measurements were all taken at 1230 daily.

Discussion and Conclusions

All analyses yielded results indicating that (a) all cows were receiving comparable environmental treatment throughout the experimental period (b) the THI during the heat stress periods was significantly higher than the THI during the thermoneutral periods, and (c) the physiological parameters expected to change during heat stress did change significantly in the expected direction. Because of this, it is not necessary to include tie stall location in the analysis as a blocking factor and it can be assumed that the heat stress applied during the relevant periods was sufficient to evoke a physiological response. Overall, ambient treatments were successfully and uniformly applied in this facility that had not previously housed mature, lactating dairy cattle.
EVALUATING PLASMA GABA CONCENTRATIONS AND THEIR RELATIONSHIP TO OTHER PHYSIOLOGICAL METRICS UNDER THERMONEUTRAL AND HEAT STRESS CONDITIONS

Introduction

γ-Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the central nervous system. It is not thought to cross the blood brain barrier (Kuriyama and Sze, 1971), but it is also produced by several peripheral systems in quantities comparable to CNS concentrations and is therefore found in systemic circulation (Erdo and Wolff, 1990). The two tissues that are best documented as sources of peripheral GABA are ovarian and pancreatic tissues (del Rio and Caballero, 1980; Erdo et al., 1982). In the pancreas, GABA is secreted by beta cells separately and independently from insulin and stimulates insulin production in a paracrine manner via the GABAA receptor (Kawai and Unger, 1983; Braun et al., 2004a). This effect contrasts with the known inhibitory action of GABA in the CNS and is due to the differential expression of chloride cation cotransporters in the two tissues (Herbert et al., 2004; Kursan et al., 2011). γ-Aminobutyric acid also has an inhibitory effect on the secretion of glucagon from pancreatic alpha cells via the GABAA receptor and insulin acts in a paracrine manner to upregulate the expression of these receptors on the alpha cell surface (Wendt et al., 2004; Xu et al., 2006). Glucagon works counter to insulin when conditions necessitate an increase in blood glucose by stimulating gluconeogenic pathways.

The secretion and actions of GABA may be of particular interest for lactating dairy cows experiencing heat stress. In lactating dairy cows, chronic heat stress induces a state of hyperinsulinemia above and beyond the effects of the reduced DMI observed during heat stress (Rhoads et al., 2009; Wheelock et al., 2010). Hypoglycemia is also observed in these cows but is observed at a similar level in pair fed animals, indicating that this effect is primarily mediated through reduced DMI during heat stress (Wheelock et al., 2010). It is currently unknown to what extent these changes are due to changes in the rate of production or clearance of insulin and glucose or how other hormonal changes observed during heat stress may play a role in mediating this altered glycemic state. We
hypothesized that GABA is involved in the upregulation of circulating insulin concentrations commonly observed during periods of heat stress.

The objectives of this study were to determine the effect of heat stress on plasma GABA concentrations in multiparous lactating Holstein cows and to identify the relationships between plasma GABA and glucose and insulin, along with other physiological parameters.

**Materials and Methods**

*IACUC Statement*

All husbandry and experimental procedures performed during this experiment were approved by the Virginia Tech Institutional Animal Care and Use Committee.

*Animals and Experimental Design*

Six multiparous lactating Holstein cows (63.33±2.35 DIM, 3.17±0.40 lactations) were randomly placed in one of two environmentally controlled rooms for four experimental periods: 1) thermoneutral (TN; d 1-5), 2) TN + hyperinsulinemic-hypoglycemic clamp (HHC; d 6-10), 3) HS (d 16-20), and 4) HS + euglycemic clamp (EC; d 21-25). Two transition periods were instated to allow the cows time to acclimate to the experimental conditions and husbandry procedures. The first was applied for three days before the start of the TN period (d -3 to -1). During this period, the cows were transported from the Dairy Science Complex to Litton-Reaves Hall and placed in their own tie stall within a room shared with one other cow. The second acclimation period was applied for five days after the termination of the HHC period until the start of the HS period (d 11-15) to ensure that the effects of chronic heat stress had sufficient time to reach maximal response levels. Cows were milked twice daily (1230 and 0030h), and milk yields were recorded at each milking. All cows were fed a TMR individually twice daily (1230 and 0030h) and refusals were weighed before the 1230 feeding each day. Prior to placing cows into the environmentally controlled rooms, two indwelling jugular catheters were placed in each cow on either side of the neck (four total per cow) for infusion of insulin and dextrose, as well as for blood sampling. These catheters were flushed with saline at least once daily to maintain patency.
Figure 14: Visual Representation of the Experimental Design. Cows were subjected to four experimental periods: 1) thermoneutral (TN; d 1-5), 2) TN + hyperinsulinemic-hypoglycemic clamp (HHC; d 6-10), 3) HS (d 16-20), and 4) HS + euglycemic clamp (EC; d 21-25).

Thermoneutral and Hyperinsulinemic-Hypoglycemic Clamp Periods

The TN and HHC periods were both performed under thermoneutral conditions for lactating dairy cattle (THI = 67.1±0.26 for TN and 64.1±0.36 for HHC). No other treatments were applied during the TN period. The HHC period began on day 6 of the experiment and lasted for 96 hours. Hand-held blood glucose monitors were used to monitor blood glucose concentrations throughout this period (Contour Next EZ, Ascensia Diabetes Care US, Inc., Parsippany, NJ). Before beginning glucose and insulin infusions, 6 baseline blood glucose measurements were recorded for each cow over 12 hours to calculate an appropriate blood glucose baseline for each cow.

The insulin infusate was prepared by adding purified bovine insulin (I5500, Sigma-Aldrich, Inc., St. Louis, MO) dissolved in 0.01 M HCl to a saline solution containing a volume of each cow’s plasma equivalent to 1.25% of the solution. This insulin solution was infused at a rate of 0.3 μg/kg of BW/h using syringe pumps (Genie Plus, Kent Scientific, Torrington, CT). A 50% dextrose solution (Nova-Tech, Inc., Grand Island, NE) was simultaneously infused (Plum A+, Hospira, Lake Forest, IL). The rate of glucose infusion varied according to each cow’s baseline blood glucose measurements in order to maintain a hypoglycemic state (90±10% of the average of the baseline blood glucose readings for each cow). Blood glucose was measured every 5 minutes at the onset of the infusions until the glucose infusion rate and blood glucose measurements stabilized, at which point blood glucose was measured at intervals of every 30 minutes or less. For cows that did not respond to the low-dose insulin infusion, the infusion rate of
insulin was slowly increased to a maximum of 0.7 ug/kg/ of BW/h until blood glucose fell within the desired range.

Heat Stress and Euglycemic Clamp

Heat stress conditions were applied for a five-day acclimation period before formally beginning the heat stress period. Environmental conditions sufficient to induce heat stress in lactating dairy cows continued to be applied throughout the HS and EC periods after that (THI = 72.2±0.36 for HS and 71.1±0.36 for EC). Heat stress conditions were set to vary diurnally to mimic a summer day. The room temperature reached a maximum of 32.2°C during the afternoon, after which it cooled slowly until it reached a minimum temperature of 21.1°C overnight. In cases where a cow failed to acclimate acceptably to the heat stress treatment (multiple and repeated intervention when rectal temperature was 40.5°C or higher), the maximum room temperature was lowered to 29.4°C.

No other treatments were applied during the HS period. The EC period commenced on day 21 of the experiment and the related infusion lasted for 96 hours. Six baseline blood glucose measurements for the heat stress period were again recorded over 12 hours prior to the start of the EC period and glucose was infused (Plum A+, Hospira, Lake Forest, IL) at variable rates to return blood glucose concentrations to 100±10% of the average thermoneutral baseline measurement for each cow. Blood glucose was initially measured every five minutes until infusion rates stabilized, at which point it was measured at intervals no longer than every 60 minutes.

Blood Sampling

Blood samples were collected once daily (1300h) via coccygeal venipuncture into heparinized evacuated tubes. Blood samples were refrigerated briefly before centrifugation at 2500 RPM for 20 minutes. Once centrifuged, plasma for GABA concentration analysis was collected and stored at -80°C. Plasma for insulin concentration analysis was collected and stored at -20°C.
Plasma GABA concentrations were determined using a competitive inhibition ELISA (LSBio Gamma-Aminobutyric Acid (Competitive EIA) ELISA Kit - LS-F10676). The inter- and intra-assay coefficients of variation were 13.9% and 6.3% respectively. Plasma insulin concentrations were determined using a sandwich ELISA (Mercodia Bovine Insulin ELISA (10-1201-01)). The inter- and intra-assay coefficients of variation were 11.1% and 5.9% respectively. Both kits were validated in our laboratory before use and all samples and standards were run in triplicate. GraphPad Prism 9.0.0 was used to interpolate the standard curves to determine the concentrations in the individual samples. Blood glucose concentrations were determined using Bayer Contour blood glucose monitors at the time of blood collection.

Statistical Analyses

The experiment was conducted using a completely randomized design. The effects of period were determined using PROC MIXED (SAS 9.4). The final model code can be found in appendix A. A first-order autoregressive covariance structure was selected for the analysis using AICc, BIC, and -2LogLikelihood as the model selection criteria. Additionally, RCORR (SAS 9.4) was used to evaluate correlational relationships between plasma GABA concentrations and the other parameters.

Because plasma GABA concentrations in heat stressed dairy cattle have never been compared to a thermoneutral control, two versions of the data were used for analysis. The first version included data from all four treatment periods. The second analysis excluded data from HHC and EC periods so that GABA concentrations during TN were directly compared to concentrations during HS.

Additionally, an RCORR analysis (SAS 9.4) was used to evaluate the relationship between the change in plasma GABA concentrations from thermoneutral to heat stress.
conditions and the corresponding change in blood glucose and plasma insulin concentrations.

Results

In the analysis including all four experimental periods, no significant period differences were found among mean plasma GABA concentrations. However, a tightening in the range of GABA levels occurred during both clamp periods as well as during heat stress (Figure 10).

![Figure 15: Plasma γ-aminobutyric acid (GABA) concentrations for all four experimental periods (TN=thermoneutral, HHC=hyperinsulinemic-hypoglycemic clamp, HS=heat stress, EC=euglycemic clamp). Different point styles represent different individual cows, as represented in the figure legend. There were no significant differences detected when all four periods were included in the analysis.](image)

Table 3: LS Means of Plasma GABA by Period (All period analysis) (TN = Thermoneutral, HHC = TN + Hyperinsulinemic-Hypoglycemic clamp, HS = Heat Stress, EC = HS + Euglycemic clamp).
In the analysis that directly compared the thermoneutral period to heat stress without including either clamp period, plasma GABA concentrations during heat stress tended to be lower than during the thermoneutral period (Figure 11).

**Table 4: LS Means for Plasma γ-aminobutyric acid by Period (TN and HS only).** Only data from the Thermoneutral (TN) and Heat Stress (HS) periods were used for this analysis.

<table>
<thead>
<tr>
<th></th>
<th>Plasma GABA (ng/mL)</th>
<th>Standard Error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN</td>
<td>16.49</td>
<td>1.7</td>
<td>0.0912</td>
</tr>
<tr>
<td>HHC</td>
<td>16.15</td>
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<td></td>
</tr>
<tr>
<td>HS</td>
<td>14.07</td>
<td>1.75</td>
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</tr>
<tr>
<td>EC</td>
<td>13.88</td>
<td>1.79</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 16:** Plasma γ-aminobutyric acid (GABA) concentrations by period for the thermoneutral (TN) and heat stress (HS) periods only. Plasma GABA during HS tended to be reduced compared to the TN period.
A correlational analysis was also conducted on this data set and plasma GABA concentrations were moderately correlated with daily milk production (r = 0.42, p = 0.0014). None of the other variables tested (daily feed intake, average daily rectal temperature, or average daily respiration rate) were significantly correlated to daily plasma GABA concentrations (Figure 12). A linear fit of milk production versus plasma GABA yielded a poor R² value, but the F-test for this simple linear model produced a p-value of 0.0006. This indicates that plasma GABA is a useful predictor of milk production but is not sufficient by itself to predict daily milk production (Figure 13).

<table>
<thead>
<tr>
<th></th>
<th>Plasma GABA (ng/mL)</th>
<th>Standard Error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermoneutral</td>
<td>16.51</td>
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<tr>
<td>Heat Stress</td>
<td>13.85</td>
<td>2.34</td>
<td></td>
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</table>

Figure 17: Correlogram showing the correlations between plasma γ-aminobutyric acid (GABA) concentration, daily feed intake, average daily rectal temperature (RT), average daily respiration rate (RR), and daily milk production in pounds. The ellipses show the shape of the points in a scatterplot of the two variables being compared. The numbers are the Pearson correlation coefficient.
for each pairing. Any box marked with an ‘X’ indicates that the correlation between the two variables being compared is not statistically significant using $p < 0.05$ as the threshold.

Figure 18: The linear fit of total daily milk production in pounds versus plasma $\gamma$-aminobutyric acid (GABA) concentration. Plasma GABA was a significant predictor of milk production, but not sufficient by itself to explain a satisfactory portion of the variability in milk production.

Baseline blood glucose for the thermoneutral and heat stress periods were compared to average plasma GABA for those periods (Figure 14). Baseline blood glucose was moderately correlated to average plasma GABA, but this correlation did not achieve significance using alpha = 0.05. These metrics may not be appropriate for comparison due to the differing amounts of time they represent. When baseline plasma GABA was used instead of the average for the period, the correlation improved slightly and became statistically significant ($r = -0.63$, $p = 0.0273$) (Figure 15).
Figure 19: The linear fit of baseline blood glucose versus average plasma γ-aminobutyric acid (GABA). The correlation for these two variables was not significant and the simple linear model of these two variables was not significantly better than the intercept only model.

Figure 20: The linear fit of baseline blood glucose versus baseline plasma γ-aminobutyric acid (GABA). This comparison yielded a moderate correlation and the model including baseline GABA was significantly better than the intercept only model.
The percent change in plasma GABA was compared the percent change in blood glucose from the thermoneutral to heat stress period (Figure 16). A comparison using the percent change in average plasma GABA over the period yielded a high correlation and the simple linear model produced a remarkably high $R^2$ value, indicating that the percent change in average plasma GABA alone can explain approximately 90% of the variation in the percent change in blood glucose. However, the small sample size available for this analysis limits the generalizability of these results. More data points must be analyzed before definitive conclusions can be made about this relationship. Additionally, as mentioned above, the comparison of an average value to a point in time value may not prove relevant so the same analysis was repeated using the baseline plasma GABA (Figure 17). Though still highly correlated, the percent change in baseline plasma GABA was slightly less correlated with the percent change in baseline blood glucose than was the average plasma GABA ($r = -0.80$, $p = 0.0585$). The $R^2$ value also fell to 0.63, indicating that the change in baseline plasma GABA from thermoneutral to heat stress conditions explains less variability in the change in blood glucose than does the change in the average.
Daily plasma insulin concentrations were compared to daily plasma GABA concentrations for all four experimental periods as well as for the thermoneutral and heat stress periods only. When all four periods are included, plasma insulin was lowly, but significantly correlated with plasma GABA (Figure 18). Additionally, the $R^2$ value yielded by this analysis indicated that plasma GABA explains less than 10% of the variability in plasma insulin concentrations. When only TN and HS are included in the analysis, the correlation and $R^2$ are improved slightly but overall, the two analyses yielded comparable results (Figure 19).
Figure 23: The linear fit of daily plasma insulin concentrations versus daily plasma γ-aminobutyric acid (GABA) concentrations including data points from all four experimental periods. The two are lowly correlated and plasma GABA is not explanatory of a substantial portion of variability in plasma insulin concentrations.

Figure 24: The linear fit of daily plasma insulin concentrations versus daily plasma γ-aminobutyric acid (GABA) concentrations including data points from only TN and HS periods. The correlation and R-squared are comparable to the analysis including all four periods.
Discussion and Conclusions

Concentrations of γ-aminobutyric acid (GABA) in the plasma of dairy cattle and the physiological consequences of changes therein are not well characterized. The only available studies pertaining to GABA in lactating dairy cows are based on supplementing a rumen-protected form of GABA to heat stressed cows and analyzing the outcome of supplementing increasing doses. These studies have documented increased feed intake, decreased rectal temperatures, modest increases in milk production, and various changes in immune molecule concentrations when GABA is supplemented (Cheng et al., 2014, Cheng et al., 2016). A similar study performed under thermoneutral conditions also documented increased feed intake and modest increases in milk production (Wang et al., 2013). This study also observed decreased serum NEFA concentrations when GABA is supplemented, however this effect may come as a result of the observed increased feed intake. In this study, we did not observe a significant correlation between plasma GABA and feed intake. This may imply that the feed intake effects documented in the studies mentioned comes as a pharmacological effect of exogenously increasing plasma GABA. However, the results of our correlational analysis of plasma GABA and daily milk production yielded a moderate positive correlation, which is concurrent with the observation that supplementing GABA may modestly increase milk production.

Because of the small sample size available for this experiment, it is not realistic to make definitive conclusions regarding the correlational relationships observed. However, four out of six cows experienced a decrease in plasma GABA during the heat stress period. Knowing that GABA is generally stimulatory for insulin secretion, this result was the opposite of what was expected. Interestingly, the cows that responded in the opposite direction also experienced a much more extreme hypoglycemic response during heat stress, as shown by the percent change in blood glucose analyses. While more samples are necessary to confirm these results, this study demonstrates a clear tendency for plasma GABA concentrations to be reduced during heat stress in lactating dairy cows and provides compelling reason to research the implications of that reduction for heat stress induced hypoglycemia and milk production loss further.
Because plasma insulin was weakly correlated to plasma GABA, this study may provide evidence that plasma GABA is not representative of the concentration of GABA in the intercellular space within the pancreas. This makes sense if most of the GABA produced in the pancreas is used locally in an autocrine or paracrine fashion as is widely considered to be the case (Caicedo, 2013). However, several studies have demonstrated that perfusing the pancreatic cells with GABA or GABA<sub>A</sub> receptor agonists does impact the release of insulin from the pancreas (Dong et al., 2006; Pizarro-Delgado et al., 2010; Bansal et al., 2011) and Feng and co-workers (2017) showed that administering GABA to mice treated with streptozotocin to induce type I diabetes largely restored insulin levels. Taking these into account, there is reason to think that alterations in plasma GABA levels, no matter their origin, should impact insulin secretion from the pancreas. However, the correlational analysis of plasma GABA and plasma insulin presented in this work suggest that the impact may be much smaller than that of other endocrine or nervous changes yet to be investigated.

In the case that plasma GABA is directly representative of pancreatic GABA, the lack of substantial correlation between plasma insulin and plasma GABA supports the findings from Braun and co-workers (2004a) that the release of GABA from the pancreas is independent of the release of insulin both temporally and spatially. It would also support the findings of Wendt and co-workers (2004) that GABA<sub>A</sub> receptor subunits are not found in substantial quantities on the surface of beta cells and therefore GABA does not have much impact on the release of insulin from the pancreas at physiological concentrations. Some studies have implicated GABA in the insulin production process via its ability to increase beta cell mass rather than beta cell action (Purwana et al., 2014; Untereiner et al., 2019). Investigating changes in beta cell mass and GABA<sub>A</sub> receptor expression on the beta cell surface during heat stress in lactating cows would provide more clarification regarding whether changes in plasma GABA truly exert any effect on insulin production.

The relationship between blood glucose and plasma GABA observed in this study is of particular interest going forward. While the action of GABA on pancreatic beta cells is somewhat contested, there is substantial unified evidence that GABA<sub>A</sub> receptors exist on the surface of alpha cells and allow GABA to mediate an inhibitory effect on glucagon
release (Rorsman et al., 1989; Wendt et al., 2004; Xu et al., 2006). Additionally, Xu and co-workers (2006) provided evidence that insulin induces GABA<sub>A</sub> receptor translocation to the alpha cell membrane and that this is the mechanism by which insulin can suppress glucagon release. Therefore, the state of heat stress induced hyperinsulinemia may increase the GABA<sub>A</sub> receptor density on the alpha cell surface. However, this does not explain why cows that showed a large decrease in plasma GABA during heat stress also appeared to be those that experienced the most severe hypoglycemia during heat stress unless chronic hyperinsulinemia ultimately causes desensitization of the alpha cells to GABA. Analysis of the daily blood samples for plasma glucose concentrations may help illuminate whether the observed relationship between plasma GABA and baseline glucose measurements holds over more data points or if the relationship is somewhat coincidental. Access to these measurements would also allow for the comparison of the hypoglycemia induced by the hyperinsulinemic hypoglycemic clamp during the thermoneutral period to that observed during heat stress. This comparison could potentially illuminate any buffering of the hypoglycemia during heat stress that would occur under the same hyperinsulinemic conditions in a thermoneutral state. GABA could serve as this kind of buffer, which would explain why a reduction in plasma GABA during heat stress appears to predominate in the group of cows included in this study. Immunohistochemical staining for GABA<sub>A</sub> receptor subunits of pancreatic alpha cells from cows exposed to heat stress versus a control would also provide more substantial information regarding what occurs within this pathway during heat stress.

Limited research is available regarding the action of GABA within mammary tissue in mammals and none exists that is specific to bovines of any kind. The available research predominantly pertains to the role of GABA in breast cancer metastasis (Opolski et al., 2000; Sizemore et al., 2014) or to regulation of prolactin secretion by GABA in the hypothalamus and pituitary gland (Lamberts et al., 1978; Mansky et al., 1982). Because GABA does not cross the blood brain barrier, more research is necessary to determine if GABA is produced within mammary tissue and to understand if it plays a role in the bovine mammary gland or elsewhere to influence milk production. A comparative analysis of mammary tissue from heat stressed cows and from cows under thermoneutral
conditions for GABA receptor expression would serve as an informative starting point for this investigation.

The results of this experiment provide preliminary indications of relationships between plasma GABA and pertinent physiological parameters in heat stressed dairy cows. Additionally, this study was the first to determine how plasma GABA changes during heat stress compared to a thermoneutral control and demonstrates a clear tendency for plasma GABA to be reduced during heat stress. Taken together, these data provide compelling support for the further investigation of the role of GABA in the heat stress response in lactating dairy cows regarding both milk production and the altered glycemic state observed during heat stress.
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APPENDIX

Appendix A

SAS code for final mixed model used for data analysis

proc import datafile="C:\Users\a4g5a\Documents\GABA Data\GABA JMP Data for Stats.jmp"
OUT=GABA_jmp
DBMS=jmp;
run;
proc print;
run;

proc print;
run;

*For this experiment, it is necessary to specify both the RANDOM and REPEATED lines because the cows served as subjects in all four periods. Therefore, the RANDOM line models the correlations of observations within a cow while the REPEATED statement models the correlations among the repeated measures (Tao et al., 2015, SAS).

TITLE 'three';
PROC MIXED data = GABA_jmp RATIO
  COVTEST;
CLASS Date Period DOP Rep Cow_ID;
MODEL Plasma_GABA = Period / ddfm=kenwardroger OUTP=resids;
RANDOM Int /
  SUB=Cow_ID;
REPEATED Date /TYPE = AR(1) SUB=Cow_ID(Period) R RCORR;
LSMEANS Period/ADJUST=TUKEY;
RUN;
QUIT;

*This model represents model 'three' above with GABA concentration transformed into log(GABA).
This transformation was executed because the distribution of plasma GABA conc. was skew left rather than normal. By transforming it in this way, the distribution is corrected and this statistical method can be appropriately applied.;

TITLE 'Log GABA.three';
PROC MIXED data = GABA_jmp RATIO
  COVTEST;
CLASS Date Period DOP Rep Cow_ID;
MODEL log_GABA = Period / ddfm=kenwardroger OUTP=resids;
RANDOM Int /
   SUB=Cow_ID;

REPEATED Date /TYPE = AR(1) SUB=Cow_ID(Period) R RCORR;
LSMEANS Period/ADJUST=TUKEY;
RUN;
QUIT;
APPENDIX

Appendix B

THI SAS Code

```
proc import datafile="C:\Users\a4g5a\Documents\THI Analysis\THI Full.jmp"
OUT=full_jmp
DBMS=jmp;
run;
proc print;
run;
TITLE 'THI';
PROC MIXED data = full_jmp RATIO
COVTEST;
CLASS Day Period Rep Cow
Location;
MODEL THI = Location Period/ ddfm=kenwardroger OUTP=resids;
RANDOM Int / SUBJECT = Cow;
REPEATED Day /TYPE = ARH(1) SUB=Cow(Period) R RCORR;
LSMEANS Location/ADJUST=TUKEY;
LSMEANS Period/ADJUST=TUKEY;
RUN;
QUIT;

TITLE 'Rectal
Temp';
PROC MIXED data = full_jmp RATIO
COVTEST;
CLASS Day Period Rep Cow
Location;
MODEL RT = Location Period/ ddfm=kenwardroger OUTP=resids;
RANDOM Int / SUBJECT = Cow;
REPEATED Day /TYPE = ARH(1) SUB=Cow(Period) R RCORR;
LSMEANS Location/ADJUST=TUKEY;
LSMEANS Period/ADJUST=TUKEY;
RUN;
QUIT;

TITLE 'Resp
Rate';
PROC MIXED data = full_jmp RATIO
COVTEST;
CLASS Day Period Rep Cow
Location;
MODEL RR = Location Period/ ddfm=kenwardroger OUTP=resids;
RANDOM Int / SUBJECT = Cow;
```
TITLE 'Milk
Production';
PROC MIXED data = full_jmp RATIO
COVTEST;
CLASS Day Period Rep Cow
Location;
MODEL TotalMilk = Location Period/ ddfm=kenwardroger OUTP=resids;
RANDOM Int / SUBJECT = Cow;
REPEATED Day /TYPE = ARH(1) SUB=Cow(Period) R RCORR;
LSMEANS Location/ADJUST=TUKEY;
LSMEANS Period/ADJUST=TUKEY;
RUN;
QUIT;

TITLE 'Feed
Intake';
PROC MIXED data = full_jmp RATIO
COVTEST;
CLASS Day Period Rep Cow
Location;
MODEL FeedIntake = Location Period/ ddfm=kenwardroger OUTP=resids;
RANDOM Int / SUBJECT = Cow;
REPEATED Day /TYPE = ARH(1) SUB=Cow(Period) R RCORR;
LSMEANS Location/ADJUST=TUKEY;
LSMEANS Period/ADJUST=TUKEY;
RUN;
QUIT;
APPENDIX

Appendix C

RT and RR Comparison SAS Code

proc import datafile="C:\Users\a4g5a\Documents\Original THI Data\HStemps.jmp"
OUT=temp2
DBMS=jmp;
run;
PROC PRINT;

TITLE 'RTemp verification';
PROC MIXED data = temp2 RATIO COVTEST;
CLASS Cow REP DOP Period Time Date HOD;
MODEL RT = Time Period Period*Time/ ddfm=kenwardroger OUTP=resids;
RANDOM Intercept/ SUB=Cow;
REPEATED HOD/Type = AR(1) SUB=Cow(Date);
LSMEANS Period Time/ ADJUST = TUKEY;
RUN;
QUIT;

TITLE 'RRate verification';
PROC MIXED data = temp2 RATIO COVTEST;
CLASS Cow REP DOP Period Time Date HOD;
MODEL RR = Time Period Period*Time/ ddfm=kenwardroger OUTP=resids;
RANDOM Intercept/ SUB=Cow;
REPEATED HOD/Type = AR(1) SUB=Cow(Date);
LSMEANS Period Time/ ADJUST = TUKEY;
RUN;
QUIT;

proc import datafile="C:\Users\a4g5a\Documents\Original THI Data\graph_data.jmp"
OUT=graph
DBMS=jmp;
run;
PROC PRINT;

DATA graph2;
   SET graph;
   IF (Period = "HHC") THEN DELETE;
RUN;
PROC PRINT;

TITLE 'graph comp RT';
PROC MIXED data = graph2 RATIO COVTEST;
CLASS Cow REP DOP Period Time Date;
MODEL RT = Time/ ddfm=kenwardroger OUTP=resids;
RANDOM Intercept Date/ SUB=Cow;
LSMEANS Time/ ADJUST = TUKEY;
RUN;
QUIT;

TITLE 'graph comp RR';
PROC MIXED data = graph2 RATIO COVTEST;
CLASS Cow REP DOP Period Time Date;
MODEL RR = Time/ ddfm=kenwardroger OUTP=resids;
RANDOM Intercept Date/ SUB=Cow;
LSMEANS Time/ ADJUST = TUKEY;
RUN;
QUIT;