Effects of Heat Stress on Blood Metabolites and Milk Quality in Lactating Holstein and Jersey Cows

by

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Abstract

Heat stress has been linked to numerous changes related to health and metabolism in dairy cattle. The effects of heat stress on milk quality factors and blood metabolites were observed in Holstein and Jersey cows under varying severities of heat stress (n = 12/ 6 = Holstein, 6 = Jersey). Milk quality was assessed using increasing somatic cell count (SCC), conductivity, and bacterial load as indicators of decreased milk quality. Concentrations of some blood metabolites such as calcium (Ca), potassium (K), sodium (Na), blood urea nitrogen (BUN), albumin (Alb), glucose, cholesterol, and non-esterified fatty acids (NEFA) were monitored for changes as well. Many significant differences relating to increases in SCC ($p = 0.0936$) and conductivity ($p = 0.0195$) were observed. There were no significant findings relating bacterial load to heat stress of breed. Furthermore, some significant differences concerning blood metabolites, such as Mg ($p = 0.0388$), will require more research to ascertain a better understanding of the underlying mechanisms. The results uncovered an overall decline in milk quality due to heat stress as well as an unclear relationship between heat stress treatments and variable concentrations of blood metabolites.

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Review of Literature

Regulating heat stress caused by relatively high ambient temperatures and humidity has become a challenge for dairy producers in the Southeastern United States, especially in the face of rising global temperatures. Dairy cows are most susceptible to heat stress during periods of high temperatures combined with high humidity due to the cows' diminished ability to utilize evaporative cooling in these environmental conditions (West, 2003). Heat stress poses a variety of problems for dairy cows including decreased immune function (Dahl, 2020), decreased dry matter intake, decreased milk yield, (Zhao, 2019) and increased somatic cell count (Hammami, 2013).

Increased somatic cell counts (**SCC**) can be an indication of subclinical or clinical mastitis (DeVires, 2012) caused by bacterial pathogens that are shed in the milk (Olatoye, 2018). Mastitis is defined as inflammation of the mammary tissues which can be caused by infection from a variety of bacteria. Unfortunately, high SCC decreases milk quality and may even result in the milk being discarded if the SCC exceeds the acceptable limit. Mastitis can be caused by a variety of environmental conditions including the excessive presence of animal waste and unclean milking machines, which allows the udder to come into contact with pathogenic bacteria (DeVires, 2012). This condition renders milk from the affected cow unsellable. This is a major detriment to dairy producers and should be avoided by maintaining low SCC and practicing high quality, hygienic milking routines.

There is a complex association between heat stress, immune function, and SCC. Somatic cell count has been shown to increase in response to stressors such as pregnancy (Nyman, 2008) and transport (Koczura, 2020). Additionally, periods of high temperature-humidity index (**THI**) have been shown to cause stress in cattle and trigger stress

associated physiological mechanisms. One such mechanism is the excessive secretion of glucocorticoids into the bloodstream (Farooq, 2010). Somatic cell count increases during periods of high THI due to increased immune response; however, the overall functionality of the immune system is compromised due to decreased circulation of immunoglobulin and inflammatory cytokines (Dahl, 2020). Furthermore, the function of these immune cells as well as many others is compromised by the increase in glucocorticoid concentration in the bloodstream (Farooq, 2010). Immune function will affect SCC in milk as the somatic cells found in the milk are mostly leukocytes, which are immune cells that concentrate in areas where infection may be occurring. A combination of the immune response and the decreased function of these leukocytes creates a higher concentration of somatic cells in the milk.

Additionally, heat stress alters the metabolic profile of cows, increasing the difficulty of maintaining a proper energy balance and good health for the affected cow (Soriani, 2013). One example of stress-induced changes in blood metabolites is the fluctuation of non-esterified fatty acids (**NEFA**) under stressful conditions. Blood concentrations of both NEFA and glucose increase for a short time after cows are transported by truck, an event that is considered stressful due to the noise, motion, and proximity of the cows (Koczura, 2020). During these periods of stress, glucose levels are expected to decline while NEFA and beta hydroxybutyrate (**BHBA**) levels increase in response (Ster, 2012). Non-esterified fatty acids and BHBA are energy-carrying molecules that occur naturally in the body to provide energy from body reserves when glucose is not readily available in the diet. Increases in these 2 blood metabolites is to be expected in heat stressed cows due to decreased dry matter intake (**DMI**), reducing the

amount of glucose in the body. However, occurrences of decreased blood NEFA concentrations during heat stress have also been noted and attributed to the increased metabolic heat produced by beta- oxidation, the process of breaking down fatty acids (Wheelock, 2010). Furthermore, the decreased DMI associated with heat stress can also affect immune function because glucose is necessary for immune function (Dahl, 2020). Other blood metabolites such as selenium and urea have not shown clear associations with either immune function or stress (Nyman, 2008).

Although many studies have focused on the effects that heat stress has on total production and aspects of milk quality (Dahl, 2020; Farooq, 2010; Hammami, 2013; West, 2003), very few have centered on the possible connection between these diminished quality factors and the metabolic changes that occur during heat stress. By evaluating the metabolic profile of these cows in tandem with their milk quality, a new connection may be established between the heat stress induced changes in blood metabolites and the diminished quality of milk among affected cows. This connection could foster a better understanding of why both milk quality and milk quantity suffer under heat stress conditions and give more specific insight to negate these ill effects.

Breed Differences

Holstein and Jersey cows, while they are both dairy cows, show a variety of differences in productions, milk content, and several other physiological factors. The high fat and protein content of Jersey milk is a major reason for the popularity of Jersey cows despite their lower overall milk production compared to other breeds of dairy cows (Lim, 2020). Alternatively, Holstein cows do not have the same fat and protein content of Jersey cows but do produce the highest yield of milk among dairy breeds. Recent studies

have also shown differences in the mineral components within the milk from these breeds. Jersey cows were shown to have more stable concentration of Calcium, Phosphorus, Potassium, Magnesium, and Zinc in their milk throughout their lactation period, whereas Holstein cows initially show greater concentrations of these minerals in early lactation but have a much greater decline in these concentrations as the annual lactation period proceeds (Lim, 2020). Holstein cows have been shown to be more susceptible to heat stress than Jersey cows with a greater decline in milk production in Holsteins than Jerseys (West, 2003). Holsteins are larger in size than Jerseys, which contributes to this increased susceptibility to heat stress.

Metabolic Profiles

Several blood metabolites are prone to changes due to several factors including season, duration of lactation, and pregnancy status (Lee, 1978). In previous studies, heat stress triggered a decrease in blood levels of glucose, total cholesterol, low density lipoproteins (LDL), albumin, blood urea nitrogen (BUN), calcium (Ca), sodium (Na), potassium (K), chlorine (Cl), and magnesium (Mg) (Joo, 2021). Heat stress can cause the loss of minerals including Ca and Na. Sodium is an essential mineral for maintaining the osmotic balance of cells and the transport of glucose into cells (Joo, 2021). Additionally, a decrease in Ca is known to be a cause of several metabolic diseases in cattle. A decrease in glucose could be expected in heat stress situations due to decreased feed intake and increased energy exerted for respiration and increased stimulation of the immune system. Furthermore, cholesterol levels would also be expected to decrease due to increased use of lipids for energy in the glucose poor environment (Joo, 2021).

Altered Energy Requirements

Cows undergoing heat stress will have a higher maintenance energy level than thermoneutral cows because of the additional energy required for increased respiration and other biological cooling mechanisms (Wheelock, 2010). Furthermore, other biological factors will require additional energy intake such as increased immune system activity and the utilization of alternative energy processing mechanisms as discussed previously (Ster, 2012). This elevated energy requirement becomes even more complicated by the decreased DMI observed in heat stressed cattle. Decreased DMI triggers a decrease in blood glucose levels which in turn will require the use of lipid metabolism to generate energy (Ster, 2012). These same lipid metabolism mechanisms are utilized extensively by thermoneutral, lactating cows with the rates of lipolysis being reported as two to five times greater than their non-lactating, pre partum counterparts (McNamara, 1994). However, these mechanisms are utilized to an even greater extent during heat stress conditions (Wheelock, 2010). Previous studies indicate that the suppressed DMI associated with heat stress only accounts for a portion, approximately 35% to 50%, of production loss while the reason for the remaining portion of this production loss is unclear (Wheelock, 2010).

Somatic Cell Counts and Mastitis

Somatic cell count varies based on many factors including age, pregnancy status, lactation status, genetics, environment, and breed (Lee, 1978). There have been some discrepancies in recent studies as to which breeds have higher somatic cell counts, although a significant difference between the SCC in Holsteins and Jerseys has been reported several times (Lim, 2020). Somatic cell count has a variety of ranges that are

considered healthy, but the International Dairy Federation set a limit of 200,000 cells/ml as an indication of mastitis. Furthermore, SCC between 100,000 and 200,000 cells/ml would be considered subclinical mastitis (Sharma, 2011). Clinical mastitis is characterized by chemical changes in the milk as well as physical changes in the mammary tissue generally observed as swelling and inflammation (Sharma, 2011). Subclinical mastitis is a form of mastitis in which physical signs, such as mammary tissue inflammation has not yet begun. Mastitis is not caused by the somatic cells alone but rather by either contagious or environmental pathogens such as *Staphylococcus aureus* or *Streptococcus uberis* (Sharma, 2011). In the case of this study, elevations in SCC were expected to be triggered by heat stress mechanisms. However, the elevated SCC indicates a decrease in the effectiveness of the immune system, thus increasing the possibility of becoming infected by these mastitis causing pathogens (DeVires, 2012; Hammami, 2013).

Animal Welfare

Heat stress itself commonly occurs as a natural phenomenon in dairy cows due to the variety of climates in which they are raised. Nevertheless, negating the negative physiological and psychological effects of heat stress should be a priority for proper animal health and welfare. A large consideration for the health of dairy cattle in heat stress conditions is the additional energy required for heat abatement in addition to the energy required for lactation and pregnancy (Polsky, 2017). If the energy balance of these animals is not closely managed, they can become susceptible to metabolic diseases and, in extreme cases, die. The affective state of the cows must also be considered. Many studies have evaluated the impacts of hunger, thirst, discomfort, and frustration

associated with heat stress situations. Two of the most notable stressors in this list are discomfort caused by increased standing time and frustration from confinement with a lack of adequate resources like shade and water (Polsky, 2017).

Impacts on Human Health

While milk is pasteurized and extensively tested for potential disease-causing contaminants, high SCC in milk poses potential health problems for humans by increasing the chances of bacterial toxin contamination (Sharma, 2011). This is particularly a problem for individuals who drink unpasteurized milk. Increased SCC also decreases the shelf life of dairy products (Sharma, 2011).

Economic Impacts

Heat stress causes significant production loss and waste, posing major economic impacts for dairy producers. The estimated economic losses for dairy producers due to heat stress in the U.S. is around $900 million. There are several factors that contribute to this economic loss including higher instances of mastitis requiring additional medical costs, compromised reproduction, increased culling, and the heat related death of approximately 10 cows out of every 1000 (St-Pierre, 2003).

Objective

The objective of this study is to determine the effects of heat stress on blood metabolites including serum calcium, phosphorus, magnesium, glucose, cholesterol, albumin, non-esterified fatty acids, and beta hydroxybutyrate as well as various aspects of milk quality including somatic cell count, milk bacterial load, and milk production of lactating Jersey and Holstein cows. This analysis of both milk quality and blood metabolites will potentially provide a new means of identifying issues with milk quality and how to rectify them. This research is meant to further investigate the foundations for producing quality dairy products and to provide guidelines for dairy producers, especially those who regularly deal with heat stress due to warmer climate.

Methodology

Two groups of cows (6 Holsteins and 6 Jerseys; 12 total) were selected from the MTSU dairy farm and evaluated during 3 experimental periods. Each cow prior to this study had a herd identification number assigned to her by the dairy farm; these numbers were used to organize and label the samples and data obtained from each cow. Three experimental periods were conducted during the months of April and May, 2021, when the Temperature-Humidity Index (THI) is variable from week to week. The THI was calculated using measurements from the farm in following equation:

THI = [0.8 × ambient temperature (°C)] + [(% relative humidity/100) × (ambient temperature -14.4)] + 46.4

The THI of each period was categorized as control (THI below 72), mild (THI between 72 and 78), moderate (THI between 78 and 88), or high (THI greater than 88) depending

on the measurement at the time of the period (Armstrong, 1994). Session 1 was used as a control period with no heat stress (THI = 66), session 2 was representative of moderate heat stress (THI = 79), and session 3 was representative of mild heat stress (THI = 74).

During each period, blood samples were collected from the coccygeal vein of each cow. These samples were collected into a sterile vacutainer collection tube that allowed the blood to clot. This clotted blood was then centrifuged at 1000 x gravitational units (g) for 10 minutes to obtain serum and subsequently cooled at -20 °C for proper storage. The samples were then shipped to Texas A & M Veterinary Medical Diagnostic Laboratories for analysis. The profile included calcium, phosphorus, magnesium, albumin, BUN, glucose, cholesterol, sodium, potassium, chloride, and beta-hydroxybutyrate.

Milk samples were obtained from each cow during the regular milking times and under normal milking procedures. The procedure for routine milking at the MTSU dairy farm includes a dry wipe to clean any dirt or debris from the udder and teats, a pre-dip solution applied to each teat, and the application of a clean mechanical milking unit. Somatic cell count was evaluated using the DeLaval Cell Counter (**DCC**). A few drops of the milk sample were placed into a specialized DCC cassette which was then inserted into the machine and a SCC value was reported.

All samples were plated on a tri-plate agar to determine if bacteria were present in the sample and, if applicable, the species of the bacteria were determined. The tri-plate agars consisted of three different mediums in which different bacteria can be cultured. These mediums include the following: Factor medium, MacConkey medium, and Focus medium. Factor medium is an optimal environment for gram-positive bacteria, while

MacConkey Medium is an optimal environment for gram-negative bacteria. Focus medium promotes the growth of *Streptococcus* and similar bacteria. The milk samples were plated following the Minnesota Easy Culture System User's Guide. In accordance with this guide, all plating took place in a designated, clean area and using clean disposable gloves in order to limit the risk of environmental contamination. To plate the sample, a sterile cotton swab was dipped into the milk sample and smeared onto the three mediums in the following order: Factor medium, MacConkey medium, Focus medium. After plating, the samples were incubated at 37°C (98.6°F) and approximately 75% humidity. Humidity was maintained by placing a pan of water in the bottom of the incubator. The plates were incubated for a total of 48 hours and analyzed at 24 and 48 hours for bacterial growth. Additionally, the milk yield, activity time, rest time, and milk conductivity of each cow was recorded throughout the study using data collected by the afimilk® system already installed and used in daily operations at the dairy farm. Dry matter intake averages for each breed were also collected by the afimilk® system. Data were analyzed using the Mixed Model in SAS with fixed effects of treatment and breed and the random effect of cow.

Results

Milk Yield and Conductivity Results

There was a significant difference between breed and kilograms (kg) of milk produced per day (d) (Table 1). Holstein cows produced significantly more milk per day than Jersey cows with an average of 30.7573 kg/d and 24.2472 kg/d respectively ($p = <0.0001$) (Figure 1). There was no significant difference between milk production and

level of heat stress. There was a significant difference between breed and conductivity with Holsteins having an average conductivity of 9.926 mS/cm and Jerseys having 9.4325 mS/cm ($p = 0.0195$) (Figure 2). Additionally, there was a significant difference between conductivity and level of heat stress with cows under no heat stress having an average conductivity of 9.3966 mS/cm and cows under mild heat stress having an average conductivity of 10.0704 mS/cm ($p = 0.0003$) (Figure 3).

Activity Results

Activity was measured in three defined measures: activity time in minutes (min) per day, rest bouts in number (n) per day, and rest time in min/d (Table 2). Activity time showed a significant difference between breeds ($p = <0.0001$) as well as a significant difference between both no heat stress and mild heat stress ($p = 0.0117$) and moderate and mild heat stress ($p = 0.0481$) (Figure 4). Jerseys had significantly more activity time than Holsteins with 126.35 and 101.81 min/d respectively (Figure 5). There was also a significant difference between breed and rest bouts per day with Jerseys having an average of 26.3762 rest bouts per day and Holsteins having an average of 22.4956 rest bouts per day ($p = <0.0001$) (Figure 6). Finally, rest time showed a significant difference with breed ($p = <0.0001$). Holsteins had an average rest time of 678.85 min/d while Jerseys has an average rest time of 820.08 min/d (Figure 7).

Somatic Cell Count and Somatic Cell Score Results

For SCC there was a trend by heat stress period when comparing the no heat stress period to the mild heat stress period ($p = 0.0936$). The no heat stress period showed an average of 94,250 cells/mL while the mild heat stress period showed an average of 380,417 cells/mL (Figure 8). There was no significant difference or trends between the no heat stress period and the moderate heat stress period. Somatic Cell Score (SCS) is mathematically determined from the somatic cell count by the formula Somatic Cell Score = log base 2 (SCC/ 100,000) + 3 (Table 1). There was a statistical trend between the no heat stress period and the mild heat stress period ($p = 0.0514$) (Figure 9).

Blood Metabolite Results

There were no statistical differences by breed or level of heat stress for phosphorus, cholesterol, sodium, chloride, or beta hydroxybutyrate. There was a trend by breed for blood calcium levels ($p = 0.0707$) with Holsteins averaging 9.5267 mg/dL and Jerseys averaging 9.7900 mg/dL. Potassium also showed a breed trend ($p = 0.0829$) with Holsteins averaging 4.4011 mEq/dL and Jerseys averaging 4.6544 mEq/dL. There was a significant difference by breed when evaluating blood glucose levels ($p = 0.0106$) with Holsteins averaging 73.5556 mg/dL and Jerseys averaging 67.6556 mg/dL. There was a significant difference in magnesium by heat stress level ($p = 0.0388$) with cows during the no heat period averaging 2.0398 mEq/dL and the cows during the mild heat stress period averaging 2.1710 mEq/dL (Figure 10). Blood Urea Nitrogen (**BUN**) showed a similar significant difference by period ($p = 0.0056$) with cows during the no heat period

averaging 16.3991 mg/dL and the cows during the mild heat stress period averaging 13.1303 mg/dL (Figure 11). Finally, albumin showed a significant breed by heat stress level interaction ($p = 0.0084$) (Figure 12).

Culture Plate Results

There were no significant differences associated with the 24-hour or 48-hour evaluation of the culture plates when compared by breed or level of heat stress. The most prevalent category of bacteria observed was *Staph* species or other non-*Strep*, Gram-Positive species making up 25% of the cultures from the no heat stress period, 50% of the cultures from the mild heat stress period, and 64% of the cultures from the moderate heat stress period (Table 3). This category was also the most prevalent in both breed making up 39% of the cultures for Holsteins and 53% of the cultures for Jerseys.

Discussion

The results present a combination of expected and unexpected outcomes. Several previous studies have identified many of the breed differences observed in this study such as the differences in total milk production between Holsteins and Jerseys (West, 2003; Armstrong, 1994; Lim, 2020). Additionally, the breed trends observed in both calcium and potassium serum levels make sense because Lim et al. (2020) previously found that Jersey cows have more stable levels of these minerals in their milk when compared to Holsteins. This means that Jerseys, who are producing less milk and maintaining more stable mineral concentrations in their milk, are contributing less minerals from their

blood to use for milk production. Furthermore, potassium is also the major cation in bovine sweat (West, 2003). Holstein cows due to their larger size and heightened susceptibility to heat could be excreting a large amount of sweat, depleting their serum potassium. The glucose difference can be explained due to the difference in DMI between the two breeds. Holsteins had a higher dry matter intake and a higher glucose concentration compared to Jerseys.

Moreover, there were also some breed differences when analyzing rest time and activity time. Holsteins had significantly less activity time per day, less rest time per day, and less rest bouts per day than Jerseys. This is supported by previous studies which found that Holsteins, due to their larger size, tend to stand in place to expose more surface area for heat dissipation (Polsky, 2017). Conversely, the Jersey cows in this study had both high activity times and frequent rest bouts indicating restlessness.

There were several unexpected results related to the varying levels of heat stress. An example is the trend observed in SCC and SCS over the length of the study. The SCC and SCS continued to increase as the study went on even though the last experimental session was a milder level of heat stress than the previous session. Previous studies report an increase of SCC and SCS in relation to the level of heat stress the cows are experiencing (Hammami, 2013). Milk conductivity has previously been linked to udder health and subsequently to milk quality. As milk conductivity increases, the chance of mastitis increases (Norburg, 2004). There was a significant increase in milk conductivity from the no heat stress period to the mild heat stress period as seen with both SCC and SCS. Serum albumin also showed an interesting difference that is contrary to the findings of Joo et al. (2021), who reported a decrease in serum albumin in response to heat stress.

The current data show the albumin in Jerseys trended up with each experimental session despite being exposed to varying levels of heat stress. Furthermore, Holsteins had a decrease in albumin from the no heat stress period to the moderate heat stress period but then had an increase in albumin from the moderate heat stress period to the mild heat stress period. One possible explanation is that both breeds experienced dehydration at some point during the study as increased albumin can be an indicator of dehydration (Alberghina D., 2015). All of these results would be expected in response to the moderate heat stress period rather than only the mild heat stress period. Additionally, BHB was expected to increase as the level of heat stress increased (H. Tian, 2016); however, there was no significant difference observed between the levels of heat stress.

Another unexpected result was the significant difference between magnesium concentration and level of heat stress. While previous studies have reported a significant decrease in serum magnesium levels in response to heat stress (Joo, 2021), this study shows a significant increase in magnesium in response to heat stress. Magnesium is known to be a cofactor for many enzymes involved in cellular metabolism as well as playing an unknown role in immune system activation (Libera, 2021). Magnesium is not easily mobilized in the body without the addition of a dietary supplement, so this increase in magnesium is more likely derived from the redistribution of the metabolite from other physiological cycles. Because neither BHB nor glucose significantly increased in response to heat stress, the krebs cycle, which relies on magnesium, is most likely being used as the primary metabolic mechanism. Magnesium is most likely not being released from this cycle as magnesium is essential for it to progress. Additionally, the increase in SCC would indicate a more active, but less effective immune system. Logically, if

magnesium is used to activate the immune system, the increased SCC would be paralleled by a decrease in serum magnesium levels. Altogether, this increase in serum magnesium cannot be easily explained by any trends in previous research or the current study. More research is needed to determine the exact role that magnesium plays in the immune system or if magnesium has some other interaction with heat stress and heat stress recovery.

One explanation for this collection of unexpected results may be the weather patterns during the study. The THI was very mild in the beginning of the study, then got high enough to trigger moderate heat stress in the cows, and finally fell to a THI that triggered mild heat stress. Because of this, these results may reflect the cows recovering from the moderate heat stress during the last experimental session instead of showing the true effects of mild heat stress. Also, the cows may not have been exposed to the heat and humidity long enough to trigger a response to the moderate heat stress when the second experimental session occurred.

The behavior of the cows during the study also fits with this explanation. Activity minutes per day decreased significantly from the moderate heat stress period to the mild heat stress period. This, alongside the increase in SCS and serum albumin, may suggest the cows were feeling unwell due to possible dehydration and other effects of heat.

This study may yield results closer to those of previous studies if the THI could be regulated as they are in many other studies. Presumably the best method would be to have a thermoregulated building for the cows to be kept in so the THI could be manipulated and controlled. While this is a very expensive method, a more cost-effective adjustment may be to ensure the cows have been exposed to the desired level of heat

stress for a minimum of three days to observe physiological effects in blood and milk. Another adjustment could include allowing more time between sessions involving moderate or high heat stress since the recovery period of short term moderate and high heat stress is unknown at this time.

Conclusion

Overall, the quality of the milk declined over the course of the study with an increase in SCC, SCS, and milk conductivity. Conversely, very minimal changes to blood metabolites were observed in relation to varying levels of heat stress. Due to the sequence of heat stress levels tested, many of the changes expected from the moderate heat stress period were seen during the mild heat stress period that followed. Presumably, the changes seen in the mild heat stress period were due to the cows recovering from the moderate heat stress period resulting in notable changes to milk quality but more subtle changes in blood metabolites. This study would need to be repeated with some necessary adjustments, such as testing only after longer exposures to heat, in order to confirm these explanations. Although the results did not align perfectly with what was expected, the core idea that heat stress should be avoided still holds true. Heat stress ultimately decreases the quality and production of milk as well as having negative effects on udder health.

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Appendix

Table 1. Production measures by identification number and experimental period.

Tag Number	Breed	Testing Period	Milk Yield (kg/d)	Conductivity (mS/cm)	Somatic Cell Score
318	Holstein	1	38	8.35	1
		2	42.2	9.3	1
		3	39.6	9.8	4
326	Holstein	1	70.9	9.65	4
		2	57	11.15	5
		3	53.4	15.4	5
504	Jersey	1	55.1	15.1	5
		2	52.7	11.75	-
		3	52.2	9.2	6
515	Holstein	1	102.2	9.25	0
		2	96.3	12.8	3
		3	-	15.1	7
603	Holstein	1	54.2	9.75	2
		2	65.3	10.1	2
		3	31.4	14.65	3
611	Jersey	1	69.7	9	4
		2	61	9.05	5
		3	39.3	8.9	3
619	Holstein	1	79.7	9.15	1
		2	48.5	8.9	1
		3	46.2	9.05	1
702	Holstein	1	55.7	9.5	2
		2	49.3	10	4
		3	42.2	9.6	3
705	Jersey	1	34.1	7.95	1
		2	38.8	8.4	4
		3	32.7	14.35	2
706	Jersey	1	50.9	8.55	4
		2	59.6	12.7	4
		3	52.6	1.46	4
801	Jersey	1	69.5	9.75	1
		2	51.5	12.6	1
		3	63.7	14.4	0
803	Jersey	1	44.2	8.7	0
		2	22.2	8.8	3
		3	20.1	9.05	7

Table 2. Measurements of activity time, rest time, and rest bout by identification number and experimental period.

Tag Number	Breed	Testing Period	Average Activity Time (min/d)	Average Rest Bouts (#/d)	Average Rest Time (min/d)
318	Holstein	1	118.5	16	810
		2	109	13	660
		3	97	13	672
326	Holstein	1	109.5	34	597
		2	106	12	195
		3	101.5	33	438
504	Jersey	1	116	9	684
		2	112.5	18	744
		3	101.5	18	645
515	Holstein	1	92.5	15	597
		2	92	16	540
		3	81.5	11	483
603	Holstein	1	90	17	822
		2	105.5	13	711
		3	92	14	774
611	Jersey	1	138.5	18	648
		2	138.5	14	570
		3	137.5	13	555
619	Holstein	1	97.5	0	0
		2	88.5	1	3
		3	92.5	1	3
702	Holstein	1	112	17	747
		2	104	13	624
		3	104	13	618
705	Jersey	1	140	22	651
		2	137.5	20	654
		3	118.5	12	729
706	Jersey	1	130.5	15	636
		2	138	17	513
		3	123	16	540
801	Jersey	1	118.5	12	645
		2	106	13	573
		3	105.5	10	588
803	Jersey	1	126	9	702
		2	126	12	531
		3	109	6	720

Table 3. Number of cultures by period after 48 hours of incubation.

Period	No Growth	Staph species or other non-Strep Gram-Positive Species	Strep or Strep-like species	Staph and Gram-negative Species	Strep and Gram-negative Species
Period 1	0 %	25 %	25 %	17 %	33 %
Period 2	0 %	64 %	18 %	18 %	0 %
Period 3	0 %	50 %	17 %	25 %	8 %

Table 4. Table of cultures by breed after 48 hours of incubation.

Breed	No Growth	Staph species or other non-Strep Gram-Positive Species	Strep or Strep-like species	Staph and Gram-negative Species	Strep and Gram-negative Species
Holstein	0 %	39 %	22 %	22 %	17 %
Jersey	0 %	53 %	18 %	18 %	11%

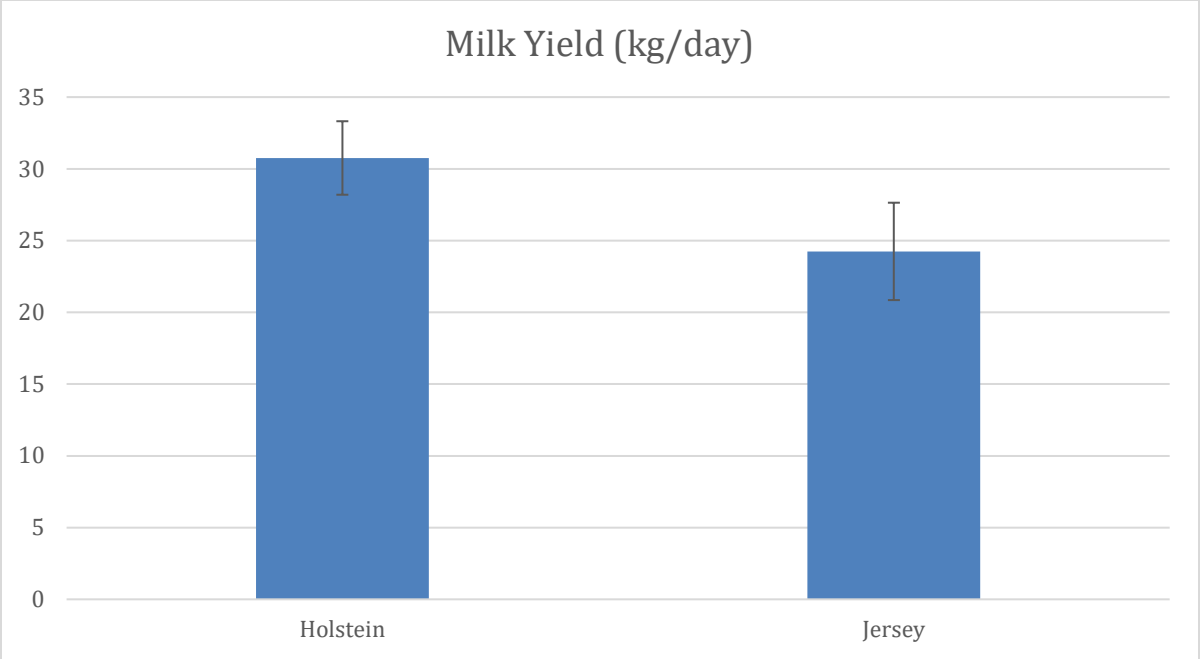

Figure 1. Average milk yield by breed. Holsteins had an average yield of 30.76 kg/day, while Jerseys had an average yield of 24.25 kg/day ($p = <0.0001$).

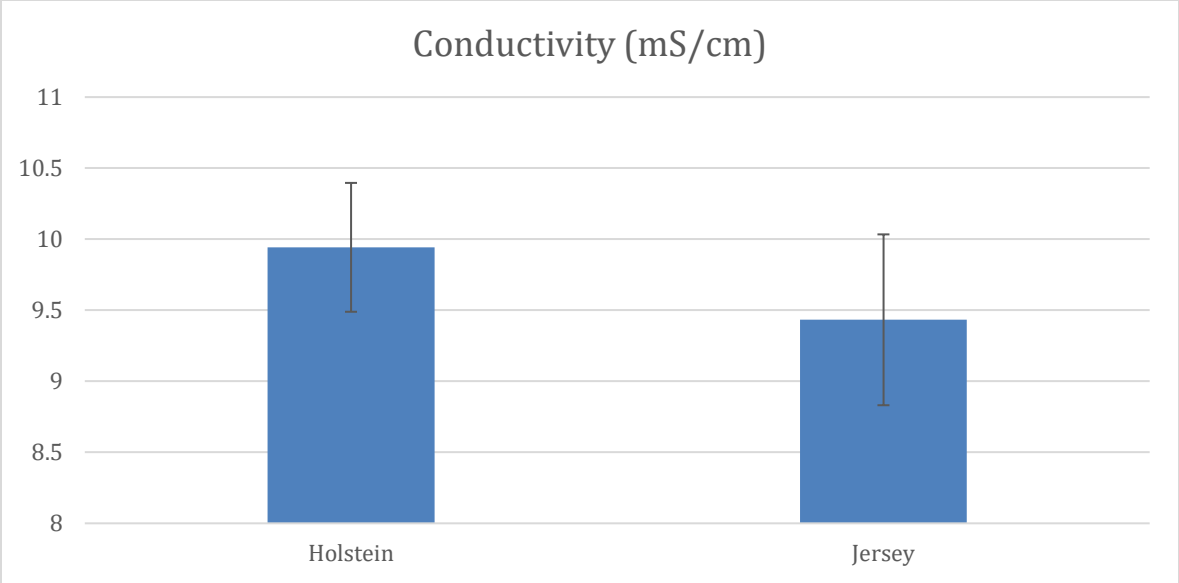

Figure 2. Average conductivity shown by breed. Average conductivity was 9.94 mS/cm for Holsteins and 9.43 mS/cm for Jerseys ($p = 0.0195$).

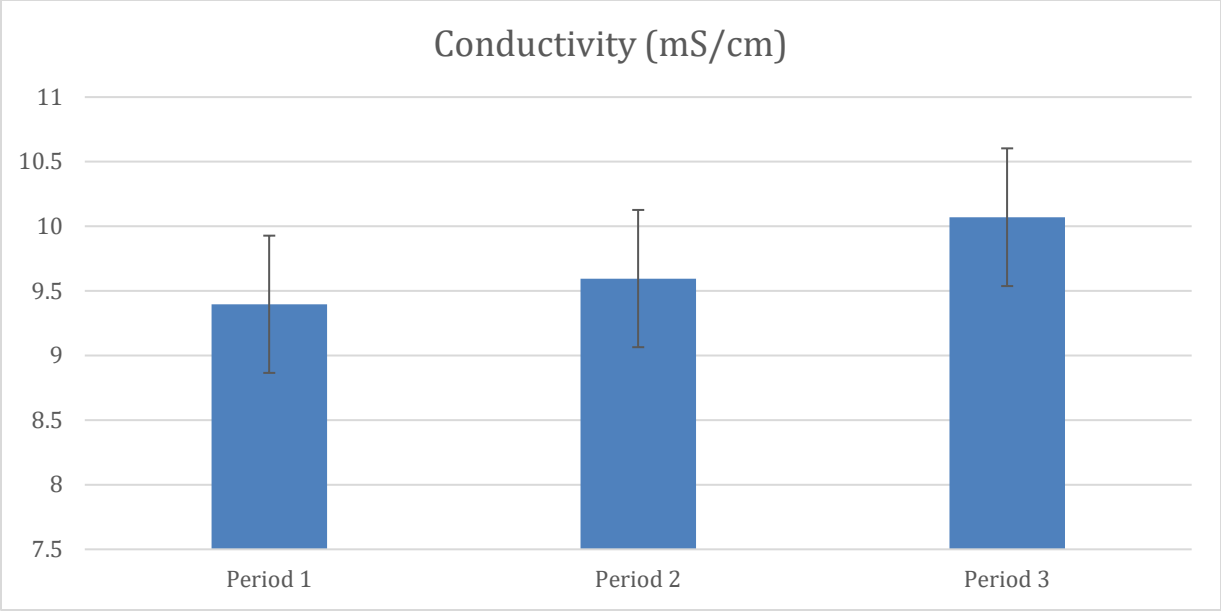

Figure 3. Average conductivity shown by testing period. Average conductivity was 9.40 mS/cm under no heat stress (Period 1), 9.60 mS/cm under moderate heat stress (Period 2), and 10.0704 mS/cm under mild heat stress (Period 3) ($p = 0.0003$).

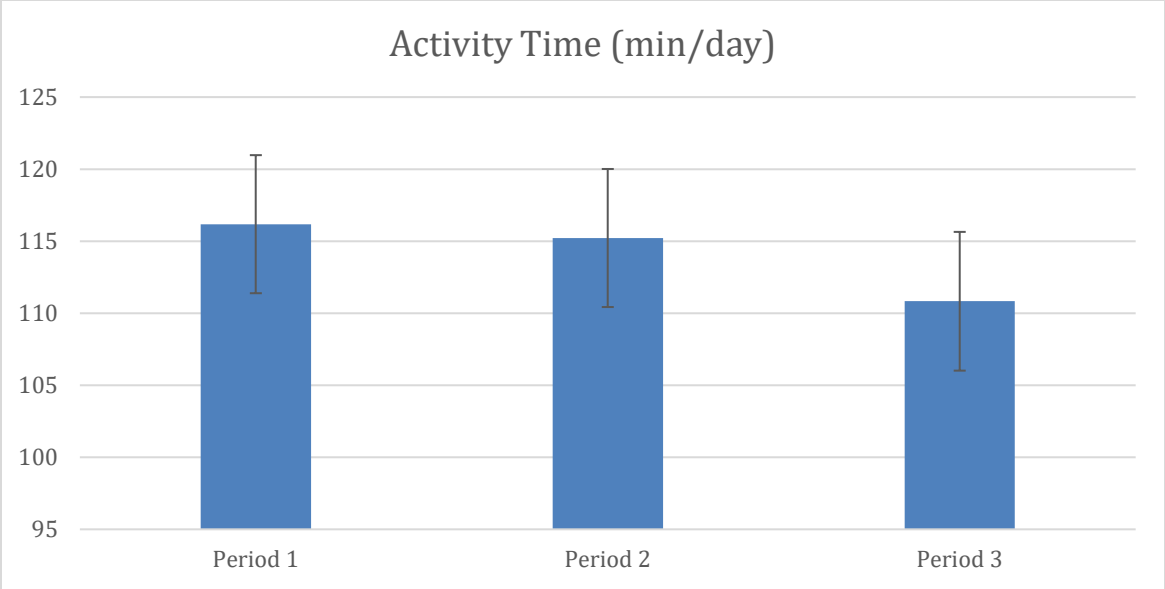

Figure 4. Average activity time shown by testing period. Average activity time was 116.18 min/day under no heat stress (Period 1), 115.22 min/day under moderate heat stress (Period 2), and 110.83 min/day under mild heat stress (Period 3) ($p = 0.0117$).

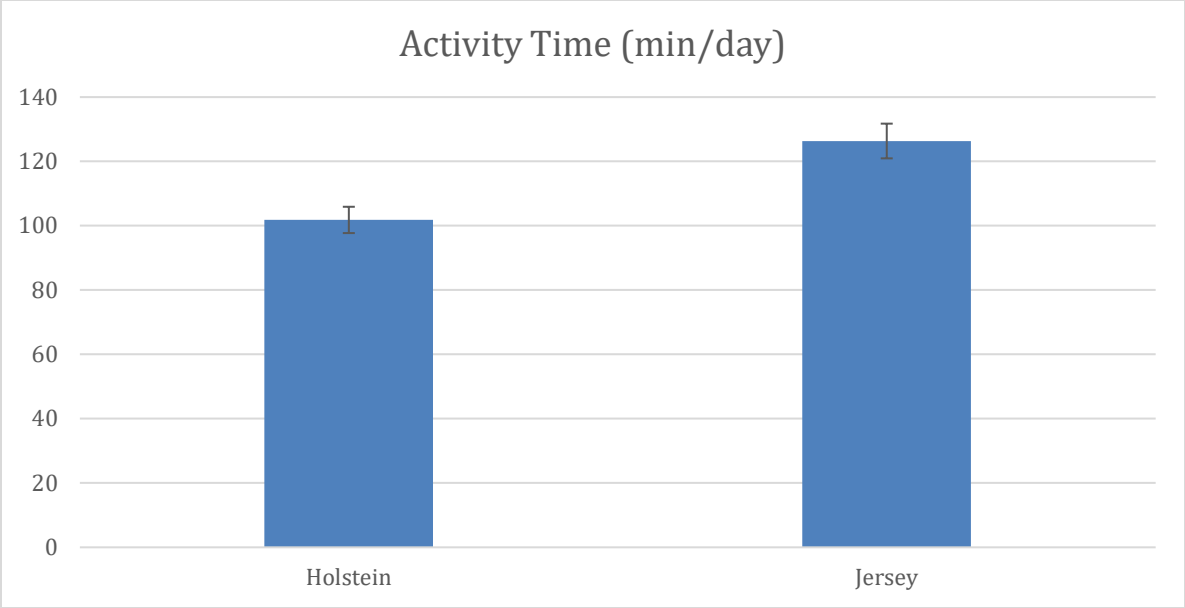

Figure 5. Average activity time shown by breed. Holsteins averaged 101.81 min/day while Jerseys averaged 126.35 min/day ($p = <0.0001$).

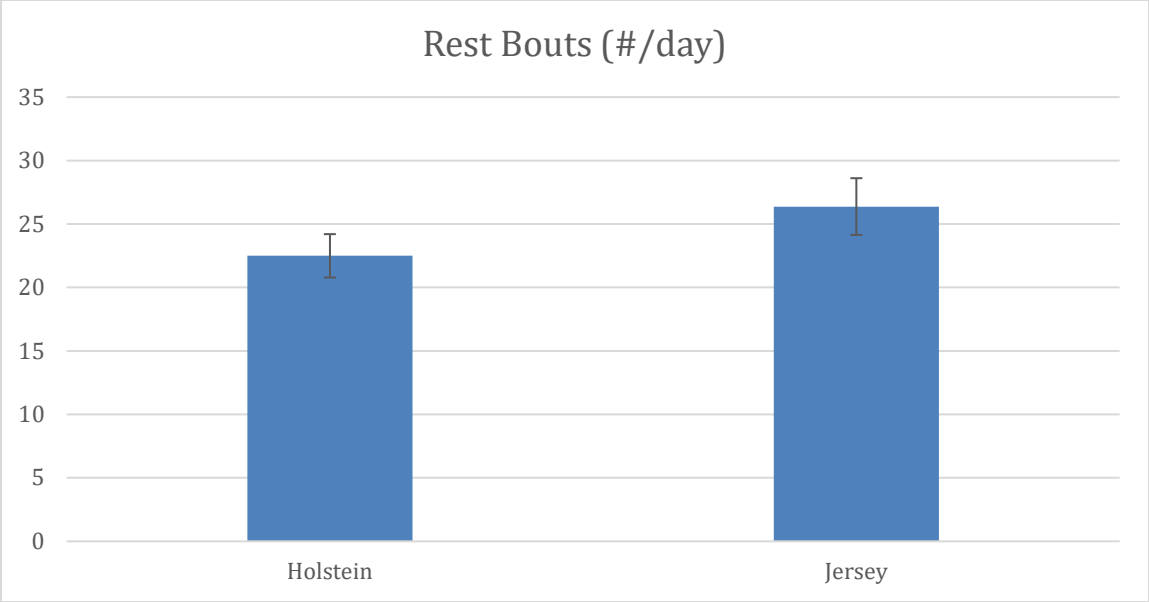

Figure 6. Average rest bouts shown by breed. Holsteins averaged 22.4956 rest bouts per day while Jerseys averaged 26.3762 rest bouts per day ($p = <0.0001$).

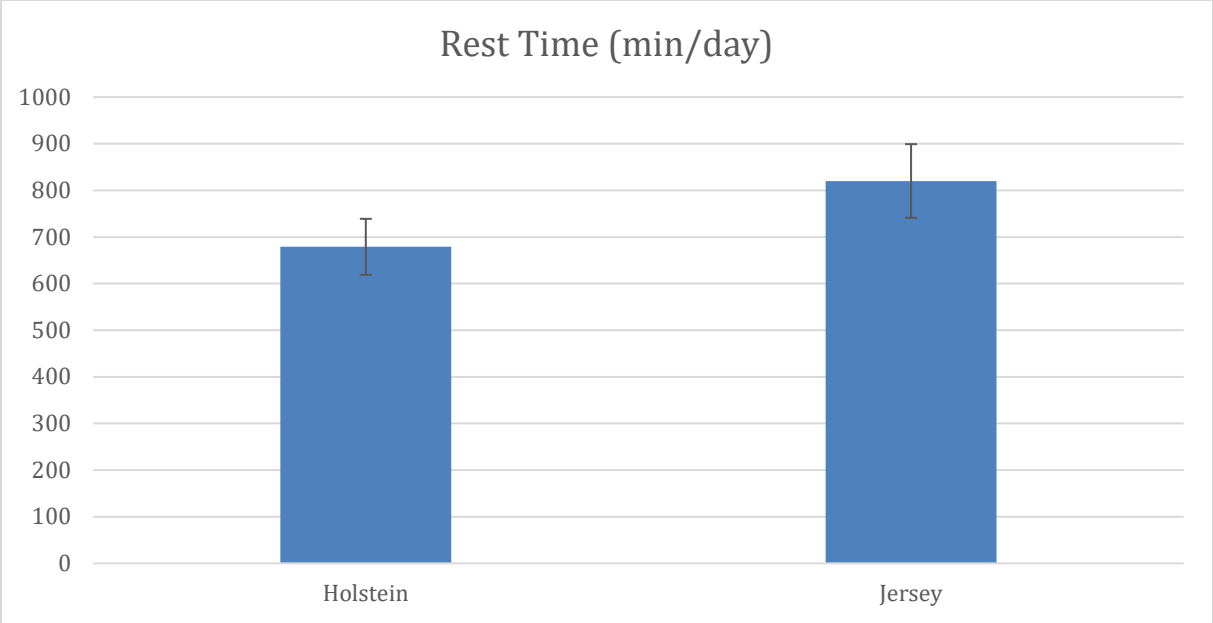

Figure 7. Average rest time shown by breed. Holsteins averaged 678.85 minutes of rest time per day while Jerseys averaged 820.08 minutes of rest time per day ($p = <0.0001$).

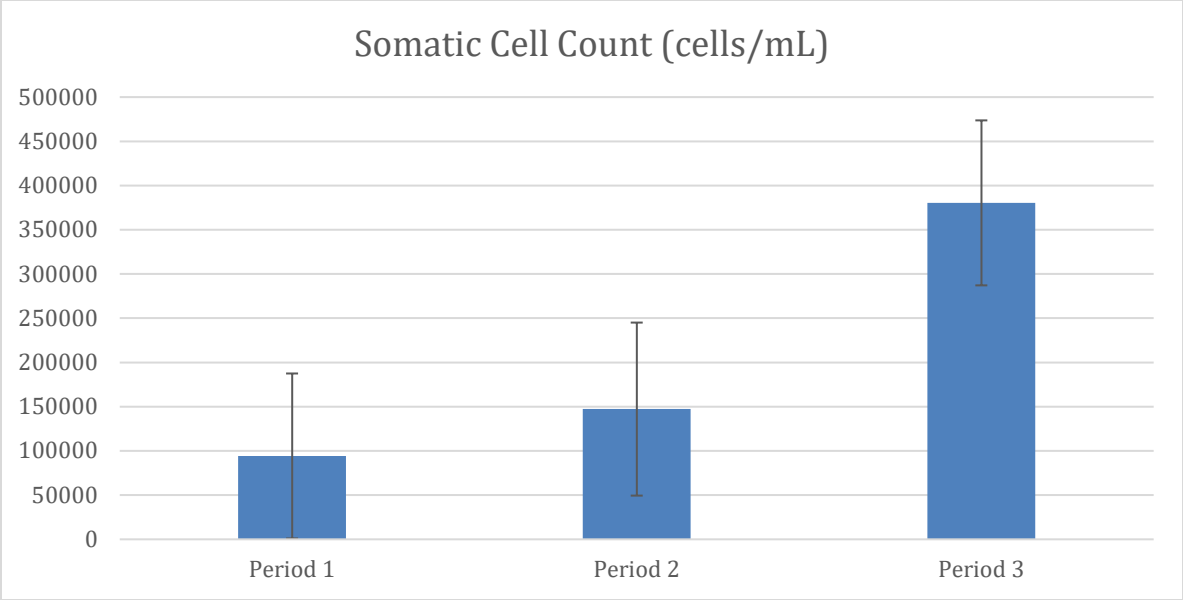

Figure 8. Average somatic cell count shown by testing period. Average SCC was 94,250 cells/mL under no heat stress (Period 1), 147,250 cells/mL under moderate heat stress (Period 2), and 380,417 cells/mL under mild heat stress (Period 3) ($p = 0.0936$).

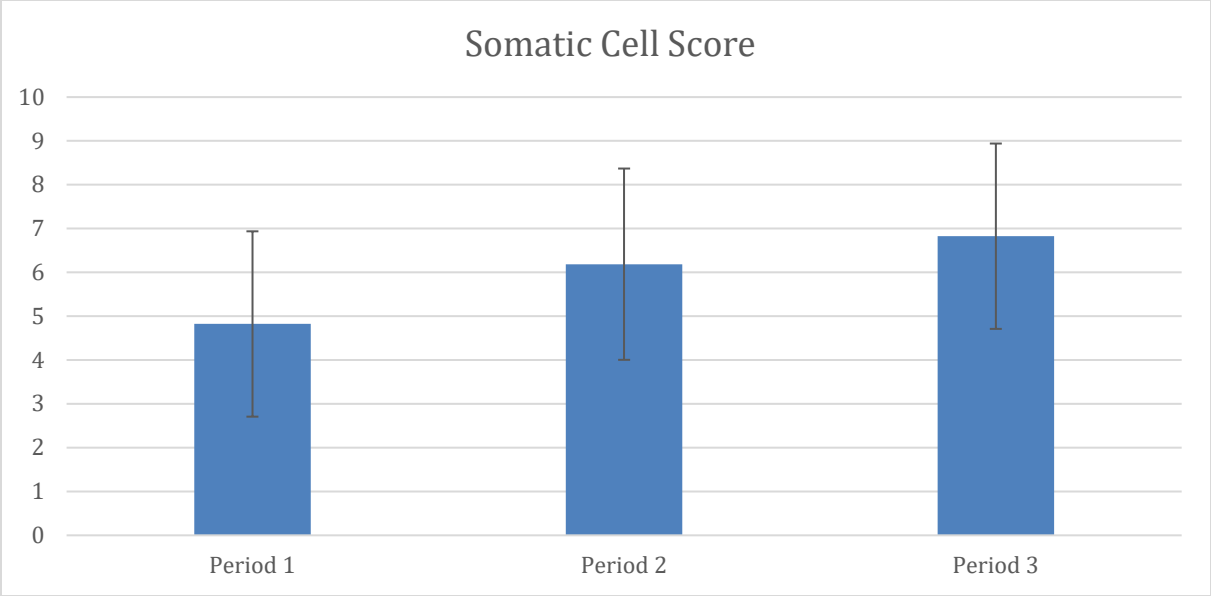

Figure 9. Somatic cell score shown by testing period. Somatic cell score was 4.8227 under no heat stress (Period 1), 6.1874 under moderate heat stress (Period 2), and 6.8257 under mild heat stress (Period 3) ($p = 0.0517$).

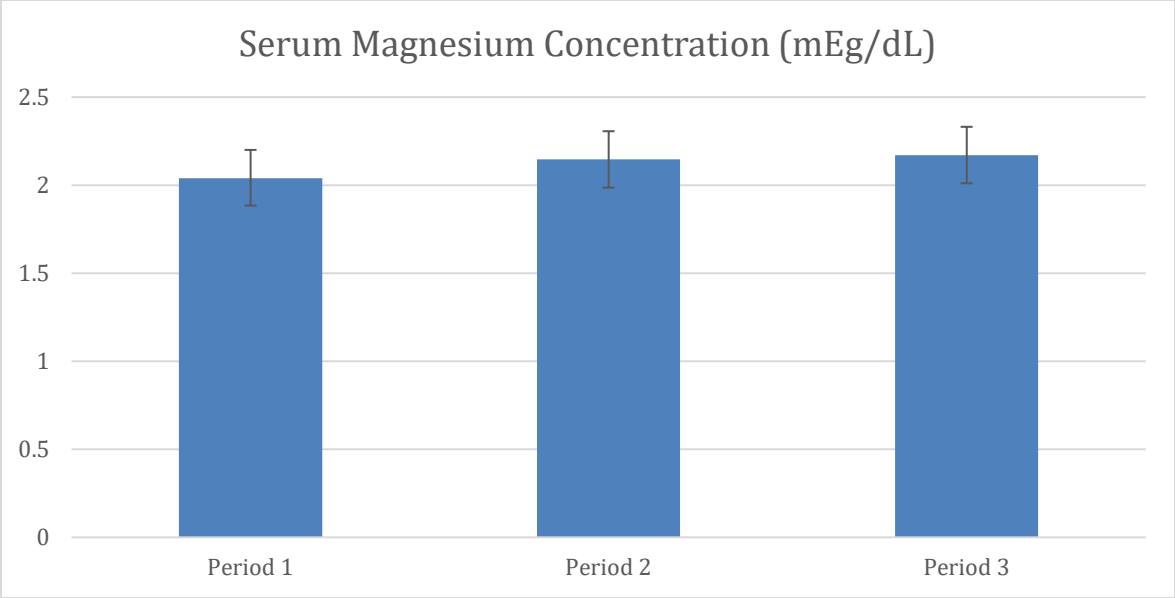

Figure 10. Average magnesium shown by testing period. Average magnesium was 2.0398 mEg/dL under no heat stress (Period 1), 2.15 mEg/dL under moderate heat stress (Period 2), and 2.17 mEg/dL under mild heat stress (Period 3) ($p = 0.0388$).

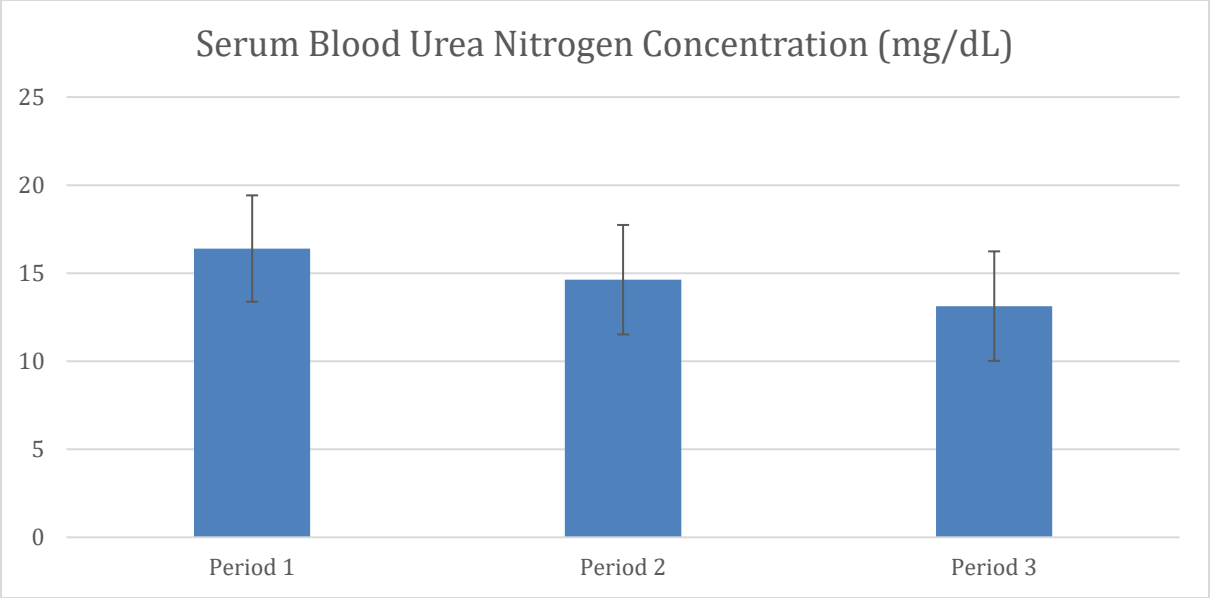

Figure 11. Average BUN shown by testing period. Average BUN was 16.3991 mg/dL under no heat stress (Period 1), 14.6303 mg/dL under moderate heat stress (Period 2), and 13.1303 mg/dL under mild heat stress (Period 3) ($p = 0.0056$).

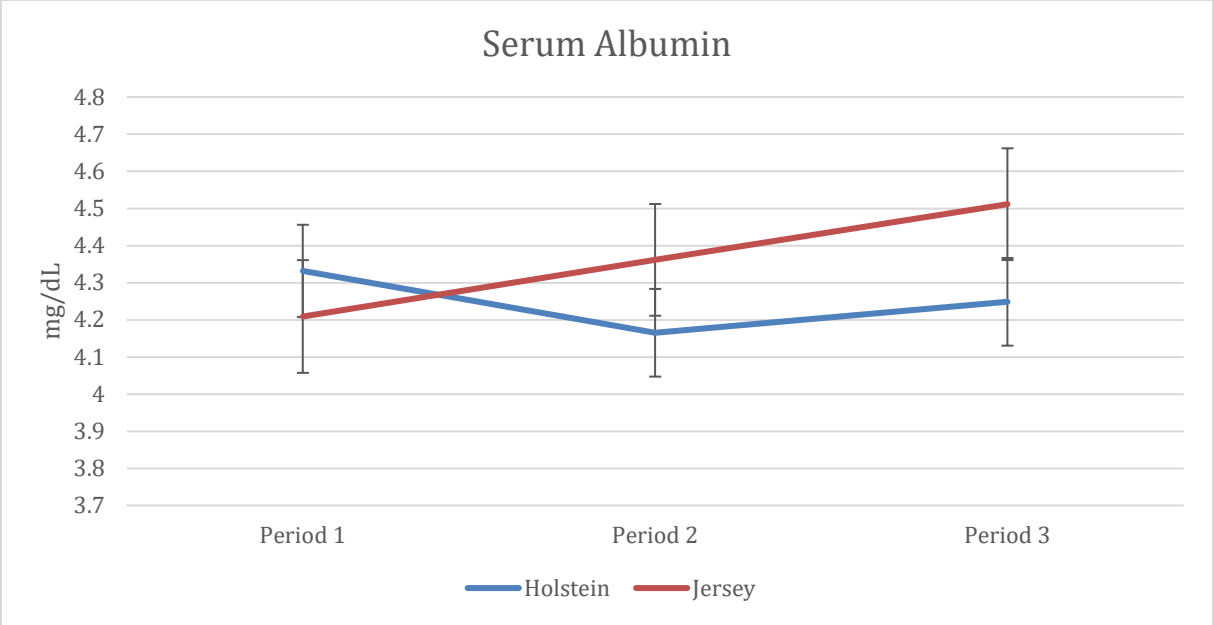

Figure 12. Breed vs. period interaction of serum albumin levels. Both Holsteins and Jerseys had significant differences from period 1 (no heat stress) to period 3 (mild heat stress) ($p = 0.0084$).

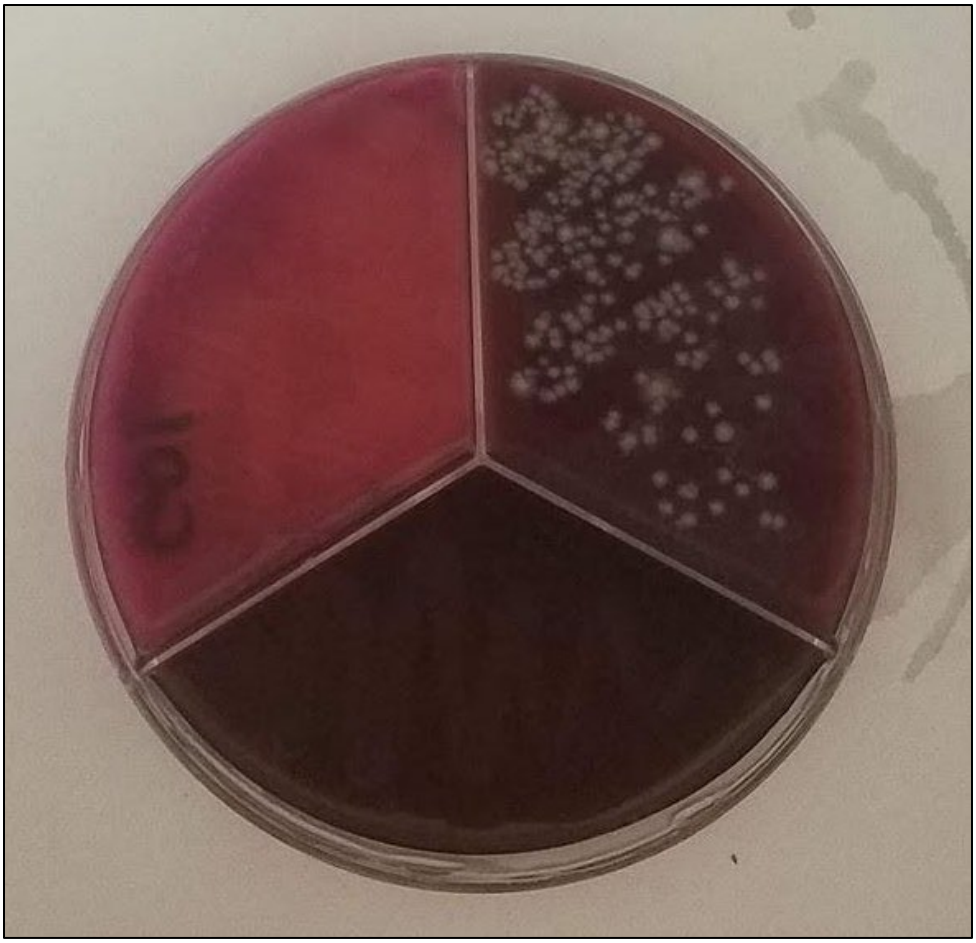

Figure 13. A tri-plate culture medium showing growth on Factor media. Growth on only factor media indicates presence of *Staph* species or other non-*Strep* Gram-positive bacteria.

Figure 14. A tri-plate culture medium showing growth on Factor, Focus, and MacConkey media. Growth on all three sections indicates presence of *Strep* or *Strep*-like species and Gram-negative bacteria.

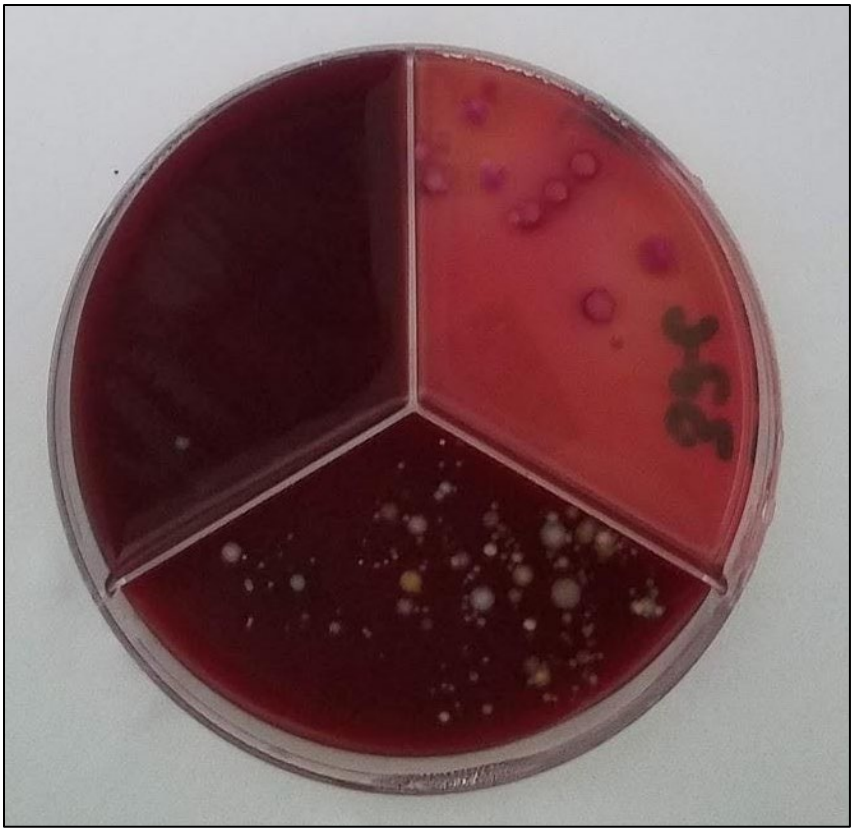

Figure 15. A tri-plate culture medium showing growth on Factor and Focus media. Growth on both Factor and Focus media indicates presence *Strep* or Strep-like species of Gram-positive bacteria.

Figure 16. A tri-plate culture medium showing growth on Factor and MacConkey media. Growth on both Factor and MacConkey media indicates presence of *Staph* species or other non-*Strep* Gram-positive bacteria and Gram-negative bacteria.

IACUC
INSTITUTIONAL ANIMAL CARE and USE COMMITEE
Office of Research Compliance,
010A Sam Ingram Building,
2269 Middle Tennessee Blvd
Murfreesboro, TN 37129

MIDDLE TENNESSEE STATE UNIVERSITY

IACUCN001: PROTOCOL APPROVAL NOTICE

Monday, March 22, 2021

Senior Investigator	**Maegan Hollis** (ROLE: Principal Investigator)
Co-Investigators	Alison Blanton (Student PI)
Investigator Email(s)	*abb6m@mtmail.mtsu.edu; Maegan.hollis@mtsu.edu*
Department	Agriculture
Protocol Title	***Effects of Heat Stress on Blood Metabolism and Milk Quality in Lactating Holstein and Jersey Cows***
Protocol ID	**21-2005**

Dear Investigator(s),

The MTSU Institutional Animal Care and Use Committee has reviewed the animal use proposal identified above under the ***Designated Member Review (DMR) mechanism*** and has approved your protocol in accordance with PHS policy. A summary of the IACUC action(s) and other particulars of this this protocol is tabulated as below:

IACUC Action	**APPROVED for one year**	
Date of Expiration	3/31/2022	Approval Date:: 3/5/2021
Number of Animals	FIFTEEN (15)	
Approved Species	**MTSU Bovine**	
Category Subclassifications	☐ **Teaching** ☐ Classroom ☐ Laboratory	☒ **Research** ☐ Laboratory ☒ Field Research ☐ Field Study ☒ Handling/Manipulation ☐ Observation
	Comment: NONE	
Approved Site(s)	MTSU Dairy (3211 Guy James Rd, Lascassas, TN)	
Restrictions	**1. Satisfy DMR requirements AND annual continuing review.** **2. Follow CDC guidelines and MTSU requirements to counter COVID-19 infection**	
Comments	Approved during COVID-19 emergency	

This approval is effective for three (3) years from the date of this notice (2/28/2024). This protocol **expires on 3/31/2022** The investigator(s) MUST file a Progress Report annually regarding the status of this study. Refer to the schedule for Continuing Review shown below; NO REMINDERS WILL BE SENT. A continuation request (progress report) must be approved

by the IACUC prior to 3/31/2022 for this protocol to be active for its full term. Once a protocol has expired, it cannot be continued and the investigators must request a fresh protocol.

Continuing Review Schedule: Refer tp the following table to request your CR:

Reporting Period	Requisition Deadline	IACUC Comments
First year report	1/31/2022	TO BE COMPLETED
Second year report	1/31/2023	TO BE COMPLETED
Final report	1/31/2024	TO BE COMPLETED

MTSU Policy defines an investigator as someone who has contact with live or dead animals for research or teaching purposes. Anyone meeting this definition must be listed on your protocol and must complete appropriate training through the CITI program. Addition of investigators requires submission of an Addendum request to the Office of Research Compliance.

The IACUC must be notified of any proposed protocol changes prior to their implementation. Unanticipated harms to subjects or adverse events must be reported within 48 hours to the Office of Compliance at (615) 494-8918 and by email – compliance@mtsu.edu.

Post-approval Protocol Amendments:

Date	*Amendment(s)*	*IRB Comments*
NONE	NONE	NONE

All records pertaining to the animal care be retained by the MTSU faculty in charge for at least three (3) years AFTER the study is completed. Be advised that all IACUC approved protocols are subject to audit at any time and all animal facilities are subject to inspections at least biannually. Furthermore, IACUC reserves the right to change, revoke or modify this approval without prior notice.

Sincerely,

Compliance Office
(On behalf of IACUC)
Middle Tennessee State University
Tel: 615 494 8918
Email: iacuc_information@mtsu.edu (for questions) and
iacuc_submissions@mtsu.edu (for sending documents)