Resilience of High Immune Responders Beef Cattle in the Context of Climate Change

by

Nasrin Husseini

A Thesis

presented to

The University of Guelph

In partial fulfilment of requirements
for the degree of

Master of Science

in

Pathobiology

Guelph, Ontario, Canada

ABSTRACT

RESILIENCE OF HIGH IMMUNE RESPONDERS BEEF CATTLE IN THE CONTEXT OF CLIMATE CHANGE

Nasrin Husseini Advisor: Dr. Bonnie Mallard
University of Guelph, 2020

Animal health and welfare are important features of highly productive herds. Animal diseases result in loss of appetite, weight loss, and ultimately lower production with economic costs to the producer. Additionally, climate change with increases in ambient temperature and humidity can decrease livestock production and reproduction potential, as well as increase disease susceptibility. High (H) immune response (IR) dairy cattle, identified using the University of Guelph's HIR™ methodology, have been reported to have fewer incidents of disease compared to average and low immune responders making immuno-phenotyped cattle an ideal model to examine the effects of global warming on health traits. Results of this thesis indicated that beef cattle can be immuno-phenotyped for antibody (AMIR) as early as 2-3 weeks of age, and cell-mediated immune response (CMIR) between 3 weeks and 9 months of age, using HIR™. Additionally, beef cows with high AMIR were able to regulate their body temperature better than average and low AMIR phenotypes.

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my advisor, Dr. Bonnie Mallard, for her constant support and guidance throughout this entire project. She continually and convincingly conveyed a spirit of adventure in regard to research and scholarship.

I also would like to thank my supervisor Dr. Douglas Hodgins; without his guidance and persistent help this dissertation would not have been possible. I would like to thank my committee members, Dr. Niel Karrow and Dr. Angela Cánovas for their helpful advice, suggestions and comments throughout the preparation of this thesis. The field work would not have been possible without the help of Tess Altvater-Hughes and Elora Beef Research Station staff. The laboratory work would not have been possible without the help of Saeid Tabatabaei, Marnie McKechnie and Julie Schmied.

I am extremely grateful to my parents for their love, prayers, caring and sacrifices for educating and preparing me for my future. I am very thankful to my dad and my mom for giving their daughter the opportunity to study while other fathers in Afghanistan make their daughters marry at a very early age; my dad broke this taboo by letting me get a higher education and MSc degree. I also would like to thank my grandfather who is not alive now but was one of my main supporters in this path.

I would like to acknowledge and thank Arrell Food Institute and the Arrell family for their support. This project was funded by Arrell food scholarship and Ontario Veterinary College scholarship.

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1 Introduction and Literature Review

1.1 Brief overview of Canadian beef industry

Canada has approximately 13 million beef cattle, which reside on approximately 60,000 beef farms and annually contribute $33 billion to the Canadian economy (Beef production 101, https://www.cattle.ca/cca-resources/animal-care/beef-production-101/).

Almost all Canadian beef cattle are *Bos taurus;* the major beef breeds include Angus, Charolais, Limousin, and Simmental. *Bos indicus* include the breeds of Brahman and Zebu, which are rare in Canada; however, the United States and Australia use these breeds for beef production. According to Wheeler et al., (1994) “meat produced from *Bos indicus* cattle was less tender than meat from *Bos taurus* cattle, regardless of marbling score”. Cattle are able to covert materials that are not digestible for humans into highly digestible protein foods.

Farmers usually breed their cows to calve in the spring so that they have nutritious grass to graze. Most calving is unassisted. Cows that are non-pregnant in the autumn maybe be culled for economic reasons. In the autumn, after return from pasture calves are separated from their mothers. By late autumn a cow is in calf or is a candidate for culling. However, they might keep some of the female calves as replacement heifers. Success in the beef industry is highly dependent on how healthy the calves are, and one key to achieve this goal is buying and maintaining healthy animals from cow-calf operations. Identifying the healthiest beef cows and their calves, particularly in the face of climate change, is the focus of this thesis. Cattle that are both healthy and adaptable

to climate change, specifically heat stress, will be defined as resilient in the context of this thesis. According to the literature a temperature humid index (THI) of approximately 68-74 is considered comfortable for cattle (Habeeb et al., 2018; Polsky et al., 2017) and therefore THI of <74 will be considered normal, whereas THI ≥ 74 will be considered above normal in this thesis.

Most beef calves, but not all, are born in the spring, and they stay with their dams on pasture until autumn weaning. After weaning, beef calves are fed higher energy diets in feedlots until they reach market weight. Beef farmer oversight of pastured cattle is minimum.

1.2 Brief overview of selection of livestock for heath and productivity

In the past, breeding systems around the world focused more on breeding for increases in production, unaware of its possible negative effects on animal health. For instance, in dairy cattle, selection usually was done to enhance milk production and change the protein and fat content of milk which resulted in increased mastitis occurrence (Heringstad, 2005), as well as in problems with fertility and reproduction (Veerkamp, 2001). Beef breeders focused on growth traits with little or no emphasis on health (Hayes et al., 2013). Tremendous improvement has been made in both dairy and beef production using selective breeding methods while producers mostly still rely on vaccination, antibiotics and management techniques to maintain animal health. However, consumer concern about food safety and animal welfare have made farmers and animal breeders think more about other alternative approaches to health, particularly those that will reduce the amount of antibiotic usage in food animals.

Increasing concern about antibiotic use in food animals and increasing cost of new drug development, as well as antibiotic resistance in humans are all concerns, which drive animal breeders' attention toward a new system of breeding in which animals are inherently healthier and can combat disease naturally (Mallard, 2007). Currently, the costliest problem the Agri-food industry is facing is infectious disease of livestock with an estimated cost of $18 billion/year in North America (Mallard & Wilkie 2007). It is now accepted that genetic selection for important health traits must accompany progress in production traits (Mallard & Wilkie, 2007). Breeding for improved health traits has been done in Scandinavian cattle for many years with breeding more for resistance to mastitis as a prime example (Heringstad, 2005). Selective breeding for resistance to specific diseases in Canada and around the world is now becoming more prevalent, including breeding for resistance to nematode infection in sheep and resistance to mastitis in cattle (Stear et al., 2001; 2017).

Active breeding for enhanced immune response in pigs demonstrated an enhancement in their general immune response, as well as their response to commercial vaccines and even their growth rate. Immune responses vary among individuals within herds and among breeds; factors such as genetics and environment dictate this variability (Emam et al., 2019). Selection for broad-based disease resistance based on immune response traits is proving commercially effective for dairy cattle and can reduce the occurrence of udder, uterine, kidney, lung, and navel infections (Larmer and Mallard, 2017). Breeding for resistance to diseases such as bovine respiratory disease complex (BRD), which is a considerable problem in North American beef feedlots, would also benefit from

genetic approaches to improve animal health. The Bovine Respiratory Disease Coordinated Agriculture Project (BRD-CAP), which was a 5-year project funded by United States Department of Agriculture (USDA), aimed to identify the genetic loci for BRD susceptibility in order to develop DNA-based selection. Neibergs et al. (2014) reported heritability estimates for BRD susceptibility in dairy calves in the range of 19–21% for New Mexico and California as individual populations, and around 13% when these areas were combined indicating that genetic improvement should be achievable.

Genetic selection based on the health disorder data recorded in an on-farm software program also has been considered as a possible tool to improve animal health (Zwald et al., 2004). However, this tool is only effective when the health data records are accurate. In general, if accurate and systematic on farm data recording can be achieved, it should help facilitate genetic selection for disease resistance of livestock. Results of this study show that it is possible to incorporate these traits into selection indices (Zwald et al., 2004). Recently in the US, Zoetis provided a “Wellness Index” for dairy producers based on a large number of producer health records (Vukasinovic et al., 2017). Holstein Canada plans to launch a similar system called Compass in 2020 (Doormaal, 2018).

Behavioral traits are also included in some breeding programs with the potential to improve animal welfare and production (Haskell et al., 2014). In beef cattle, this trait is potentially beneficial to ease the handling of animals. However, it is difficult and time-consuming to measure this trait in a reliable manner and include it in a breeding program (Haskell et al., 2014). Adding disease resistance traits into breeding programs

reduces monetary losses, undesirable genetic correlations between high production and disease, responds to consumers' request for high quality food from healthier animals, and reduces antibiotic usage in farm animals (Detilleux et al., 2001). In dairy farms, mastitis and lameness are the costliest diseases dairy farmers face (Bennett, 1999). The cost of milk loss production from lameness in dairy cattle has been estimated to be 1.5-2.8 kg/day in first two weeks after the diagnosis (Amory et al. 2008; Cartwright et al. 2017). In beef cattle production, BRD is the most expensive health trait, costing North American producers USD $54.12 million to treat sick animals; this does not include the production and mortality costs (Johnson, 2017). Breeding goals for cattle, as well as other livestock, will no doubt continue to strive to balance important traits such as production with health, fertility and feed efficiency (Olesen, 2000).

1.3 Selective breeding of beef cattle

1.3.1 Selection for feed efficiency

It is estimated that by 2050, agricultural products will be needed to feed 10 billion people (Morris, 2007). Methods need to be developed to produce more animal protein using fewer material resources. Therefore, selection for feed efficiency will help to improve the constancy and longevity within herds. Growing calves and mature cattle both show genetic variation in feed efficiency (Archer, 1999). Advances in genetic, nutritional and transcriptomic beef research have led to improvement in feed efficiency of beef cattle (Myer, 2017). Gastrointestinal tract microorganisms are not only important for synthesis of protein in the rumen but also are vital for immunological, metabolic, and physiological processes. Researchers, by re-examining these processes and

developing innovative methods, are able to mitigate methane emissions, enhance fibre digestibility and increase feed efficiency. Continuing investigations of microbial communities may permit identification of microbial markers, which will help to predict high production phenotypes (Myer, 2017).

1.3.2 Selection for behavior

Selection for behavior of farm animals is expected to help to improve production as well as product quality, labor costs and safe handling of the animals (Haskell et al., 2014). Behavioural traits have a heritability in the range of 0.23-0.36 similar to other production traits, suggesting that selection for this trait is possible. Farmers would hope to use behaviour traits on their farms with the goal to improve the characteristics of adaptability and decrease fearfulness and over reactivity in their cattle. However, there is some concern, from the perspective of the public, that modifying behaviour traits genetically is interfering with nature (Haskell et al., 2014).

1.3.3 Selection for resistance to diseases such as Bovine Respiratory Disease Complex (BRD)

Infections of the respiratory tract are a substantial problem in beef cattle, causing economic losses from death, medication costs, veterinary visits, low production, and increased labour costs (Neibergs, 2014). Beef industry success heavily depends on the health of the cattle, especially on their resistance to BRD, also called “shipping fever” due to its increased prevalence during and following shipping to feedlots. Estimated costs from treatment and lower gains in a 1000-cattle feedlot from BRD infection have been estimated at US $13.90 per animal (Snowder, 2006; Johnson, 2017).

There are a group of microorganisms, both viral and bacterial, which play a role in the pathogenicity of BRD making it a difficult disease to tackle. Parainfluenza-3 virus (PI3V), bovine viral diarrhea virus (BVD), bovine respiratory syncytial virus (BRSV), *Mannheimia hemolytica* (*M. hemolytica*), *Mycoplasma bovis, Pasterulla multocida, Histophilus somni* all contribute to BRD. BRD causes death, decrease in feed efficiency, reproduction, growth and productivity. The most economical and widely agreed upon opinion is that prevention is better than treatment to keep BRD costs low. Treatment of disease is costly, and it also contributes to public concerned about the overuse of antibiotics in food animals. Poor management and pathogens are the direct causes of BRD, and different approaches have been done in those areas. Lately, new approaches have been made; The BRC-CAP program was a 5-year-program with the goal to find cattle with less susceptibility to BRD. Identifying genetic associations with BRD and building a breeding system to increase the resistance of cattle to BRD can be beneficial. Neibergs et al. (2014) have reported different estimates of the heritability of resistance to BRD, depending on the method of analysis. In general, the estimates of the heritability in beef cattle were 17.7% for the binary case-control phenotype, and 29.2% when using disease scoring numerical values of the McGuirk system (that ranged from 0 to 12) as a semiquantitative definition of BRD (Neibergs, 2014). To date, most studies agree that genetic selection to reduce BRD should be possible.

1.4 The immune system

1.4.1 Brief overview of the immune system

Our environment contains a large number of pathogens, as well as antigens, that challenge our immune systems. It is therefore not surprising that the immune system uses a complex set of responses and protective mechanisms to combat various types of disease. The immune system is the main system in the body to control disease. Resistance, tolerance, robustness and resilience are terms commonly used to describe characteristics of immune systems, but they are often poorly defined. According to Colditz, disease resistance is the ability of the host immune system to limit or completely eliminate pathogens using behavioural, physiological, and immunological responses (Colditz, 2008).

Mammalian immune systems have two main arms commonly referred to as the innate and adaptive immune systems which are in actual fact integrated systems of host defence. These two arms work together and are highly connected as they cooperate using an elaborate array of cells and molecules to provide host protection. In general, the innate immune system is the first to respond to a foreign pathogen and since the recognition molecules utilized by this system are broadly expressed on cells and recognize common molecular patterns of pathogens the innate system is able to act rapidly. The innate immune system also includes physical barriers (e.g. epithelial cell layers with tight junctions), the mucus layer that covers the epithelium of the gastrointestinal, respiratory and genitourinary tracts, and epithelial cilia. There are also soluble proteins and molecules that are constitutively present as part of the innate

immune system, including complement proteins, and ficolins, as well as other molecules that are released after the cells producing them are activated such as cytokines, chemokines, inflammatory leukocytes, and enzymes.

The innate immune system recognizes a pathogen using pattern recognition receptors (PRRs) such as TLR1, TLR2, TLR4, TLR5, expressed on plasma membranes plus TLR3, TLR7, TLR8 and 9, RIG, NOD, which are in the cytosol (Hoebe, 2004). These receptors are able to bind to molecular patterns on the surface, as well as RNA or DNA inside of the pathogens. Macrophages and neutrophils are some of the leukocyte subsets that provide a first line of defense and are essential for combating common bacterial infections. However, there are pathogens that the innate immune system does not deal with efficiently. This may relate to the nature of the pathogen, the dose or site of entry. This is when the adaptive immune system is needed to clear the pathogen and provide greater protection against reinfection. It takes around 4 to 14 days for adaptive immune responses to start working effectively depending on primary or secondary exposure and the genetics of the host; during this period innate responses have important roles in controlling infection (Roth, 1993; Thompson-Crispi & Mallard, 2012).

The adaptive immune system needs specialized antigen presenting cells including dendritic cells (DCs) in order to be activated. These cells tend to be long-lived and reside in most tissues. DCs migrate to the regional lymph nodes where they meet naïve lymphocytes. As with neutrophils and macrophages, immature DCs have receptors that identify the common features of pathogens and after binding to pathogens, immature DCs engulf and degrade them intracellularly. Activated DCs also secrete cytokines and

chemokines that impact both innate and adaptive immune responses. These cells are critical for antigen presentation to lymphocytes. Adaptive immune responses to pathogens are more specific than the earlier innate responses. The adaptive system is composed of T- and B-lymphocytes with exquisite specificities that enable them to identify individual pathogens or invading microorganisms. These cells proliferate and mount an effective adaptive immune response in response to the specific antigen. A key feature of this adaptive system is long-lived memory cells that can replicate rapidly on re-exposure to the same antigen. Protective responses to vaccines, are at least in part, results of antibody-mediated immune response (AMIR, sometimes termed humoral immunity) and/ or sensitized T cells (cell-mediated immune responses, CMIR) (Janeway, 2002; Netea et al., 2019).

Activation of naïve T cells by antigen-presenting cells is the first crucial step in activation of adaptive immune responses, which occurs in lymphoid tissues. Antigen-specific naïve T cells only respond and become activated when antigen-presenting cells present specific antigen, and co-stimulatory signals occur via receptors B7.1 and B7.2. Macrophages, B cells and dendritic cells are the three cell types that can function as professional antigen presenting cells; dendritic cells activate naïve T cells the most potently; however, degradation of ingested antigen is less efficient in DCs compared to macrophages and neutrophils; reduced degradation of antigens by DCs help them conserve antigenic peptides and enhances their presentation on class I and II major histocompatibility complex (MHC) receptors (Hoebe, 2004). DCs take up the antigen, migrate to local lymphoid tissue, and during migration, they differentiate into mature

dendritic cells that express co-stimulatory molecules that enable them to activate antigen-specific naïve T cells; after activation naïve T cells become effector CD4+ T helper cells or CD8+ cytotoxic T cells that directly or indirectly clear the pathogen. CD8 cytotoxic T cells go to the site of infection and kill the infected cells directly. They bind to MHC class I and directly attack the cells that are infected with the pathogen. (Janeway, 2002; Netea et al., 2019). This CMIR is predominantly important in protection against intracellular pathogens. The presence of a CMIR after vaccination or infection indicates that expanded T-cell clones were able to recognize the specific antigen. There are several assays that can be used to identify CMIR, such as delayed type hypersensitivity (DTH) tests, cytotoxic T cell assays, and antigen specific T-cell cytokine response to name a few (Roth, 1993; Mallard et al., 2007; Hernandez, 2003).

Antibodies are globular immune proteins (immunoglobulin), specific for antigens in their native unprocessed conformation, occurring in plasma and extracellular fluid. They can bind to pathogens to assist in their destruction and can neutralize toxic products. B-cells produce antibodies in response to pathogens, but often require the assistance of antigen-specific T cells. Further to occur, helper T cells identify the antigenic peptide fragments presented by B cells. CD40L on T cells binds to CD40 on B cells leading to direct release of cytokines to support the ensuing immune response by the B cells. Helper T cells induce and direct the naïve B cells to differentiate to plasma cells and secrete antibody or differentiate to memory B cells, and throughout this differentiation antibody isotypes as well as antigen-binding properties of the antibody can be changed. CD4 helper T cells help to activate macrophages, T cells and B cells by secreting

cytokines. Some of these cells will stay in secondary lymphoid tissue, and others go to the site of infection to enhance macrophage and cytotoxic cell activity (Hoebe, 2014; Netea et al., 2019). After activation by T cells, B cells begin to respond by secreting IgM but quickly switch to different isotypes. IgM antibodies are pentameric and mainly reside in blood. IgM by binding to antigen efficiently initiate the complement cascade. IgG antibodies are bivalent and are found in blood and extracellular fluid; they have higher affinity and are capable of binding, and neutralizing toxins, viruses and bacteria, activating the complement system and opsonizing them for uptake by phagocytic cells. IgA antibodies have monomeric structure in blood and dimeric structure in the lamina propria of epithelial surface. For example, in the GI tract IgA cross the epithelia and enter the lumen of the gut to neutralize toxins and viruses. IgE antibody is able to reside on the surface of mast cells and eosinophils via Fc receptors; when antigen binds to the IgE، it triggers their degranulation and local defense or type I hypersensitivity reactions (Hoebe, 2014). The immune system is complex and only the briefest overview has been provided here.

1.4.2 Disease resistance, tolerance, robustness and resilience

These terms, disease resistance, tolerance, robustness and resilience have various definitions depending on the context in which they are used. For that reason, only a few examples will be provided here, as well as how these terms will be used in the context of disease and stress.

Animals that are able to maintain their productivity, even during a stress situation or disease, are defined as resilient (Bishop and Morris, 2007), and if they maintain resilient

during stress, such as a disease challenge then they are considered robust (Kitano, 2007). Disease resilience is a component of general livestock resilience, and as an animal is able to maintain its productivity and normal physiological functions in case of disease, it may also able to do the same in the case of environmental challenges, such as management and social challenges (Colditz, 2016). Disease tolerance, on the other hand, has been defined as those individuals that exhibit little damage from disease in spite of substantial pathogen load (Bishop and Morris, 2007). These individuals may continue to harbour or spread the disease but remain undetected as carriers of the disease unless some sort of diagnostic testing is performed.

According to Wilkie and Mallard, immuno-competence is “the ability of the body to produce an appropriate and effective immune response when exposed to a variety of pathogens” (Wilkie and Mallard, 1999). It is important that an individual mount the most efficient immune response to control a pathogen in order to limit pathological consequences of too strong or too weak a response. In terms of adaptive immunity, in order to efficiently clear intracellular pathogens, such as viruses or facultative intracellular bacteria, cells of the immune system have to attack infected cells and the pathogens residing inside - a process described as a CMIR. In contrast, to efficiently clear extracellular pathogens, such as bacteria, the body predominantly uses antibodies of various isotypes - a process described as an AMIR (Colditz, 2016). However, it should be noted that AMIR and CMIR generally work in combination to control and clear pathogens depending on the stage and nature of the infection. For example, during viremia AMIR may be the most important in controlling the infection, but once the virus

has established itself inside the host cell, then CMIR is required for clearance. Therefore, it is critical for the immune system to be able to have balanced responses consisting of both AMIR and CMIR. Disease resistance in this context, is when the immune system has controlled and cleared the pathogen. From a management perspective, this is the ideal situation rather than having individuals that carry or shed the pathogen but are not sick themselves. That is the reason that the High Immune Response (HIR™) technology strives for disease resistance versus disease tolerance. However, if an individual can be disease resistant and at the same time be adaptable to stressors, such as climate change, they are referred to as resilient in this context.

Consumer demand and changes in community attitudes have led to pressure to breed simultaneously for improvements in animal productivity, health and welfare. Ideally, these animals will have the ability to maintain high animal productivity and at the same time increase their health and welfare. Since both immune response and stress responses are under genetic control (Emam et al., 2019) it may be feasible to identify and select livestock that have both enhanced disease resistance and are adaptable to stressors such as climate change. This hypothesis will be addressed as part of this thesis.

1.4.3 The High Immune Response (HIR™) methodology

Selection based on general immune responsiveness first was done in Yorkshire pigs by measuring innate and adaptive immune responses and generating estimated breeding values (EBVs) that could be used to rank pigs as high, average and low immune responders (Mallard et al., 1992). The HIR™ technology was developed at the

University of Guelph and it is designed to identify animals with both high AMIR and CMIR, as well as aspects of innate host defence (figure 1). This technology has been applied to dairy cattle in order to identify the HIR animals that should be kept for future breeding to improve herd health, while the low immune responders, with their increased risk of disease, should be culled from the herd or managed according to their IR phenotype and if possible, not be used to breed future generations (Mallard et al., 2011).

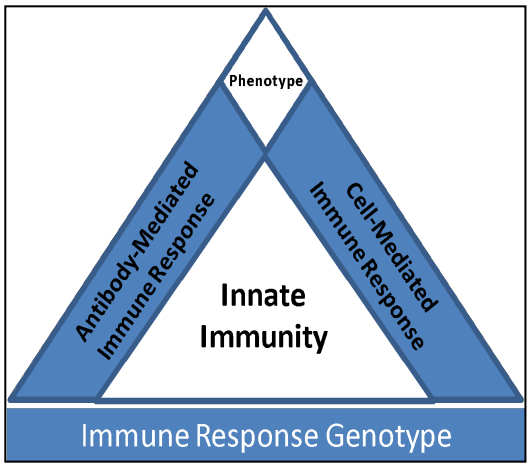


Figure 1.1: Genetic variation in the ability to resist disease is due to a large number of additive genetic effects, which together regulate innate and adaptive immune responses (Source: adapted from Wilkie and Mallard 1999)

1.4.4 HIR™ methodology and its effects on health

In subsequent breeding of high, average and low immune responder porcine lines it was possible to demonstrate that HIR pigs had greater antibody responses to the test antigen as well as to several commercial vaccines, and lower risk of disease (Wilkie and Mallard, 1999). Recent studies by the Mallard lab have confirmed that HIR pigs have

less mortality and also require less antibiotic treatment (Schmied et al., 2019, internal report). However, in one study, HIR pigs challenged with *Mycoplasma hyorhinis* had more severe arthritis compared to low responder pigs, suggesting that HIR pigs may be more susceptible to inflammatory responses (Magnusson et al., 1998). Nonetheless, it is important to consider that the same study also showed that high immune responder pigs had less severe pleuritis, pericarditis and peritonitis, and produced higher antibody responses faster to *M. hyorhinis* in comparison to low immune responder pigs (Magnusson et al, 1998). Overall, the mortality rate of the low responders was greater than that of the HIR pigs. In this case, some trade-offs needed to be considered in achieving better overall pig survival.

Studies also have been performed to assess immune responses of dairy cattle on both research and commercial dairy farms to investigate associations between immune response phenotypes and disease incidence (Heriazon, 2009; Thompson Crispi et al., 2014; Larmer and Mallard, 2017; Emam et al., 2019). Although this test system primarily evaluates adaptive immune responses, some features of the innate immune system required to initiate adaptive immunity may also be enhanced in HIR. Assays of macrophage phagocytic activity and nitric oxide production are currently under investigation in the Mallard lab and indicate that cattle can also be ranked based on nitric oxide production (Emam et al., 2019). HIR dairy cows have disease incidences of about half that of LIR (Mallard and Wilkie, 2007; Thompson Crispi et al., 2014; Larmer and Mallard, 2017). Dairy cattle classified as high responders experience improved quality of life, fewer incidents of the disease, and save producers a minimum of $125

per animal per lactation in health costs, including reduced use of antibiotics (Mallard et al., 2015).

Additionally, Cartwright et al. (2014) reported that high AMIR dairy cattle had a significantly lower occurrence of bacterial digital dermatitis in comparison to those with average and low AMIR. However, there was no significant difference among high, average and low CMIR cows (Cartwright et al., 2014). These benefits led Canada largest dairy genetics company, the Semex Alliance, to obtain a license from the University of Guelph in 2012, to utilize the HIR™ technology in its breeding program. Semex successfully markets this technology under the trade name Immunity+ (Larmer and Mallard, 2017). A genomics test for HIR™/Immunity+ is also now available as part of the Semex Elevate Program (Thompson-Crispi et al., 2012, Emam et al., 2019, https://www.dairybusiness.com/new-products-semexs-elevate-program-delivers-female-immunity-test/). Given the general success of the HIR™ technology as a natural method to improve inherent disease resistance of pigs and dairy cattle, there is increasing interest to determine if the technology could be adapted for use in beef cattle. Therefore, this will be one of the objectives of this thesis.

1.4.5 HIR™ methodology and effects on production traits

HIR pigs are reported to be more robust and reach market weight 10-12 days before low and average immune responders (Wilkie et al., 1999). More recently, Holstein heifers identified as HIR showed increased average daily gain (Aleri et al., 2016). These studies demonstrate that it is not only possible to improve livestock health traits by selecting for enhanced immune response, but that the high responders may have

various other fitness benefits including increased growth. Importantly these immune response traits are heritable (H^2= 0.25-0.35), and the beneficial genetic variants are passed to the next generation (Willkie et al., 1999; Mallard et al., 2016).

Genetic selection for improved livestock production has resulted in significant improvements in various production parameters. For example, large gains in milk production of dairy cows, muscle mass of beef cattle or leanness in pigs have been attributed to genetic selection (Miglior et al., 2005; Mallard et al., 2016). Additionally, it has been established that it is possible to improve both health and certain production and reproduction traits by selecting for enhanced immune responsiveness in pigs and dairy cattle (Larmer and Mallard, 2017; Thompson-Crispi et al., 2013). At the very least, it appears that there are no major detrimental associations between HIR™ and productivity.

1.5 Resilience of livestock in the face of climate change

A layer of natural gases such as water vapour, carbon dioxide, methane, nitrous oxide, and ozone, which trap heat and keep the surface of the earth warm, surrounds the earth. Among these gases carbon dioxide, nitrous oxide and methane have direct effects on climate change. Carbon monoxide, and the rest of these gases can influence climate change indirectly by participating in formation of greenhouse gases. However, with the population growing and more industrialization happening, more gases are being added to this layer, challenging the earth's capability to remove them; this consequently leads to what is called 'global warming'. According to the Intergovernmental Panel on Climate Change (IPCC), methane is the most powerful (20-fold higher) of these gases

to trap heat, and has increased in concentration 143% over the last two centuries (Ishler, 2008).

Cattle devote 2% - 12% of their gross energy intake to methane production (Uemoto et al. 2020). The cattle industry is intent on reducing this figure by improving cattle efficiency. According to Agriculture and Agri-Food Canada, 10% of Canada's greenhouse gases are emitted from crop and livestock production with methane and carbon dioxide being the largest proportion of these gases (Greenhouse gases, https://www.epa.gov/ghgemissions/sources-greenhouse-gas-emissions). Cattle gain heat from their environment and from heat generated during fermentation (digestion). Enteric fermentation is the main source of methane emission in Canadian livestock. This is not only important from a global warming perspective, but also has a significant value from a production perspective. Specifically, it illustrates how animals can be inefficient in use of their feed in terms of meat or milk production and waste their energy resources through methane emission. Recent research demonstrates the potential to improve feed efficiency in dairy cattle through genetic selection of those animals with inherently greater efficiency potential (Manafiazar et al., 2015).

Heat stress can have negative effects on production, performance and health of animals, when body heat cannot be dissipated to maintain normal body temperature (thermoregulation). According to Blackshaw, cattle are able to acclimatize to hot conditions, however, this process takes 2-7 weeks (Blackshaw, 1994), but some breeds such as Holsteins and Black Angus are particularly prone to heat stress and do not easily adapt (Blackshaw, 1994). The core body temperature of cattle is higher than the

ambient temperature and thus heat will flow from the animal to its surroundings. On hot days, animals dissipate heat through evaporation. Thus, panting and open-mouthed breathing can be a sign of heat stress in cattle (Samali, 1999).

High environmental temperatures, extreme weather, intense solar radiation and humidity can all have a negative influence on cattle performance and may make them susceptible to diseases (Hahn, 1985). Animals cope with hot weather better if the humidity is low. However, animals may experience heat stress in less extreme temperatures if the humidity is sufficiently high (Samali et al., 1999). After short exposure to heat, cells will enter a thermotolerant state and this helps them to be less sensitive to the otherwise lethal effects of a higher temperature (Samali et al., 1999), heavy metals, and anti-cancer drugs (Bhanuprakash et al., 2016). Animals exposed to heat stress will show a decrease in their food intake, productivity and growth efficiency. Gestation length and birth weight are also significantly reduced by hot weather. Heat stress also can activate latent viruses and make the environment suitable for a secondary bacterial infection (Hahn, 1985).

Cattle can regulate their body temperature; they can balance the heat produced through metabolism by the heat they dissipate to the surrounding environment (Bhanuprakash, 2016). Transcriptional activation of heat shock proteins (HSP) is one of the mechanisms suggested to alleviate heat stress at the cellular level. Among heat shock protein families, HSP70 is thought to have a critical role in cell heat stress responses (Bhanuprakash, 2016). HSP70 plays an important role in protection against environmental and physiological stress. HSP70 improves the overall cellular protein

integrity and inhibits apoptosis directly (Bhanuprakash, 2016). Some members of this family are constitutively expressed, while others are only expressed when there is hyperthermia, oxidative stress or a change in pH (Bhanuprakash, 2016). Animals within a herd will have different responses to heat stress; some are expected to be better able to adapt to the heat, whereas some will show heat stress signs; this raise the possibility of genetic differences in heat stress adaptation. Livestock with different immune response phenotypes have not previously been examined in the context of climate change and this will be the focus of the proposed research.

1.6 Summary of the problem and proposed research

BRD is a critical health and welfare issue in North America's beef industry. However, attempting to breed cattle only for resistance to this disease may leave them at risk of susceptibility to other diseases, such as economically important enteric infections. Selecting dairy cattle using the HIR™ methodology has been shown to produce improvements in broad-based disease resistance which may also benefit the beef industry if this method of selection can be adapted for use in the beef context. Furthermore, global climate warming and volatility is having negative effects on animal health and production. Public concerns about food safety and animal welfare are issues that need to be addressed, particularly in light of global warming. Therefore, adapting the HIR™ methodology to improve beef health and evaluating the high, average and low immune responder phenotypes in the context of the heat stress associated with climate change may lead to more resilient animals that are well adapted to shifting environmental challenges.

1.6.1 Hypotheses

1- The high immune response (HIR™) methodology can be applied to beef cattle allowing them to be classified as high, average or low immune responders.

2- Beef cattle classified as high immune responders are able to maintain their body temperatures better than average and low immune responders under heat stress condition.

3- Blood mononuclear cells (BMCs) from high immune responder beef cattle are more heat tolerant than those from average and low immune responders based on their *in vitro* production of HSP70 and nitric oxide.

1.6.2 Objectives

1- To determine if beef cattle of various ages and mixed breeds can be ranked based on their immune responsiveness using the high immune response (HIR™) methodology.

2- To determine the youngest age that beef cattle can be immune response phenotyped using the HIR™ method.

3- To determine if high immune responder beef cattle are able to maintain body temperatures (BT) better than average and low immune responders when the temperature humid index (THI) is 74 or greater.

4- To determine if BMCs *in vitro*, from high immune responder beef cattle, are more heat tolerant than average and low immune responders based on their production of HSP70 and nitric oxide.

1.6.3 Brief overview of methods

1- Measuring serum antibody-mediated immune response (AMIR) and cell-mediated immune response (CMIR) based on Delayed-Type Hypersensitivity (DTH) will be based on the University of Guelph's patented HIR™ methodology described previously (Thompson Crispi et al., 2012). Experiments will focus on adapting this method for use in the context of beef cattle production systems.

2- Rectal temperatures of beef cows at the University of Guelph Elora Research Station with different immune response phenotypes will be measured every other week (n=12 per week) from June – September 2018 (twice a day am and pm) to determine if variation exists within this population to regulate body temperatures when THI is above normal (74 or greater).

3- Heat Shock Protein 70 and nitric oxide concentrations will be used here to evaluate the effects of heat stress on cells from high, average and low immune responders under *in vitro* conditions using commercial test kits.

4- Statistical analysis: SAS or other appropriate statistical packages will be used to identify different immune phenotypes and evaluate any differences between these phenotypes.

2 Immuno-phenotyping of Canadian Beef Cattle: Adaptation of the High Immune Response (HIR™) Methodology for Utilization with Beef Cattle

2.1 Abstract

Animal health and welfare are important features of highly productive herds. Animal diseases result in loss of appetite, weight loss, and ultimately lower production with economic costs to the producer. Different management approaches have been used to reduce livestock disease occurrence; however, few focus on improving animal health genetics. The High Immune Response (HIR™) methodology measures the genetic performance of the two arms of the adaptive immune system to identify and breed animals with balanced and robust immunity. The HIR™ methodology has previously been used in dairy and swine to improve disease resistance but it has not been investigated in beef cattle. Therefore, the objective of the current study was to examine whether the HIR™ technology could be adapted for use in Canadian beef cattle industry and to determine the earliest age for immune response phenotyping of beef calves. In the dairy industry, cows are usually bred throughout the year and their calves are accessible year-round. However, in North America, beef cattle are typically bred so that their calves are born in the Spring during a narrow calving period and then dispersed to pasture where they are no longer conveniently accessible. Consequently, testing calves before they go to pasture is one option. In this study, beef calves (n = 295) of various ages, as well as mature beef cows (n=170) of mixed breeds, were immunized using type 1 and type 2 test antigens to assess their antibody-mediated immune response

(AMIR) and cell-mediated immune response (CMIR). Results of this study indicated that the patented HIR™ technology can be adapted for use in beef cattle, and that beef calves as young as 2-3 weeks of age are capable of mounting a comparable AMIR response to mature cows. However, at 3 weeks of age, the CMIR response had not matured to that of older cattle, although by 9 months of age the CMIR was equivalent to that of mature cows. Therefore, HIR™ testing can be used to measure AMIR as early as 2-3 weeks of age, but calves should be older before testing CMIR.

Key words: beef cattle, high immune response, health, antibody and cell-mediated immune response

2.2 Introduction

In the past, selection of livestock mainly focused on production traits with less attention on health traits. However, more attention is paid to animal health and welfare recently, particularly in light of consumer concern about food management systems and antibiotic treatment of livestock (Tirado et al., 2010). Vaccination and prevention management are solutions to help improve animal health. Nonetheless, vaccine efficacy can still be a challenge for certain complex diseases, such as bovine respiratory disease (BRD) (Anholt et al., 2017). Genetic selection is another approach to enhance livestock health. Studies show selective breeding of dairy cattle for balanced, superior and robust immune responses not only reduces the incidence of disease but also improves the quality of their milk and colostrum (Thompson-Crispi et al., 2014; Fleming et al., 2016; Stear et al., 2017; Emam et al., 2019), as well as certain reproduction and growth traits (Mallard and Wilkie, 2007; Aleri et al., 2015; Thompson-Crispi et al., 2012). Additionally,

Mallard and Wilkie (2007) reported that high immune responder (HIR™) pigs reach market weight (100 kg) 10-12 days faster than low immune responders. Aleri et al. (2015) reported similar findings in their study of Australian Holstein heifers in that high immune responders had higher daily weight gain compare to low immune responders.

Using HIR™, dairy cattle and pigs with superior immunity have been identified and bred for these heritable health traits (Mallard et al., 2015). This technology ranks animals by measuring the response of both arms of the adaptive immune system - antibody (AMIR) and cell-mediated immune responses (CMIR), and classifies individuals as High (H), Average (A) or Low (L) immune responders. Thompson-Crispi et al. (2012, 2014) reported that the incidence of disease in HIR™ dairy cattle is about half of that of low immune responders. Additionally, these HIR are able to pass this fitness trait to the future generation with a heritability similar to the trait of milk production (approximately 0.20 to 0.35, Thompson-Crispi et al., 2012; Larmer and Mallard, 2017). The HIR™ technology is utilized by the Semex Alliance under the trade-name of Immunity+. Daughters of the HIR™/Immunity+™ dairy sires have lower incidence of disease with no adverse effects on production (Mallard et al., 2015; Larmer and Mallard, 2017).

Given the health benefits of selecting dairy cattle using the HIR™ method, the objective of the current study was to determine if the HIR™ methodology could be adapted for use in Canadian beef cattle of mixed breeds, as well as to determine the earliest age for immune-phenotyping beef calves. It is expected that adapting the HIR™ methodology and breeding for inherited health traits in beef cattle will lower the incidence of important production diseases, such as BRD. The hypothesis of this study was that mature beef

cattle of mixed breeds, as well as young calves, could be immune-phenotyped for both AMIR and CMIR using HIR™ methodology.

2.3 Materials and Methods

2.3.1 Immuno-phenotyping of beef cows and calves

In 2016, 151 crossbred beef calves (Black Angus, Red Angus, Simmental and Piedmontese crosses) born at the Elora Beef Research Station (operated by the University of Guelph) were immune phenotyped at either 3 weeks, or 9 months of age. The dams of calves in this study were part of a concurrent nutritional study (6 different treatment groups). To evaluate AMIR and CMIR, calves were tested according to the patented HIR™ method (patent # US7258858B2) using an antigen preparation containing both type 1 and type 2 antigens in adjuvant as described previously (Thompson-Crispi et al., 2013) given at approximately 3 weeks of age (n= 76 calves at 21-27 days of age, mean of 24 days, standard deviation (SD) of 1.68 days) OR at approximately 9 months of age (n= 75 calves at mean of 8.5 months, SD of 0.54 months). Calves were selected to be tested at 3 weeks or 9 months of age using systematic random allocation within each of the maternal nutritional groups. HIR™ testing can be performed only once in the life of an animal, to avoid anamnestic responses. The objective of this experiment was to evaluate whether calves could be tested using the established HIR™ protocol as young as 3 weeks of age by comparing their AMIR and CMIR responses to those of older calves of about 9 months of age, as well as to those of mature cows.

Specifically, heifer and steer calves beginning at either 3 weeks or 9 months of age were immunized intramuscularly on day 0 to induce CMIR and AMIR to type 1 and 2 test antigens. CMIR was measured based on cutaneous delayed-type hypersensitivity (DTH) response to the type 1 test antigen with an intradermal injection (into skin of the tail fold) on day 14, and double skin fold thickness (DSFT) measured on day 15 (24 hours after intradermal injection). AMIR was measured using an enzyme-linked immunosorbent assay (ELISA) measuring serum antibodies to the type 2 test antigen on days 0 and 14. The positive control for the ELISA was pooled sera from previously immunized cows and the negative control was fetal bovine serum. DTH data were considered in models of CMIR response as previously described by Thompson-Crispi et al. (2012).

In 2017, 144 crossbred beef calves (Black Angus, Red Angus, Simmental and Piedmontese crosses) born at the Elora Beef Research Station were immune phenotyped at either 1 week, 2 weeks, 3 weeks, or 9 months of age. The objective of this experiment was to evaluate whether calves could be tested using the established HIR™ protocol at a younger age than 3 weeks of age, by comparing their AMIR and CMIR to those of older calves of around 9 months of age, as well as those of mature cows. Briefly, heifer and steer calves were tested according to the patented HIR™ method as described above using an antigen preparation containing both type 1 and type 2 antigens given at 1week of age (n= 48 calves at 2-7 days of age, mean of 4 days and SD of 1.77 days), 2 weeks of age (n= 16 calves at 11-16 days of age, mean of 13 days and SD of 2 days) OR 3 weeks of age (n= 49 calves at 21-26 days, mean of 22.55

days and SD of 1.35 days), OR 9 months of age (n= 47 calves at mean of 8.5 months and SD of 0.54 months).

In both years, 2016 and 2017, some cow-calf pairs were on pasture over the summer and some remained in open-shed housing. These effects were evaluated in the statistical model as described below.

Additionally, in order to immune phenotype fully mature cows as another point of comparison to younger animals, 170 mixed breed (Black Angus, Red Angus, Simmental and Piedmontese crosses) mature beef cows were tested, 126 of them being the dams of the calves born in 2017. Cows were tested as described above during October-November 2017, 5-6 months after their calves were tested with the HIR™ system.

The dams of both the 2016 and 2017 calves were also part of a nutritional study with the objective to keep cows on a less nutritious diet in winter and add supplements to their feed during spring. There were 6 nutrition groups, and, in each year, calves born to cows within each nutritional group, were allocated to HIR test groups by systematic random allocation. Nutritional effects on immune response of the dams were evaluated as part of the statistical model as described below.

Finally, historical immune response data on mature Canadian Holstein cows (n=3304) from 71 herds across Canada, phenotyped as part of a previous study (Thompson-Crispi et al., 2012, 2014) were used to compare AMIR and CMIR among age groups and between breeds.

2.4 Statistical Methods

The Kruskal-Wallis non-parametric test in GraphPad Prism version 8 for macOS (GraphPad Software, La Jolla California USA, www.graphpad.com) was used to calculate p-values for descriptive statistics. An optical density from the ELISA test for detection of antibody, of less than 0.089 was considered a non-response based on 2 SD above the mean of day 0 of beef cows' AMIR responses. For CMIR, an increase in double skin-fold thickness of less than 40% was considered as a non-response based on intradermal testing of non-sensitized calves.

A SAS (SAS®/STAT, 1999) General Linear Model (GLM) was used to examine fixed effects influencing AMIR and CMIR of beef cattle of varying ages as follows:

$$y_{ijklmnopq} = \mu + \alpha \times c_i + ba_j + ra_k + si_{l.} + pi_m + a_n + n_o + g_{p+} p_q + e_{ijklmnopq},$$

where $y_{ijklmnopq}$ = CMIR or AMIR; μ = population mean; α is the regression coefficient; c_i= control site of CMIR or day zero background of AMIR at day 0 as a covariate; ba_j = effect of black Angus (number of Black Angus out of 32 possible progenitors (data from bioTract-http://agsights.com/what-is-go360-biotrack/, was used to generate the progenitors going back 5 generations); RA_k = effect of Red Angus (number of Red Angus out of 32 possible progenitors); SI_l = effect of Simmental (number of Simmental out of 32 possible progenitors); PI_m = effect of Piedmontese (number of Piedmontese out of 32 possible progenitors); a_n = effect of age (where k goes from 1-5 ; 1-week, 2-week, 3-week, 9-month and mature beef cows); n_o = effect of nutrition (where l is

nutrition group and goes from 1 to 6 depending on the diet); g_p = effect of gender (where m is 1 or 2 as male or female) ; p_q = effect of pasture (where n is 0 or 1 as barn versus pasture), and $e_{ijklmnopq}$ = residual error.

Variables with P > 0.05 were removed from the model. Results were considered to be statistically significant if P ≤ 0.05. Interactions were tested and remained in the model if P < 0.05. Least-squares means (LSmeans) were used to indicate the different AMIR and CMIR responses of beef calves, and mature beef cows.

Subsequently, another SAS (SAS®/STAT, 1999) GLM was used to examine fixed effects influencing AMIR and CMIR of beef cattle of varying ages with Holstein cow data added to the model as follows:

$y_{ijklmn} = \mu + \alpha \times c_i + b_j + a_k + n_l + g_m + p_n + e_{ijklmn}$, with variables described as above with Holstein added to the age effect.

Proc Univariate (SAS®/STAT, 1999) was used to check the normality of all data sets (optical density and the double skin fold thickness measurements were $\log_{10}$ transformed).

2.5 Results

2.5.1 Descriptive statistics and analysis of variance

Immuno-phenotyping of mature beef and dairy cows

Mature beef cows (n = 170) of mixed breeds, immuno-phenotyped during October – November 2017, utilized for age and breed comparisons had minimal antibody to the type 2 test antigen on day 0. (Mean Optical Density=0.029, Median Optical Density=0.021, SD=0.030) indicating that these animals had not been previously exposed to this test antigen.

On day 14, there was a wide range of serum antibody responses in mature cows, including 43.52% of cows that were considered non-responders (an optical density below a cut off of 0.089 was considered to indicate non-response to immunization, based on 2SD above the mean serum antibody concentration on day 0 of mature beef cows). The remaining responder cows had a range of different responses to the test antigen from OD = 0.089 - 3.067 (Mean OD= 0.695, Median=0.406, SD=0.667). Results comparing AMIR of all mature beef cows (responders and non-responders) with Holstein dairy cows (responders and non-responders) showed that Holsteins had significantly higher AMIR than beef cows ($P< 0.0001$, Kruskal-Wallis non-parametric test, Holsteins mean = 0.578, median = 0.340, SD = 0.624, beef cows mean = 0.417, median = 0.128, SD = 0.601, Table 2.1).

Table 2.1: Descriptive statistics (arithmetic mean, median and standard deviation of responders plus non-responders animals, as well as the percent non-responders) of serum IgG antibody-mediated immune response (AMIR) in 3-week- and 9-month-old mixed breed beef calves (born in 2016) compared with mature beef and Holsteins on day 14 following immunization with an HIR™ type 2 antigen

AMIR **2016 calves**	3-week-old[1] beef calves n = 76	9-month-old[2] beef calves n = 75	Mixed breed mature beef cows[3] n = 170	Holstein cows (historical data set)[4] n = 3308
Percent non-responders[5]	27.60	20.00	43.50	38.30
Mean optical density[6]	0.586	0.673	0.417	0.578
Median optical density[7]	0.318[A]	0.434[A]	0.128[B]	0.340[A]
Standard deviation	0.689	0.658	0.601	0.624

[1]The 15-day test protocol was begun at 21 to 27 days of age
[2]The 15-day test protocol was begun at a nominal age of 9 months
[3]Beef cows tested in October and November of 2017

[4] Holstein cows tested in 71 herds in Prince Edward Island, Nova Scotia, New Brunswick, Quebec, Ontario, Alberta and British Columbia in November 2012-November 2016 as part of previous study (Mallard et al., 2018)

[5] Percentage of tested cattle with a corrected optical density less than background value of 0.089

[6] Optical density (enzyme-linked immunosorbent assay, with corrections for blank wells, and plate effects)

[7] Groups with different superscripts are significantly different ($P < 0.05$, Kruskal-Wallis non-parametric test)

In response to the CMIR type 1 test antigen, 7.64 % of beef cows were considered non-responders based on DTH responses that did not differ from background (increase in DSFT < 40% is considered background based on intradermal injection of non-sensitized calves) (Hernández et al., 2003). The remaining responder cows had a wide range of response from 40 - 503.2% (Mean Percent Increase of DSFT = 120.45, Median = 104.1, SD = 63.13). Results comparing CMIR of all beef cows of mixed breeds (responders and non-responders) with Holstein dairy cows (responder and non-responders) showed that beef cows had significantly higher CMIR responses ($P< 0.0001$, Kruskal Wallis non-parametric test, Holstein Mean Percent Increase of DSFT = 74.13, median = 62.0, SD = 58.89, Table 2.2).

Table 2.2: Descriptive statistics (arithmetic mean, median and standard deviation of responders and non-responders animals) of cell-mediated immune response (CMIR) in 3-week- and 9-month-old mixed breed beef calves (born in 2016) compared with mature beef cows of mixed breeds and Holstein dairy cows on day 15 following challenge with an HIR™ type 1 antigen

CMIR 2016 calves	3-week-old[1] beef calves n=76	9-month-old[2] beef calves n = 75	Mixed breed beef cows[3] n = 170	Holsteins (historical data set)[4] n = 3308
Percent non-responders[5]	23.68	36.00	7.64	23.97
Mean percent increase[6]	87.20	99.51	113.04	74.13
Median percent increase[7]	72.65^{A}	76.20^{A}	99.00^{B}	62.00^{A}
Standard deviation	70.74	85.47	66.01	58.89

[1]The 15-day test protocol was begun at 21 to 27 days of age

[2]The 15-day test protocol was begun at a nominal age of 9 months

[3]Beef cows tested in October and November of 2017

[4] Holstein cows tested in 71 herds in Prince Edward Island, Nova Scotia, New Brunswick, Quebec, Ontario, Alberta and British Columbia as part of previous study (Mallard et al., 2018)

[5] Percentage of tested cattle with an increase in double skin fold thickness less than 40 percent

[6] Double skin fold thickness measured before type I test antigen injection and 24 hours after that

[7] Groups with different superscripts are significantly different ($P < 0.05$, Kruskal-Wallis non-parametric test)

Immuno-phenotyping test results of calves born in 2016

Antibody-mediated immune responses of calves born in 2016

At 3 weeks of age all calves (n = 76) had minimal antibody to the type 2 test antigen on day 0 prior to immunization (Mean Optical Density (OD) = 0.018, median = 0.014, SD =

0.015, Figure 2.1). On day 14, there was a wide range of AMIR response, including 27.63% of calves that were considered non-responders. Day 14 antibody response to the test antigen, based on OD, for the rest of the calves (responders) ranged from OD = 0.091 - 2.858 (Mean OD = 0.792, Median = 0.582, SD = 0.709, Table 2.1).

Nine-month-old calves born in 2016 (n = 75), also had minimal antibody concentration on day 0 (Mean OD = 0.025, Median = 0.022, SD = 0.022). On day 14, there was a range of AMIR with 20.0% classified as non-responders based on 2SD above the mean of day 0 of mature beef cows (0.089). Day 14 antibody to the test antigen, based on optical densities, for the rest of calves (responders) ranged from OD = 0.089 - 2.179 (Mean OD = 0.829, Median = 0.529, SD = 0.648, Table 2.1).

Serum antibody responses on day 14 of calves tested at 3 weeks of age (responders and non-responders) did not differ significantly from those calves tested at 9 months of age (P = 0.28, Kruskal – Wallis nonparametric test, Mean OD of 3-week-old calves = 0.586, Mean OD of 9-month-old calves = 0.673, Table 2.1, Figure 2.8 appendix). No significant difference was observed between responses of heifers and steer calves tested at 3 weeks (P = 0.52) and 9 months of age (P = 0.40).

Comparing 3-week-old, 9-month-old calves and mature beef cows (responders and non-responders) indicated that there were higher AMIR responses in 3-week-old calves (P = 0.01), and 9-month-old calves (P = 0.0001) compared to mature beef cows (Table 2.1, Figure 2.8 appendix). However, when the historical data from Holstein cows (n = 3308, phenotyped using the same method) was used as a point of reference to

compare immune responses of 3-week- and 9-month-old beef calves with Holstein cows, there were no significant differences among these groups. Among these 3 age groups AMIR values were as follows on day 14 (responder and non-responder): (P = 0.320, calves tested at 3 weeks old mean OD= 0.586, median = 0.318, SD = 0.689; calves tested at 9 months of age mean = 0.673, median = 0.434, SD = 0.658, and Holstein cows mean = 0.578, median = 0.340, SD = 0.624, Kruskal – Wallis nonparametric test, Table 2.1, Figure 2.8 appendix appendix).

Cell-mediated immune responses of calves born in 2016

Three-week-old calves' CMIR results using the type 1 test antigen indicated that 23.68% of calves had DTH responses that did not differ from background. The remaining calves (responders) exhibited a wide range of DTH from 40-348.8% (mean percent increase of DSFT = 106.36, Median = 84.95, SD = 70.5) (Table 2.2).

Nine-month-old calf CMIR results indicated that 36% were non-responders. The remaining calves had a wide range of DTH responses from 40-333.8% (mean percent increase of DSFT = 129.60, Median = 109.15, SD = 78.06, Table 2.2).

There was no significant difference between calves tested at 3 weeks of age (responder and non-responder) with those tested at 9 months of age (responder and non-responder) in CMIR response (P = 0.54, Kruskal – Wallis nonparametric test). Results comparing 3-week-old and 9-month-old beef calves, with mature beef cows indicated that 3-week- and 9-month-old calves had significantly lower CMIR (P < 0.0001, Kruskal-Wallis non-parametric test, Table 2.2). However, results comparing CMIR among 3-

week- and 9-month-old calves with Holstein cows indicated that there was no difference in CMIR response among them (Table 2.2). There were also no significant differences in CMIR between heifer and steer calves tested at 3 weeks and 9 months of age.

Immuno-phenotyping test results of calves born in 2017

Antibody-mediated immune responses of calves born in 2017

In 2017, all calves tested for AMIR response regardless of age had minimal serum antibody to the type 2 test antigen on day 0 (Mean OD of calves tested on day 0 at 1-week-old = 0.018, Median = 0.011, SD = 0.017).

One-week-old calves on day 14 (n = 32) had a range of antibody responses, including the highest proportion of calves that were non-responders (53.12%). The rest of the calves tested at 1 week of age had relatively low amounts of antibody and a narrow range of responses from OD = 0.089-1.051 (Mean OD = 0.334, Median = 0.252, SD = 0.268) that were significantly ($p > 0.05$) lower than those of older animals (Table 2.3).

Two-week-old calves on day 14 (n =16) also had a relatively narrow range of antibody responses, including 33.3% of calves that were non-responders. The rest of calves tested at 2 weeks of age had OD ranging from 0.089-0.608 (Mean OD = 0.656, Median = 0.649, SD = 0.351) which were similar to those of older animals but with lower SD (Table 2.3).

Table 2.2: Descriptive statistics (arithmetic mean, median and standard deviation of responders and non-responders animals) of serum IgG antibody-mediated immune response (AMIR) in 1-, 2-, and 3-week- and 9-month-old mixed breed beef calves (born in 2017) compared with mature beef cows of mixed breeds and Holstein cows on day 14 following immunization with an HIR™ type 2 antigen

AMIR 2017 calves	1-week-old[1] beef calves n = 32	2-week-old[2] beef calves n = 16	3-week-old[3] beef calves n = 49	9-month-old[4] beef calves n = 47	Mixed breed beef cows[5] n = 170	Holsteins (historical data set)[6] n = 3308
Percent non-responders[7]	53.10	33.30	12.20	25.50	43.50	38.30
Mean optical density[8]	0.173	0.507	0.520	0.564	0.417	0.578
Median optical density[9]	0.059[A]	0.409[B]	0.366[B]	0.346[B]	0.128[C]	0.340[B]
Standard deviation	0.237	0.101	0.492	0.653	0.601	0.624

[1] The 15-day test protocol was begun at 2 to 7 days of age

[2]The 15-day test protocol was begun at 11 to 14 days of age

[3]The 15-day test protocol was begun at 21 to 27 days of age

[4] The 15-day test protocol was begun at a nominal age of 9 months

[5] Beef cows tested in October and November of 2017

[6]Holstein cows tested in 71 herds in Prince Edward Island, Nova Scotia, New Brunswick, Quebec, Ontario, Alberta and British Columbia as part of previous study (Mallard et al., 2018)

[7] Percentage of tested cattle with a corrected optical density less than or equal to background value of 0.089

[8]Optical density (enzyme-linked immunosorbent assay, with corrections for blank wells, and plate effects)

[9]Groups with different superscripts are significantly different ($P < 0.05$, Kruskal-Wallis non-parametric test)

Three-week-old calves on day 14 (n = 49) had a range of AMIR, including the 12.25% of calves that were non-responders. The rest of the calves tested at 3 weeks of age had antibody responses ranging from OD = 0.089-1.012 (Mean OD = 0.587, Median = 0.418, SD = 0.490) which were similar to those of older animals (Table 2.3).

Results comparing 1, 2 and 3-week-old calves (responders and non-responders) indicated that there were significant differences between 1-week-old and 2-week-old beef calves ($P = 0.003$), and 1-week and 3-week-old calves ($P < 0.0001$) in antibody response, with 1-week-old calves having the lowest AMIR. However, responses of 2-week-old and 3-week-old calves did not differ significantly ($P > 0.99$, Kruskal-Wallis non-parametric test, Table 2.3, Figure 2.9 appendix).

Nine-month-old calves (n=47) tested in 2017 had a range of AMIR, including 25.53% of calves that were non-responders. The rest of the calves had antibody responses from OD = 0.089-2.488 (Mean OD = 0.743, Median = 0.470, SD = 0.669, Table 2.3) and these responses were greater than those of mature beef cows but not different from those of mature Holsteins (Table 2.3).

Combined responses across both years are shown in Figure 10 appendix. There were no significant differences between 3-week-old and 9-month-old beef calves in either year compared to mature Holstein cows, but mature beef cows continued to have the lowest AMIR among these groups.

In 2017 calves, there was a trend toward higher AMIR in heifers than steers tested at 3 weeks of age ($P = 0.06$, mean OD = 0.606, median = 0.490, SD = 0.478 for heifers;

mean OD = 0.422, median = 0.232, SD = 0.500 for steers). Additionally, heifers tested at 9 months of age had significantly higher AMIR compared to 9-month-old steers (P = 0.003, heifer calves tested at 9 months Mean OD = 0.760, Median = 0.473, SD = 0.677, steers tested at 9 months Mean OD = 0.375, Median = 0.125, SD = 0.582, Figure 2.11 appendix).

Overall, these results show that 1-week-old calves had immature antibody responses that were significantly lower than those of cattle tested at other ages. However, there were no significant differences in AMIR between calves tested at 2 and 3 weeks of age or 9 months of age. Nonetheless, since calves born in 2017 (but not 2016) and immuno-phenotyped at 2 weeks of age (2-week-old calves were not tested in 2016) had the lowest variation in AMIR (OD SD = 0.101) it is best to wait to evaluate antibody responses until beef calves are 3 weeks of age using this test system.

Cell-mediated immune responses of calves born in 2017

One-week-old calf CMIR responses to the type 1 test antigen indicated that 34.3% of calves (n = 32) had DTH responses that did not differ from background and were considered non-responders. The remaining calves had a limited range of DTH responses from 40-177.2% (mean percent increase of DSFT =89.97, Median = 84.59, SD = 36.74 of all 1-week-old calves) (Table 2.4). These calves had significantly lower CMIR than 9-month-old calves and mature beef animals, but their responses were not different from mature dairy cows (Kruskal – Wallis nonparametric test) (Table 2.4).

Table 2.4: Descriptive statistics (arithmetic mean, median and standard deviation of responders and non-responders animals) of cell-mediated immune response (CMIR) in 1-, 2-, and 3-week old, and 9-month-old mixed breed beef calves (born in 2017) compared with mature beef cows of mixed breeds and Holstein cows on day 15 following challenge with an HIR™ type 1 antigen

CMIR 2017 calves	1-week-old[1] beef calves n = 32	2-week-old[2] beef calves n = 16	3-week-old[3] beef calves n = 49	9-month old[4] beef calves n = 47	Mixed breed beef cows[5] n = 170	Holsteins (historical data set)[6] n = 3308
Percent non-responders[7]	34.37	68.75	44.90	4.25	7.64	23.97
Mean percent increase[8]	66.40	38.90	44.80	172.67	113.04	74.13
Median percent increase[9]	59.00^{A}	33.80^{A}	42.20^{A}	150.10^{B}	99.00^{B}	62.00^{A}
Standard deviation	7.91	7.72	36.80	102.76	66.01	58.89

[1] The 15-day test protocol was begun at 2 to 7 days of age

[2]The 15-day test protocol was begun at 11 to 14 days of age

[3]The 15-day test protocol was begun at 21 to 27 days of age

[4] The 15-day test protocol was begun at a nominal age of 9 months

[5] Beef cows tested in October and November of 2017

[6]Holstein cows tested in 71 herds in Prince Edward Island, Nova Scotia, New Brunswick, Quebec, Ontario, Alberta and British Columbia as part of previous study (Mallard et al., 2018)

[7] Percentage of tested cattle with an increase in double skin fold thickness less than background value of 40

[8]Double skin fold thickness measured before type I test antigen injection and 24 hours after that

[9]Groups with different superscripts are significantly different ($P < 0.05$, Kruskal-Wallis non-parametric test)

-week-old calves' CMIR results indicated that 68.75% of calves (n = 16) had DTH responses that did not differ from background and were considered non-responders. The remaining calves had a limited range of DTH responses from 40- 108.3 (mean percent increase of DSFT = 75.2, Median = 70.1, SD = 26.9). Similar to the 1-week-old calves, these calves had significantly lower CMIR than 9-month-old and mature beef animals, but their response was not different from mature dairy cows (Kruskal – Wallis nonparametric test) although the SD was lower than dairy (SD 7.7 versus 59%) (Table 2.4).

Three-week-old calf CMIR results indicated that 44.89% of calves (n = 49) had DTH responses that did not differ from background and were considered non-responders. The remaining calves had a wider range of DTH responses than 1- and 2-week-old calves from 40-219.5 (mean percent increase of DSFT = 68.99, Median = 59.7, SD = 35.35). Similar to the 1- and 2-week-old calves, these calves had significantly lower CMIR than 9-month-olds calves and mature beef animals, but the variation in response was beginning to increase (SD = 37% versus 7.9 and 7.7% in 1-and 2-week- old calves). Similar to 1- and 2-week-old calves, CMIR in 3-week-old calves did not different from responses of mature dairy cows (Kruskal – Wallis nonparametric test) (Table 2.4).

Nine-month-old calf CMIR results indicated that very few animals (4.24% of 47 calves) were considered non-responders. The responding calves had a wider range of DTH responses than 1-, 2- and 3-week-old calves, from 40-217.9 (mean percent increase of DSFT = 179.52, Median = 156.60, SD = 99.70). The CMIR of 9-month-old beef calves did not differ significantly from those of mature beef cows, but their responses were

significantly higher than those of mature dairy cows (Kruskal – Wallis nonparametric test) (Table 2.4).

Overall, comparing results of CMIR in 1-week, 2-week, and 3-week-old calves (responders and non-responders) in 2017 indicated that there were no significant difference in CMIR responses among them, but these young calves all had significantly lower CMIR compared to the beef calves tested at 9 months of age ($P < 0.0001$) and mature beef cows ($P < 0.0001$, Kruskal-Wallis non-parametric test), although by 3 weeks of age the standard deviation in CMIR was beginning to increase (Table 2.4). In 2016, CMIR did not differ significantly between 3-week- and 9-month-old calves suggesting that it might be possible to test CMIR in calves as young as 3 weeks of age, but it is better to wait until the animals are older to ensure maturity of this response.

In both 2016 and 2017, there were no significant differences in CMIR between heifers and steers tested at 3 weeks of age ($P = 0.55$) and heifers and steers tested at 9 months of age ($P = 0.44$). The only difference between heifer and steers was for AMIR in 2017 where heifers had significantly higher antibody at both 3-week- and 9-month-old immune-phenotyping.

2.5.2- General Linear Model Analyses

Comparing AMIR and CMIR of beef cattle of various ages

Antibody-mediated immune response

The model for AMIR was significant ($p<0.0001$) and accounted for ($R^2 = 0.16$) 16% of the total variation in this trait. In agreement with the descriptive statistics, results comparing least squares means (LSmeans) of calves tested at 1, 2, 3 weeks, and 9 months of age with mature beef cows of mixed breeds indicated that 1-week-old calves had lowest AMIR responses. Specifically, the AMIR response at 1 week of age was significantly lower than those of 2-week-, 3-week-, and 9-month-old-calves (Figure 2.2). The AMIR of 2-week-, 3-week-, and 9-month-old-calves were significantly higher than those of mature beef cows. However, there was not any significant difference between 1-week-old calves with mature beef cows even though the 1-week-old calves had the lowest mean antibody. The standard deviation of AMIR in mature beef cattle was relatively high (0.097-3.067) and likely accounts for the lack of significance with 1-week old calves (Table 2.5, Figure 2.2).

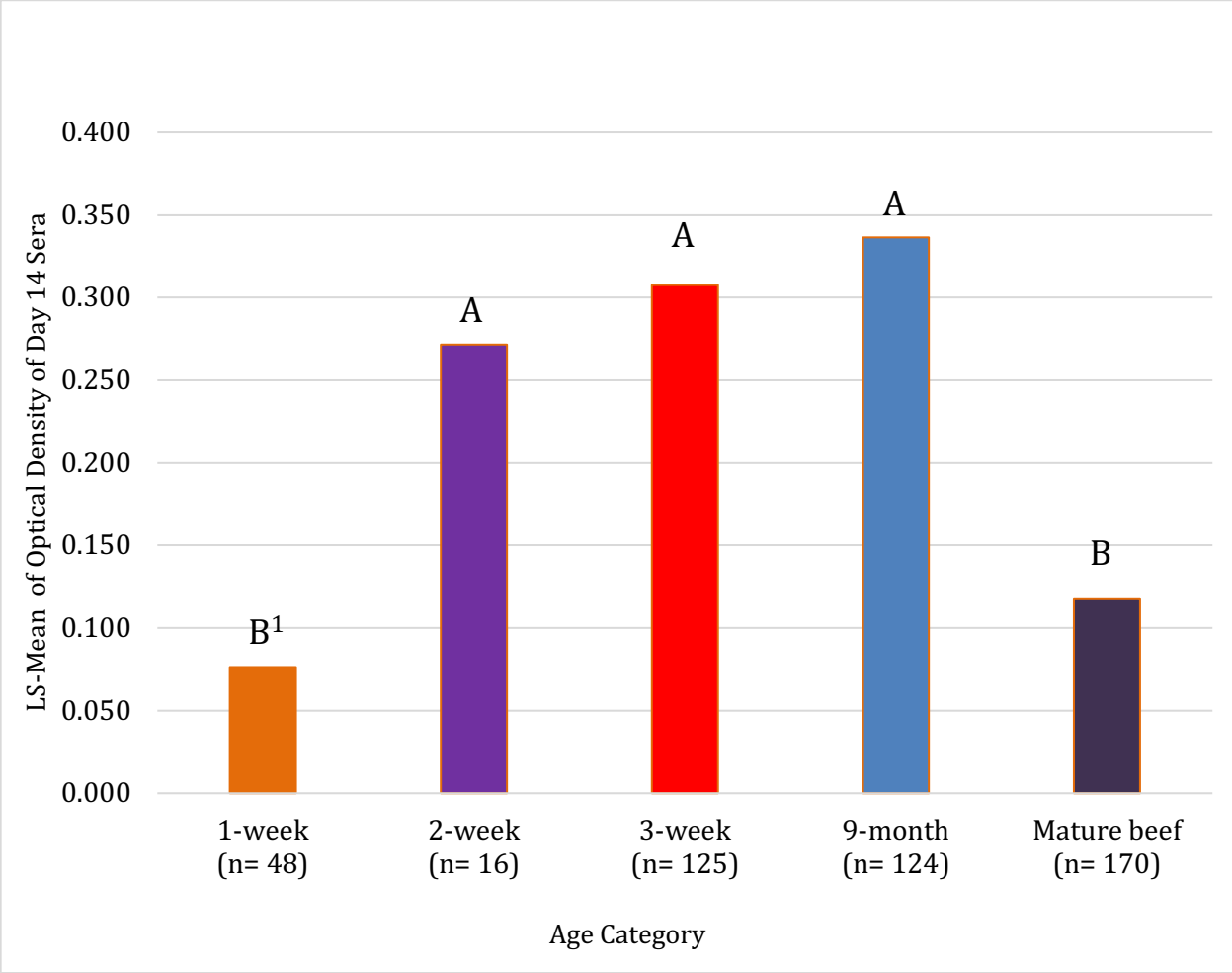
Table 2.5: General linear model for antibody-mediated immune response (AMIR) on day 14, in all age groups (1-, 2-, 3- weeks, and 9-months-old-calves with mature beef cows) by number of Angus progenitors, out of 32 possible progenitors (data from bioTrack* for the progenitors going back 5 generations, with low (0-9 progenitors), medium (10-19 progenitors)

P-Values

Variable	Model	AMIR Day 0	Age	Angus Category	Angus Category* Age	R^2
Log_{10} AMIR Day 14	< .0001	< .0001	<.0001	0.5543	0.0100	16%

*bioTract is designed to collect and help analyze different types of data generated on farm (http://agsights.com/what-is-go360-biotrack/)

Figure 2.2: Least squares mean comparisons of antibody-mediated immune response (AMIR), based on ELISA optical density of day 14 sera, in 1-, 2-, 3-week-old, and 9-month-old mixed beef breed calves born in 2016 and 2017 with mature beef cows following immunization with an HIR™ type 2 antigen

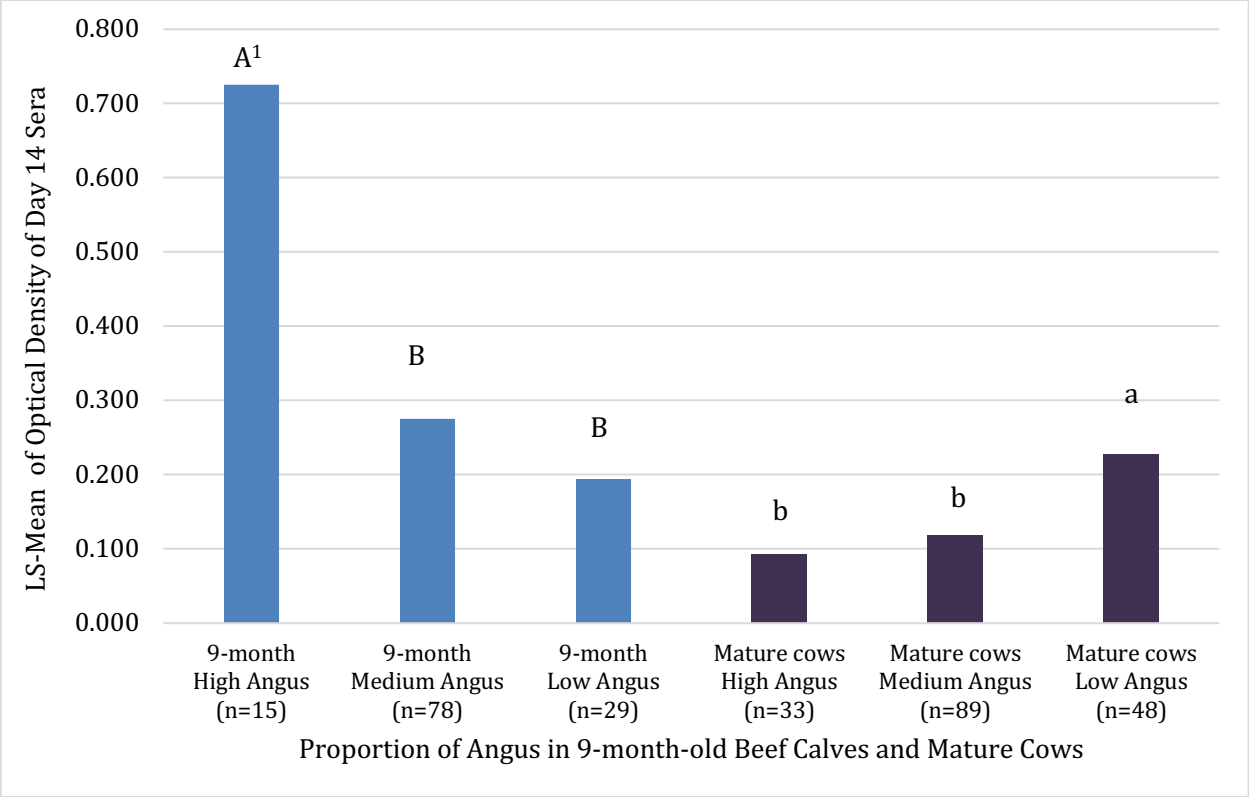


[1] Columns with the same letter do not differ significantly; columns with different letters differ significantly at $p<0.05$ (SAS General Linear Model)

After observing a significant interaction between the age of testing and proportion of Angus (P = 0.009, number of Black Angus progenitors, out of 32 possible progenitors (data from bioTract for the progenitors going back 5 generations, with low (0-9 progenitors), medium (10-19 progenitors), high (20 – 29 progenitors)), LSmeans for the interaction were examined within each age category separately (1, 2, and 3 weeks, 9-month, mature beef cows).

No effect of proportion of Angus was observed in 1- week, 2-week, and 3-week-old calves. However, 9-month-old calves with the High proportion of Angus had significantly higher AMIR response compared to those with Medium (P = 0.01) and Low (P = 0.003). In contrast, mature cows with Low proportion of Angus had significantly higher AMIR response compared to High (P = 0.005) and Medium (P = 0.01, Figure 2.3). There was no interaction with age and proportion of other breeds in the model.

Figure 2.3: Least squares mean comparisons of antibody-mediated immune responses (AMIR), optical density day 14 in sera, in 9-month-old mixed breed beef calves (born in 2016 and 2017) and mature beef cows with number of Angus progenitors, out of 32 possible progenitors (data from bioTrack[2] for the progenitors going back 5 generations, with low (0-9 progenitors), medium (10-19 progenitors) and high (20-29)



[1] Capital letters indicate comparison among 9-months old beef calves; lower case letters indicate comparison among mature beef cows. Within each group, columns with the same letter do not differ significantly; columns with different letters differ significantly at $p<0.05$. (SAS General Linear Model)

[2]bioTrack is designed to collect and help analyze different types of data generated on farm (http://agsights.com/what-is-go360-biotrack/)

Cell-mediated immune responses

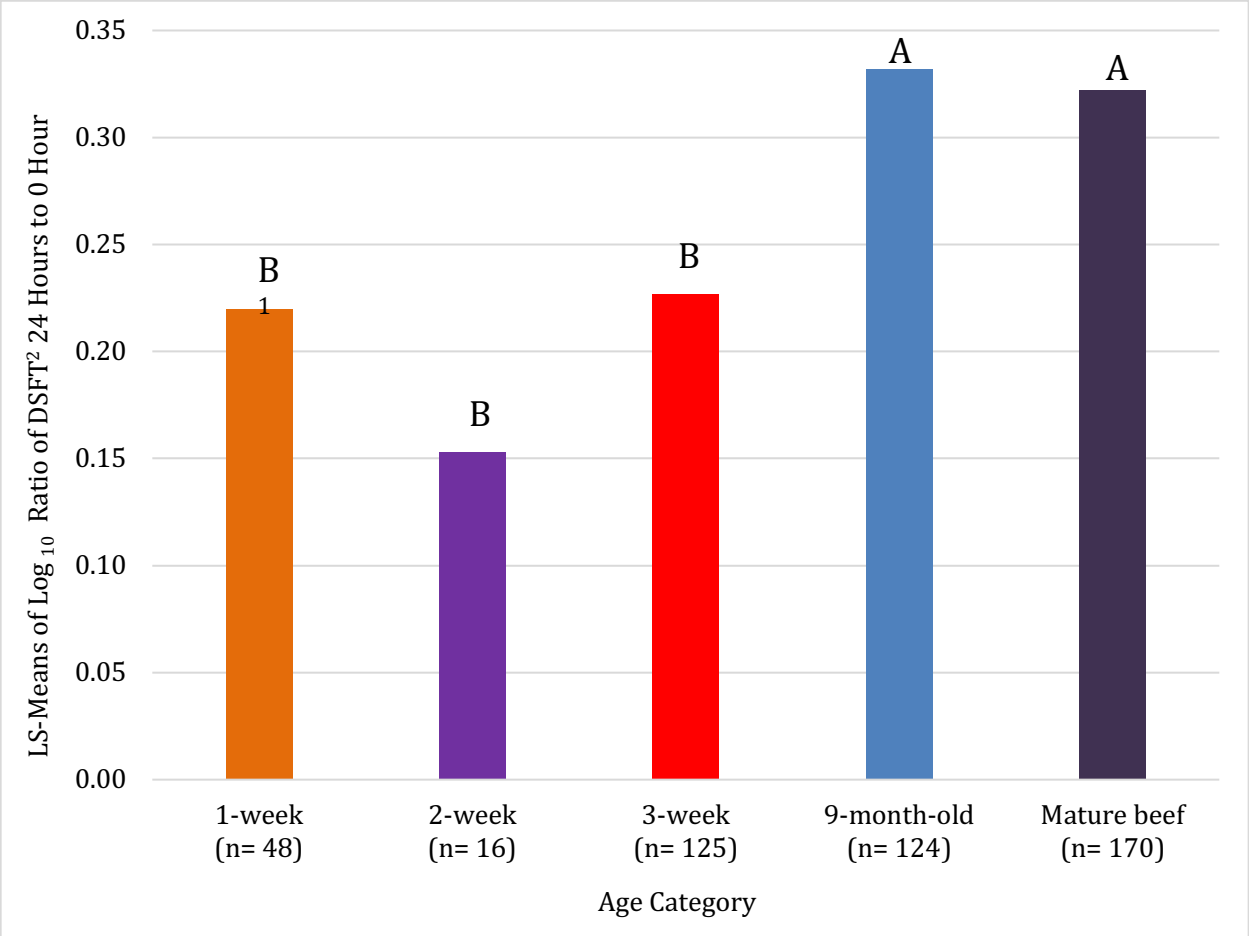
The model for CMIR was significant ($p<0.0001$) and accounted for ($R^2= 0.18$) 18% of the total variation in this trait. Age was significant in the CMIR analysis ($P < 0.0001$). LSmeans of CMIR results indicated that 1, 2, and 3-week-old calves had similar CMIR, however the responses were significantly lower than those of 9-month-old and mature beef cows (Table 2.6, Figure 2.4). There was a significant interaction between age and birth year (age category * birthyear); among calves born in 2016, CMIR in calves tested at 3 weeks and 9 months of age were similar, but among calves born in 2017, calves tested at 9 months of age had significantly higher CMIR than those tested at 3 weeks of age (Figure 2.7).

Table 2.6: General linear model for cell-mediated immune response (CMIR) on day 15, for age effect (1-, 2-, 3- weeks, and 9-months-old-calves with mature beef cows)

P-Values

Variable	Model	log_{10} control	Age	R^2
Log_{10} increase double skin fold thickness	<.0001	<.0001	< .0001	19%

Figure 2.4: Least squares mean comparisons of cell-mediated immune responses (CMIR). CMIR was measured based on cutaneous delayed-type hypersensitivity (DTH) to the type 1 test antigen with an intradermal injection (into skin of the tail fold) on day 14, and double skin fold thickness (DSFT) measured on day 15 (24 hours after intradermal injection), in 1-, 2-, 3-week-old, and 9-month-old mixed breed beef calves (born in 2016 and 2017) and mature beef cows on day 15 following immunization with an HIR™ type 1 antigen



[1] Columns with the same letter do not differ significantly; columns with different letters differ significantly (SAS General Linear Model)

[2]DSFT = Double Skin Fold Thickness as indicator of cell-mediated immune response

Comparing AMIR and CMIR of beef cattle of various ages with Holstein cows

Antibody-mediated immune response

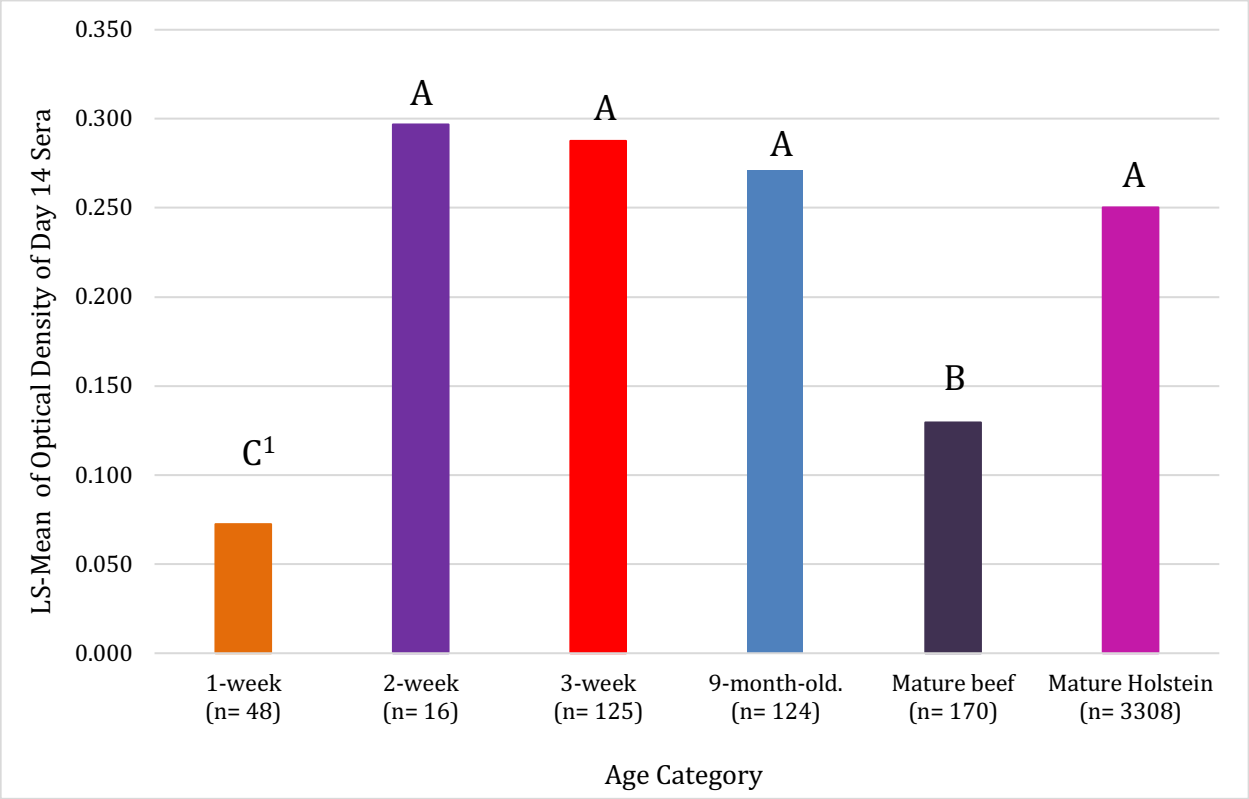
The model for AMIR was significant ($p<0.0001$) and accounted for ($R^2= 0.089$) 9% of the total variation in this trait. Results comparing LSmeans of calves tested at 1, 2, 3 weeks, and 9 months of age with mature beef cows of mixed breeds and Holstein cows indicated that 1-week-old calves had lowest AMIR response. Specifically, the AMIR response at 1 week of age was significantly lower than those of 2-week-, 3-week-, and 9-months-old-calves, as well as mature beef cows and Holstein dairy cows (Table 2.11, Figure 2.5). The AMIR of 2-week-, 3-week-, and 9-month-old-calves were significantly higher than mature beef cows, however similar to those of the Holstein cows. Additionally, there was a significant difference between mature beef cows and Holstein cows ($P<0.0001$). Holstein cows had significantly higher AMIR response than mature beef cows. Overall, AMIR of 1-week old calves was low, and it is recommended to test AMIR after calves are at least 3 weeks of age (Table 2.7, Figure 2.5).

Table 2.7: General linear model for antibody-mediated immune response (AMIR) on day 14, for age effect (1-, 2-, 3-weeks, and 9-months-old-calves with mature beef cows and mature Holstein cows)

P-Values

Variable	Model	AMIR Day 0	R^2
AMIR Day 14	<.0001	<. 0001	8%

Figure 2.5: Least squares mean comparisons of antibody-mediated immune response (AMIR), optical density day 14 sera, of 1-, 2-, and 3-week-old, and 9-month-old mixed breed beef calves (born in 2016 and 2017) with mature beef and Holstein cows on day 14 following immunization with an HIR™ type 2 antigen



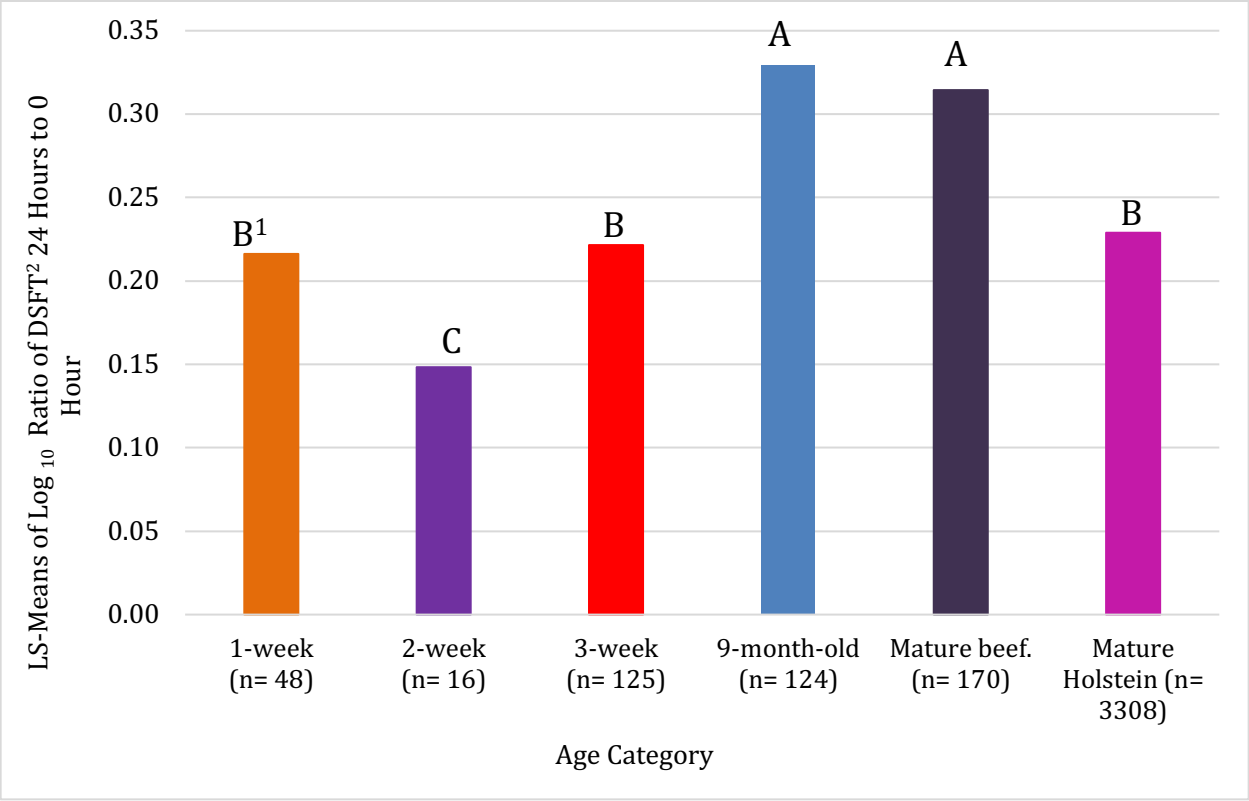
[1] Columns with the same letter do not differ significantly; columns with different letters differ significantly (SAS General Linear Model)

Cell-mediated immune response

The model for CMIR was significant ($p<0.0001$) and accounted for ($R^2= 0.088$) 9% of the total variation in this trait. Testing age was significant ($P<0.0001$). LSmeans of CMIR results indicated that 1 and 3-week-old calves had similar CMIR; 2-week-old calves had a significantly lower CMIR response compared to 1-week and 3-week-old calves (Figure 2.6). However, they all had a significantly lower CMIR from 9-month-old calves and

mature beef cows indicating that the calf immune system is not mature enough to mount a CMIR response similar to older animals. CMIR of mature beef cows was significantly higher than CMIR of Holstein cows (Table 2.8, Figure 2.6). Overall, and in agreement with the descriptive statistics, the CMIR response of calves had not fully matured by 3 weeks of age and therefore immuno-phenotyping of this trait should be performed in older animals.

Figure 2.6: Least squares mean comparisons of cell-mediated immune responses (CMIR), log_{10} ratio of double skin fold thickness 24 hours to 0 hour, in 1-, 2-, and 3-week-old, and 9-month-old mixed breed beef calves (born in 2016 and 2017) with mature beef and Holstein cows on day 15 following immunization with an HIR™ type 1 antigen



[1] Columns with the same letter do not differ significantly; columns with different letters differ significantly (SAS General Linear Model)

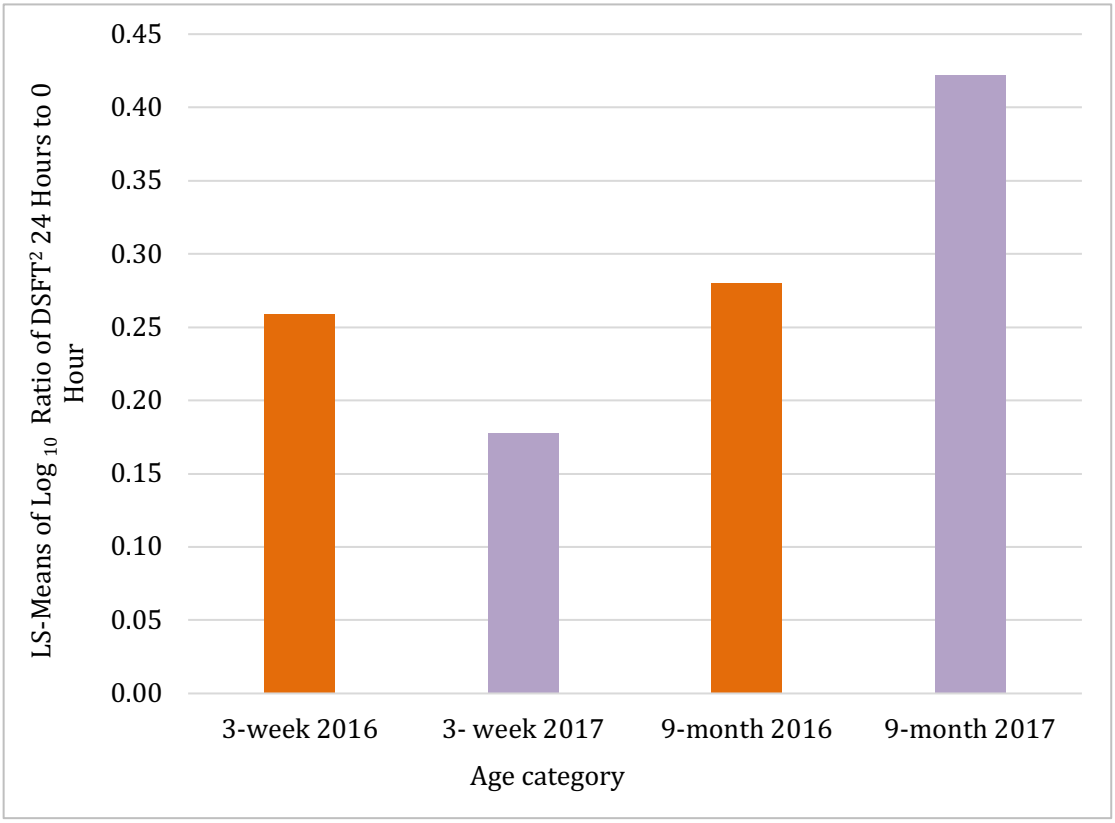
[2] DSFT = Double Skin Fold Thickness

Table 2.8: General linear model for cell-mediated immune response (CMIR) on day 15, for age effect (1-, 2-, 3-weeks, and 9-months-old-calves with mature beef cows and mature Holstein cows)

P-Values

Variable	Model	Log_{10} control	Age	R^2
Log_{10} increase double skin fold thickness	<.0001	<.0001	< .0001	8%

Figure 2.7: Least squares mean comparisons of cell-mediated immune responses (CMIR), log_{10} ratio of double skin fold thickness 24 hours to 0 hour, in 3-week-old, and 9-month-old mixed beef breed calves born in 2016 and 2017 on day 15 following immunization with an HIR™ type 1 antigen



[1] Columns with the same letter do not differ significantly; columns with different letters differ significantly (SAS General Linear Model)

[2] DSFT = Double Skin Fold Thickness

2.6 Discussion

Various approaches have been taken to reduce livestock disease incidence, some of them focusing on improving health using genetics, facilitating permanent improvements that can be passed on to future generations. The HIR™ methodology utilized in both research and commercial dairy herds permit selection of individuals with robust and balanced immune responses in order to improve health and welfare of animals (Mallard et al., 2015; Larmer and Mallard, 2017). Although the HIR™ test method has previously been applied in dairy, this test method has not been fully validated for beef cattle. Therefore, the objective of this study was to determine if the HIR™ method could be applied and optimized for use in beef cattle of mixed breeds at various ages. In dairy, the youngest age the HIR™ testing has been carried out is 2 months of age and therefore the current study had an objective to determine how young beef calves could be immuno-phenotyped. Since dairy cows are bred throughout the year and their calves are born year-round, testing in dairy cattle can be performed at any time. However, in traditional beef management systems, beef calves are born in springtime and are maintained on pasture during the summer making testing, particularly of older animals a challenge. Consequently, the goal of finding the youngest age that beef calves could be immuno-phenotyped prior to their departure to pasture was a relevant one. According to the literature, calves of younger ages are often not able to mount an effective mature immune response (Hodgins and Shewen, 2012; Barrington, 2001) which was another reason for evaluating calves of various ages, even as young as 1 to 3 weeks of age. Results from the current study found that calves in the first week of life are too young to

generate mature AMIR and CMIR utilizing the HIR™ test system. However, 2 -and 3-week-old beef calves generated AMIR responses comparable to 9-month-olds and mature animals, but CMIR should be measured in calves older than 3 weeks of age.

Following birth, calves depend on the passive immunity they receive from their dams to activate and regulate their immune responses, as well as to passively fight infection (Chase et al., 2008). Passively transferred colostrum may have immuno-regulatory roles in neonates, and the presence of antigen-specific antibodies is known to suppress active antibody responses of neonatal calves (Chase et al., 2008). The results of the current study indicated that calves tested at 1 to 3 weeks and 9 months of age had minimal serum antibody concentration to the test antigens prior to immunization on day 0, thus maternal antibodies were unlikely to affect antibody responses in these calves. Additionally, dams of these calves that were tested with the HIR™ protocol in 2017; (5 to 6 months after calving) also did not have antibodies to the test antigen in day 0 sera. Furthermore, the type 2 antigen was chosen so that environmental or immunization exposure of cattle to this antigen is unlikely.

Based on the literature, fetal calves have all the components of the adaptive immune system (Wilson et al., 1995). However, their immune system is still naïve; the number of immune cells is low as well as their functionality (Wilson et al., 1995; Simon et al., 2015). Additionally, in late gestation and near to birth, concentrations of corticosteroids increase in the fetus to initiate parturition. Because of higher concentrations of cortisol in neonates, near birth and after, the phagocytic activity and other immune functions remain low for up to 10 days after birth (Barrington and Parish 2001; Hodgins and

Shewen, 2012). Corticoid levels reach a maximum (193-331 nmol/l) at birth and return to basal levels approximately 10 days after birth (normal level of corticosteroids in lactating cows is 14-28 nmol/l) (Hodgins and Shewen, 2012).

At the outset of this study, 3 weeks was chosen as the age for testing young beef calves for practical reasons to help ensure calves would not be on pasture in a commercial herd. The age at which calves can respond to a particular antigen will vary with the antigen, the dose and the adjuvant (Kirkpatrick et al. 2008). Therefore, immune responses were evaluated in the context of the established HIR™ test system. Immuno-phenotyping results indicated that calves tested at 3 weeks were able to mount AMIR similar to those of 9 months old calves and Holstein cows, and comparable or higher than those of mature beef cows. The ANOVA confirmed that beef calves as young as 2-3 weeks of age could be effectively tested for AMIR using the HIR™ method.

Results of CMIR in 2016 calves (responders and non-responders), indicated that those animals tested at 3 weeks of age had similar responses (Mean DSFT 87%) to 9-month-old (Mean DSFT 99%) and Holstein cows (Mean DSFT 74%), but that the responses were significantly lower than those of mature beef animals (Mean DSFT 113%). In the 2017 cohort of calves, 1- week (Mean DSFT 66%), 2- week (Mean DSFT 39%) and 3-week-old calves (Mean DSFT 45%) calves had significantly lower CMIR than 9-month-old calves (Mean DSFT 173%) and mature beef cows (Mean DSFT 113%), but these responses were not different from mature dairy cows (Mean DSFT 74%). Mature beef animals in commercial testing for Immunity+™ have been seen to have higher CMIR DSFT than dairy (Semex Alliance, Personal Communication), and this was also seen

here. This is likely why the beef calf responses were similar to mature dairy but lower than mature beef cattle. To ensure that beef calf CMIR is fully mature it is recommended to test animals older than 3 weeks of age.

Differences in CMIR between years may be due to various environmental effects, including nutrition. For example, since this study was conducted in the university research herd parallel studies are not uncommon. In the current study, there was a nutritional study in progress in the dams of calves born in 2016 and 2017, with the nutrition that their dams received being different in these years. However, the ANOVA indicated that the effects of nutrition were not significant and were removed from the final model. Nonetheless, other studies have reported dietary effects on immune response, especially cell-mediated immunity (Marcos et al. 2003).

Immuno-phenotyping of beef calves, particularly those as young as 1 week of age for antibody or 1 to 3 weeks of age for CMIR, emphasized the concern of an immature immune system since these responses were consistently lower than those of other age groups. These results are consistent with the literature regarding the immature and naïve immune system of neonates. Although lymphocytes from fetal calves can respond to stimulation with mitogens by 188 to 253 days gestation (Wilson et al., 1995), yet the immune system does not appear to be fully functional until 2-4 weeks after birth to make a mature-like immune response (Tierney, 1997). Additionally, during the neonatal period (0-10 days after birth) lymphocytes and monocytes are substantially lower in numbers ($3.5x10^9$/litre and $0.4x10^9$/liter respectively) than in mature cows (7.8×10^9 / liter, and 0.8×10^9 / liter) (Knowles et al., 2000). Neonatal calves also have approximately 30%

lower B cell counts, and only by 20 days after birth do counts reach adult levels (Barrington and Parish, 2001). The type of antigen exposure and adjuvant will no doubt also be influencing factors.

Additionally, cytokines and other immuno-regulatory factors such as microRNAs, transferred with colostrum to neonates may also modulate the immune system (Okada et al., 2010; Hodgins and Shewen, 2012; Emam et al., 2019). The results of the current study confirm the immaturity of the bovine neonatal immune system, specifically at 1 week of age for AMIR, and 1-3 weeks of age for CMIR using the HIR™ immuno-phenotyping method.

Finally, a difference in AMIR was noted between heifers and steers tested in 2017, with females having a higher AMIR than males. However, this sex difference was not observed in 2016, nor were they significant in the GLM. Heifer calves tested at 3 weeks and 9 months of age had significantly higher antibody to the type 2 test antigen compare to the steers. No sex difference was observed in CMIR in either year. These differences between males' and females' antibody responses have been reported previously and are likely influenced by hormonal differences (Furman et al., 2013; Dasgupta et al., 2008; Bouman et al., 2005; Xia et al., 2009).

Results also indicated a significant interaction between age category and proportion of Angus. Nine-month-old calves with High proportion of Angus had significantly higher AMIR compared to those with Medium and Low. In contrast, mature cows with Low proportion of Angus had significantly higher AMIR compared to those with Medium and

High. Engle et al. (1998) in their experiment, determining the effects of breed on immune response of Angus and Simmental calves inoculated with infectious bovine rhinotrachitis virus via the intranasal route, indicated that production of cytokines and consequently fever was higher in Angus calves compared to those of Simmental calves. They also observed a breed*time interaction in antibody production following injection of these calves (Angus and Simmental) with pig red blood cells after which the total IgG titer in Angus calves reached its peak 7 days after injection compared to Simmental with peak response at 14 days after injection (Engle et al., 1998). Together these results indicate that differences in immune responses among beef breeds is not unprecedented.

2.7 Conclusion

New and novel approaches that do not rely on antibiotics are essential to improve animal health and wellbeing. The HIR™ methodology has been shown effective for immuno-phenotyping of dairy and swine. This method can be used to classify individuals based on their ability to make AMIR and CMIR, with those having the highest adaptive immune responses having the lowest occurrence of infectious disease. However, the HIR™ methodology had not previously been evaluated in beef cattle. Therefore, the objective of this study was to test the ability of this system to immuno-phenotype beef cattle of mixed breeds and determine the youngest age at which HIR™ testing could be used to assess immune function of beef calves. The results indicated that immuno-phenotyping for AMIR can be performed as early as 2-3 weeks of age, but evaluation of CMIR needs to be done in beef calves older than 3 weeks of age. If the

goal is to immuno-phenotype simultaneously for both of these adaptive immune response traits it needs to be done in calves older than 3 weeks of age. In this study, AMIR responses at 3 weeks and 9 months old calves were significantly higher than mature cattle. Testing for CMIR may be possible before 9 months of age but ages between 1 to 9 months would need to be evaluated. Therefore, more study is required to evaluate CMIR in calves older than 3 weeks and younger than 9-months of age to determine the earliest time point for immuno-phenotyping.

2.8 Acknowledgements

The field work would not have been possible without the help of University of Guelph Elora Beef Research Station staff. The Arrell Food Institute is gratefully acknowledged for their support. Nasrin Husseini was funded by an Arrell Food Scholarship and an Ontario Veterinary College Scholarship. The research was support by Canadian First Research Excellence Fund (CFREF) and Food from Thought (FFT) funding to Dr. Mallard.

3 Regulation of Body Temperature in Canadian Beef Cattle of Various Immune Response Phenotypes when the Temperature Humidity Index is Above Seasonal

3.1 Abstract

Climate change with increases in ambient temperature and humidity can decrease livestock production and reproduction potential, as well as increase disease susceptibility. Previous research confirms that as the temperature-humidity index (THI) increases animals are not able to regulate their body temperature efficiently, or to effectively dissipate body heat. This leads to an increase in their core body temperature and respiration rate, which in turn not only makes them uncomfortable, but also decreases their production, reproduction, and immune function. The High Immune Response (HIR™) technology, which has been developed at the University of Guelph ranks animals based on their immune response capacity as high, average, and low immune responders. HIR™ Holstein cattle have been reported to have fewer incidents of disease compared to average and low immune responders. Besides having a robust immune response, high immune responders have been reported to have better colostrum and milk quality making immune-phenotyed cattle an ideal model to examine the effects of global warming on health traits. In this study, rectal temperatures of 36 beef cows with known immune phenotypes were recorded during normal THI (THI < 74) and above normal THI (THI ≥ 74) once in the morning and once in the afternoon to determine effects of heat stress. Results indicated that 64.29% and 39.52% of cows showed increases in rectal temperature above normal based on 1 or 2 SD from the mean respectively, when THI was 74 or greater (a body temperature of 39.3°C and

higher was considered above normal in the current study based on 2SD above the mean rectal temperature on days when THI was less than 74). A significant interaction was observed between antibody-mediated immune response (AMIR) and THI (AMIR*THI). Average and Low AMIR beef cows had significantly higher rectal temperatures during THI ≥ 74. No significant difference was observed in rectal temperatures of High AMIR beef cows during THI < 74 and THI ≥ 74, indicating that the high AMIR beef cows in this study were better able to regulate their body temperatures when THI ≥ 74. There were no significant differences among cows with different cell-mediated immune response phenotypes. Beef cows with heavier weight had significantly higher rectal temperatures. Furthermore, young beef cows also had significantly higher rectal temperatures compared to older cows.

3.2 Introduction

Increased outdoor temperature, particularly when accompanied by high humidity, has been reported to adversely affect animal health making cattle more susceptible to disease resulting in loss of appetite, weight loss, and ultimately lower production, with economic costs to the producer (Bhanuprakash et al., 2016). Global warming is expected to intensify this effect. Cattle like other mammals are endotherms; they have the ability to generate their own heat and should be able to maintain a stable internal body temperature regardless of the external temperature. They regulate their internal body temperature by balancing the amount of heat produced during metabolism with the amount of heat dissipated to the surrounding environment through sweat and

respiration (Bhanuprakash et al., 2016). The normal body temperature of cattle (38.6-39.1°C) usually is higher than the environmental temperature (Hansen 2015; Horowitz 1998) and this results in heat flowing from the body to the environment (Collier et al., 2006; Webster 1974). Radiation, conduction, and convection are means that cattle use to dissipate body heat. However, as the environmental temperature rises these methods are less effective and evaporation will be the only effective way to dissipate heat in a hot and humid environment (West 2003; Blackshaw and Blackshaw, 1994).

In tropical parts of the world, some cattle breeds such as *Bos indicus* Brahman cattle are well adapted to heat stress (Bhanuprakash et al., 2016). However, in cooler climates such as Canada, where global warming is occurring faster than in the southern hemisphere, cattle are not well adapted to heat stress (Sidortsov et al., 2015). Poor ability to regulate body temperature results in diversion of energy resources toward thermoregulatory processes to decrease body temperature thereby reducing the energy resources for production, reproduction and growth (Howard et al., 2014). Even a few degrees of deviation in body temperature can have deleterious effects on cattle (Rolf, 2015).

Temperature and humidity both impact heat stress and are often combined as the temperature-humidity index (THI). Animals are able to endure higher temperatures if the humidity is low, however as the humidity increases, it also dramatically increases the risk of heat stress (Mader et al., 2006). The literature indicates that a temperature-humidity index (THI) ≥ 74 for beef cattle is considered detrimental (Nardone et al., 2010; Gaughan et al., 2002). Lower THI values of 68-72 have been reported to adversely

affect Holstein dairy cattle (Habeeb et al., 2018; Polsky et al., 2017). Dikmen and Hansen 2009 also showed that THI is a reliable indicator of heat stress. Animals that produce more or absorb more heat are at the highest risk (Rolf 2015; West 2003). According to Blackshaw and Blackshaw (1994), it takes 2-7 weeks for an animal to acclimatize to hot conditions and cattle are more susceptible than humans to high temperature and humidity.

During heat stress, open-mouthed breathing, as well as panting, are obvious signs of cattle affected by heat. These cattle will seek shade and will have excessive salivation and foam around the mouth (West, 2003). Planning ahead to identify and mitigate heat stress can improve animal well-being and performance. Therefore, identifying cattle that are able to maintain core body temperature during heat stress is advantageous, particularly in areas of the world where animals are not well adapted to this type of stress.

Effects of high THI have not been thoroughly investigated in Canadian beef cattle, particularly in the context of cattle with diverse immune response phenotypes that effect disease resistance. Therefore, the objective of this study was to examine the effects of heat stress on core body temperature among beef cattle classified as High, Average, and Low immune responders during Canadian summers when THI is highest.

3.3 Material and Methods

Thirty-six beef cows of mixed breeds (Black Angus, Red Angus, Simmental and Piedmontese crosses) with diverse immune response phenotypes were selected for this

in vivo study to examine the effects of THI on body temperatures of beef cattle housed at the Elora Beef Research Station (operated by the University of Guelph). Previously these cattle were classified based on both their antibody (AMIR) and cell-mediated immune responses (CMIR) using an antigen preparation containing both type 1 and type 2 antigens in adjuvant using the University of Guelph patented High Immune Response (HIR™) method (patent # US7258858B2) as described previously (Hernández et al., 2003). Included in the 36 animals, there were 8 High (H), 18 Average (A) and 10 Low (L) AMIR phenotypes, and 8 H, 24 A and 4 L CMIR phenotypes ranked based on standardized residuals generated in SAS (SAS®/STAT, 1999) using a General Linear Model (GLM). Cattle used in this experiment varied in age (2- 9 years old) and weight (512-990 kg). They were divided into 3 testing groups with 12 beef cows in each group. The rectal temperature of each cow in each group was measured manually twice a day (8 am and 3 pm, Figure 3.1) during normal seasonal summer temperatures (23.5°C – 25.5°C, www.theweathernetwork.com), and during high THI when Environment Canada issued a heatwave alert for this region of Ontario on very hot days in June 2018 to August 2018.

To help assure the accuracy of thermometers, two thermometers (Fisher Scientific) were used to record the temperature concurrently in 5 beef cows. Additionally, the reproducibility of the data was also checked by checking the temperature twice with the same thermometer with a 5-minute interval in these 5 beef cows. On the day of the experiment, beef cows were taken to the chute and were restrained by barn staff once in the morning and once in the afternoon. Thermometer was placed in contact with the

rectum wall. Subsequently, after 1 minute the temperature was read and recorded. The outdoor temperature and humidity were recorded from the weather station located near Elora, Ontario within 1 km of the University of Guelph research farm where the cattle were housed. THI was calculated using the standard formula: THI = F°-(0.55-(0.0055*RH)) *(F°-58) (El-Tarabany et al., 2015). In this study THI of equal to or greater than 74 was considered as above normal (Nardone et al., 2010; Gaughan et al., 2002).

3.4 Statistical Methods

An overall baseline rectal temperature was calculated using all the temperature data collected on days when THI was less than 74, both in the morning and afternoon. The mean value was 38.8 with a standard deviation (SD) of 0.23. One SD above the mean (Mean + 1SD = 39.1) was considered a slightly elevated body temperature and 2 SD above the mean (Mean+ 2SD = 39.3) was considered elevated body temperature for this study.

Analysis of Variance

Repeated measures mixed models were run in SAS using Proc mixed (SAS®/STAT, 1999) to examine the effects influencing rectal temperature of beef cattle as follows:

Statistical Model:

$y_{ijklmnopqrstuv} = \mu + \text{THI category}_i + T_j + \text{AMIR}_k + \text{AMIR*THI category}_l + \text{CMIR}_m + \text{CMIR*THI category}_n + C_o + W_p + A_q + BA_r + RA_s + SI_t + PI_u + R_v + e_{ijklmnopqrstuv}$, where:

$y_{ijklmnopqrstuv}$ = rectal temperature; μ = overall mean; THI category_i = fixed effect of THI category (THI ≥ 74 or THI < 74); T_j = fixed effect of time of day (AM or PM); AMIR_k =

fixed effect of AMIR category; AMIR*THI category $_l$ = fixed effect of AMIR category by THI category (H,A,L phenotype by THI category); $CMIR_m$ = fixed effect of CMIR category; CMIR*THI $category_n$ = fixed effect of CMIR category by THI category (H,A,L phenotype by THI category);

C_o = effect of colour (black, dark brown, light brown); W_p = effect of weight continuous (512-990 kg); A_q = effects of age continuous (2-9 years old); BA_r = effects of black Angus (number of Black Angusout of 32 possible progenitors (data from BioTract(Go 360 bioTrack, AgSights, agsights.com) for the progenitors going back 5 generations); RA_s = effects of Red Angus (number of Red Angus out of 32 possible progenitors); SI_t = effects of Simmental (number of Simmental out of 32 possible progenitors); PI_u = effects of Piedmontese (number of Piedmontese out of 32 possible progenitors); R_v random effect (group, and morning/afternoon by Id cows number) , and $e_{ijklmnopqrstuv}$ = residual error.

3.5 Results

Descriptive Statistics

Comparing rectal temperatures of 36 mixed breed beef cows of diverse AMIR and CMIR phenotypes during normal THI and above normal THI from June 2018 to August 2018

In 2018, Group 1 (n = 12) rectal temperatures were measured from June 25 to July 6 and again from August 6 to August 10. The highest THI recorded in the morning was

76.9 and the highest THI recorded in the afternoon was 81.9, well above the cutoff THI of 74.0 (Table 3.1a, Figure 3.5 appendix). During the first period (from June 25 to July 6) the average rectal temperatures of beef cows in the morning and afternoon were 39.0°C and 39.3°C, respectively; the highest rectal temperatures recorded for individual cows in the morning and afternoon were 40.2°C and 40.5°C, respectively (individual data not shown). On June 28, even though the THI was low (66.6 in the morning and 73.3 in the afternoon), beef cows had higher rectal temperatures in the morning and afternoon. All 12 of these cows were in estrus on this day since they all were treated to synchronize breeding. The rectal temperature for this day for this group were excluded from further analyses due to the well-known effects of estrus on body temperatures (Wrenn et al., 1958).

The second time the rectal temperatures of this group were measured was from August 21 to August 24. During this period, the highest THI in the morning and afternoon were 66.9 and 70.3, respectively. The average rectal temperatures recorded in the morning and afternoon were 38.7°C and 39.0°C, respectively (Table 3.1a, Figure 3.6 appendix). The highest rectal temperature recorded for an individual cow in the morning was 39.0°C and the highest recorded in the afternoon was 39.5°C (individual data not shown).

Table 3.1a: Average rectal temperatures of 12 mixed breed beef cows (Group1) recorded in June-August of 2018 during normal temperature-humidity index (THI < 74) and above normal THI (THI ≥ 74)

Group(G)/week	Date	THI < 74	THI ≥ 74	Rectal Temperature
G1-week1	June 25 am	61.0	-	38.8
	June 25 pm	65.5	-	39.0
	June 26 am	61.9	-	38.8
	June 26 pm	67.4	-	39.3
	June 27 am	67.6	-	39.1
	June 27 pm	69.1	-	39.3
	June 28 am	66.6	-	39.4
	June 28 pm	73.3	-	39.1
	June 29 am	71.9	-	38.9
	June 29 pm		76.8	39.4
	June 30 am	-	75.5	38.9
	June 30 pm	-	81.9	39.4
	July 01 am	-	76.9	39.0
	July 01 pm	-	79.3	39.6
	July 02 am	-	76.3	39.2
	July 02 pm	-	77.6	39.5
	July 04 am	72.7	-	38.9
	July 04 pm	-	74.9	39.4
	July 05 am	-	76.2	39.2
	July 05 pm	-	78.5	39.5
	July 06 am	64.2	-	38.8
	July 06 pm	66.1	-	38.8
G1-week2	August 21 am	66.9	-	38.7
	August 21 pm	70.2	-	39.0
	August 22 am	63.2	-	38.7
	August 22 pm	63.9	-	39.1
	August 23 am	63.6	-	38.7
	August 23 pm	69.2	-	38.8

	August 24 am	63.8	-	38.7
	August 24 pm	70.3	-	38.9

Group 2 (n=12) had rectal temperatures recorded from July 9 to July 13. The highest THI recorded in the morning and in the afternoon during this period were 73.0 and 74.6, respectively. Average rectal temperatures recorded in the morning and afternoon were 38.7°C and 38.9°C, respectively (Table 3.1b, Figure 7 appendix). The highest rectal temperature recorded for an individual cow in the morning was 39.2°C which is within normal range and the highest recorded in the afternoon was 39.3°C which is 1 SD above normal for this study (individual data not shown). The second time the rectal temperatures of this group were measured was from August 6 to August 10. During this period, the highest THI in the morning and afternoon were 75.4 and 74.5, respectively. The average rectal temperatures recorded were 38.7°C in the morning and 39.0°C in the afternoon (Table 3.1b, Figure 8 appendix). The highest rectal temperature recorded for an individual cow in the morning was 39.6°C and the highest recorded in the afternoon was 40.3°C (data not shown).

Table 3.2b: Average rectal temperature of 12 mixed breed beef cows (Group 2) recorded in June-August of 2018 during normal temperature-humidity index (THI < 74) and above normal THI (THI ≥ 74)

Group(G)/Week	Date	THI < 74	THI ≥ 74	Rectal Temperature
G2- week1	July 09 am	73.0	-	38.9
	July 09 pm	-	74.6	38.9
	July 10 am	68.8	-	38.8
	July 10 pm	70.8	-	38.9
	July 11 am	68.6	-	38.6
	July 11 pm	70.0	-	39.0
	July 12 am	70.4	-	38.7
	July 12 pm	72.8	-	38.9
	July 13 am	69.7	-	38.7
	July 13 pm	-	74.0	38.8
G2-week 2	August 06 am	-	75.4	38.7
	August 06 pm	-	74.4	39.1
	August 07 am	69.4	-	38.8
	August 07 pm	-	74.5	39.0
	August 08 am	67.0	-	38.8
	August 08 pm	72.1	-	39.2
	August 09 am	66.0	-	38.7
	August 09 pm	73.4	-	38.9
	August 10 am	65.5	-	38.6
	August 10 pm	70.7	-	38.9

Group 3 (n=12) had rectal temperatures recorded from July 23 to July 27 and from August 27 to August 30. During the first period, the highest THI recorded in the morning and afternoon were 70.3 and 74.8 respectively. The average morning rectal temperature was 38.8°C and the average afternoon rectal temperature recorded was 39.0°C (Table 3.1c, Figure 9 appendix). The highest rectal temperatures recorded for

individual cows in the morning and afternoon were 39.5°C and 39.7°C, respectively, both of which were 2 SD above the mean (individual data not shown). The second time the rectal temperatures were measured (August 27-August 30) the lowest and highest THI were 74 and 77.7, respectively. The average rectal temperature recorded in the morning was 38.7°C and the average rectal temperature recorded in the afternoon was 39.2°C (Table 3.1c, Figure 10 appendix). The highest rectal temperature recorded in an individual cow in the morning was 39.3°C and the highest temperature in the afternoon was 40.3°C (individual data not shown).

Table 3.3c: Average rectal temperature of 12 mixed breed beef cows (Group 2) recorded in June-August of 2018 during normal temperature-humidity index (THI < 74) and above normal THI (THI ≥ 74)

Group (G) /Week	Date	THI < 74	THI ≥ 74	Temperature
G3- week1	July 23 am	69.9	-	38.9
	July 23 pm		74.8	39.1
	July 24 am	70.3	-	38.9
	July 24 pm	-	74.5	39.1
	July 25 am	68.4	-	38.9
	July 25 pm		74.0	39.0
	July 26 am	70.0	-	38.7
	July 26 pm	69.6	-	38.9
	July 27 am	65.1	-	38.7
	July 27 pm	68.1	-	39.0
G3- week 2	August 27 am	69.3	-	38.8
	August 27 pm	-	77.4	39.4
	August 28 am	-	74.0	38.8
	August 28 pm	-	77.7	39.4
	August 29 am	72.3	-	38.8
	August 30 am	59.2	-	38.6
	August 30 pm	64.2	-	38.9

On days when THI was less than 74, on average 33.14% of cows had rectal temperatures greater than or equal to 39.1℃ (the mean plus one standard deviation of baseline rectal temperatures, Table 3.2). On days when THI was greater than or equal to 74, on average 64.29% of cows had rectal temperatures greater than or equal to 39.1℃, Table 3.3.)

On days when THI was less than 74, on average 9.51% of cows had rectal temperatures greater than or equal to 39.3℃ (the mean plus two standard deviation of baseline rectal temperatures, Table 3.4). On days when THI was greater than or equal to 74, on average 39.52% of cows had rectal temperatures greater than or equal to 39.3℃, Table 3.5.

Table 3.2: Percent of mature beef cows in all groups with rectal temperature greater than or equal to the mean plus one standard deviation of baseline temperatures in THI < 74

Date	Percent
June 25	75.00
June 26	91.67
July 06	8.33
August 21	41.67
August 22	58.33
August 24	25.00
July 10	41.67
July 11	33.33
July 12	25.00
August 08	50.00
August 09	30.00
July 26	33.33
July 27	41.67

August 29	8.33
August 10	0.00
August 23	0.00
August 30	0.00
Mean	**33.14**

Table 3.3: Percent of mature beef cows in all groups with rectal temperature greater than or equal to the mean plus one standard deviation of baseline temperatures in THI ≥ 74

Date	**Percent**
June 30	83.33
July 01	100
July 02	91.67
July 04	91.67
July 05	91.67
July 09	41.67
July 13	8.33
August 06	50.00
August 07	50.00
July 23	58.33
July 24	50.00
July 25	16.67
August 27	83.33
August 28	83.33
Mean	**64.29**

Table 3.4 - Percent of mature beef cows in all groups with rectal temperature greater than or equal to the mean plus two standard deviation of baseline temperatures in THI < 74

Date	Percent
June 25	16.67
June 26	50.00
July 06	8.33
August 21	16.67
August 22	25.00
June 12	8.33
August 08	20.00
July 27	16.67
August 23	0.00
August 24	0.00
July 10	0.00
July 11	0.00
August 09	0.00
August 10	0.00
July 26	0.00
August 29	0.00
August 30	0.00
Mean	**9.51**

Table 3.5- Percent of mature beef cows in all groups with rectal temperature greater than or equal to the mean plus two standard deviation of baseline temperatures in THI ≥ 74

Date	Percent
June 30	75.00
July 01	83.33
July 02	91.67
July 04	58.33
July 05	58.33
July 09	8.33
July 13	0.00
August 06	10.00
August 07	10.00
July 23	8.33
July 24	16.67
July 25	8.33
August 27	50.00
August 28	75.00
Mean	**39.52**

Table 3.6- Repeated measures mixed models of rectal temperatures of 36 mixed breed beef cows with known antibody-mediated immune response (AMIR) phenotypes during normal temperature-humidity index (THI < 74) and above normal THI (THI ≥ 74)

P-values

Variable	AMIR p-value	Time (am/pm) p-value	THI range ≥74 and <74 p-value	AMIR*THI range p-value*
Rectal Temperature	.009	<.0001	< .0001	.015

*Beef cows with high AMIR phenotypes had significantly lower rectal temperature compared to average and low AMIR phenotypes.

Analysis of Variance

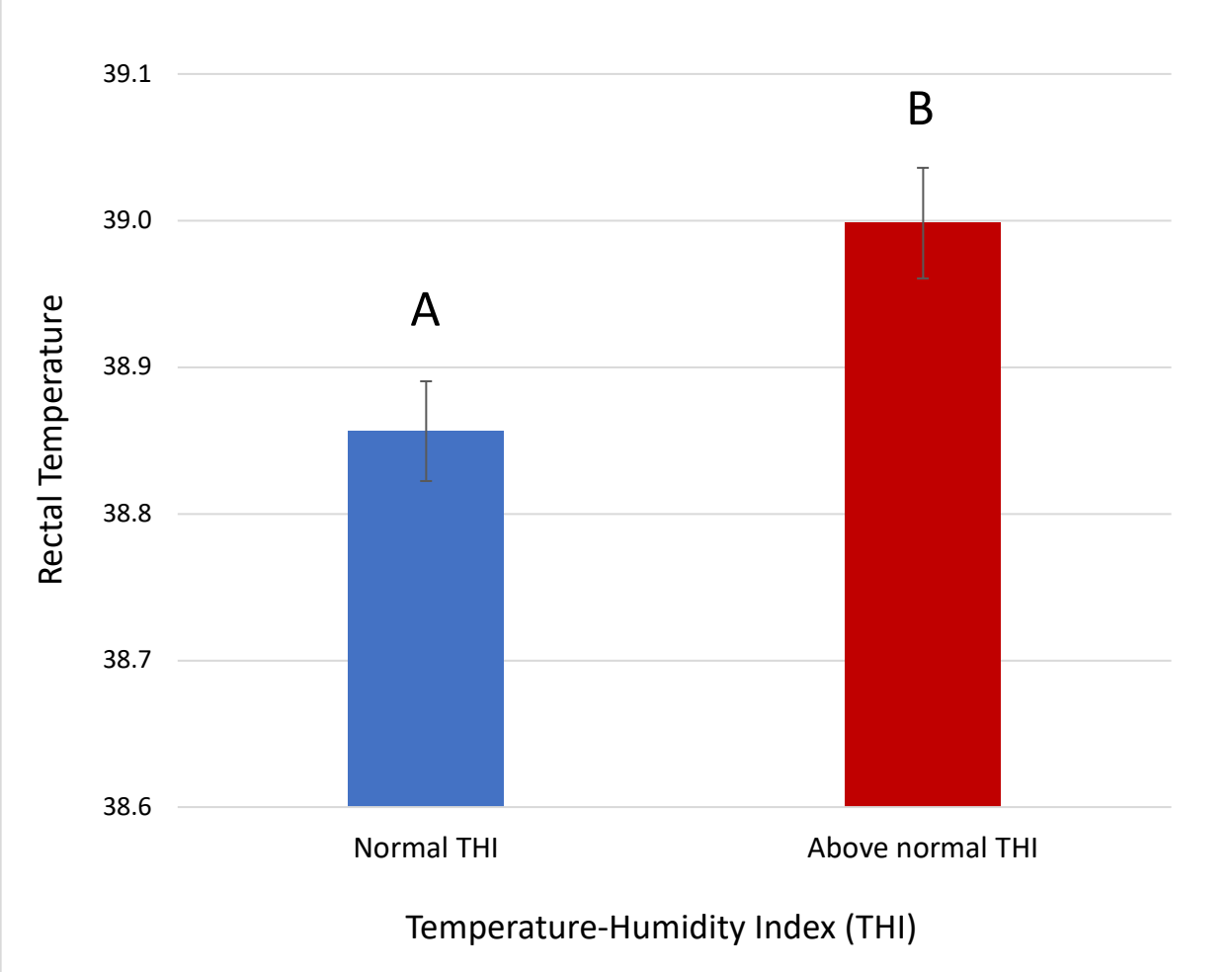
A summary of the ANOVA results is provided in Table 3.6.

Comparing rectal temperatures of 36 mix breed beef cows with different AMIR and CMIR immune phenotypes during normal THI and above normal THI using repeated measures mixed models in SAS

Results comparing rectal temperature during THI < 74 and THI ≥ 74 showed a relationship between THI and rectal temperature in that as THI increased, LSmean rectal temperature of these animals increased by 0.14°C, $p < 0.0001$, Figure 3.2). Additionally, a circadian rhythm was observed in rectal temperature with a significantly

lower LSmean rectal temperature in the morning (38.82°C), and a higher rectal temperature in the afternoon (39.04°C, $p < 0.0001$, Figure 3.3)

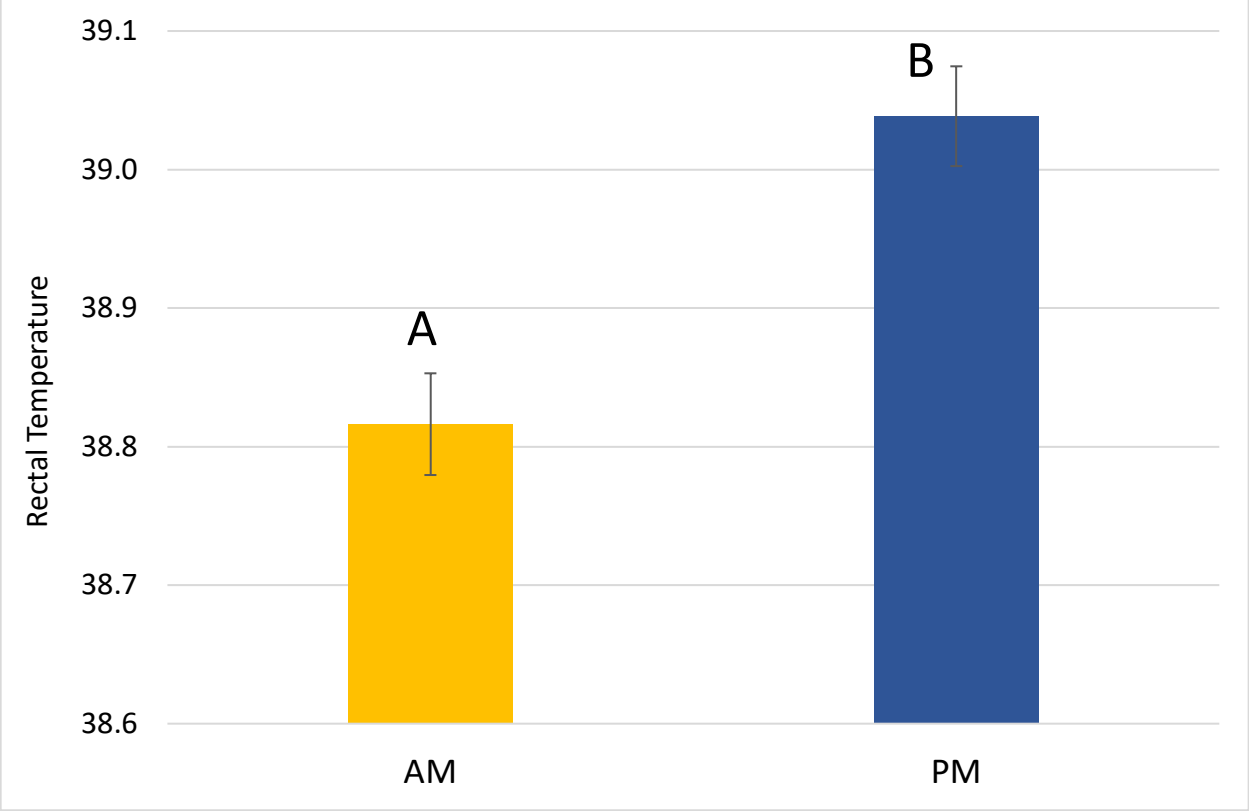
Figure 3.2: A comparison of least square means of rectal temperatures on 36 beef cows of mixed breeds during normal THI (< 74) and above normal THI (≥ 74) with standard errors from the mean



[1] columns with different letters differ significantly at $p<0.05$

[2] The mean body temperature of cattle in this study during normal THI was 38.8 +/- 0.23

Figure 3.3: A comparison of least square means of rectal temperatures of 36 mixed breed beef cows in the morning and in the afternoon with standard errors from the means



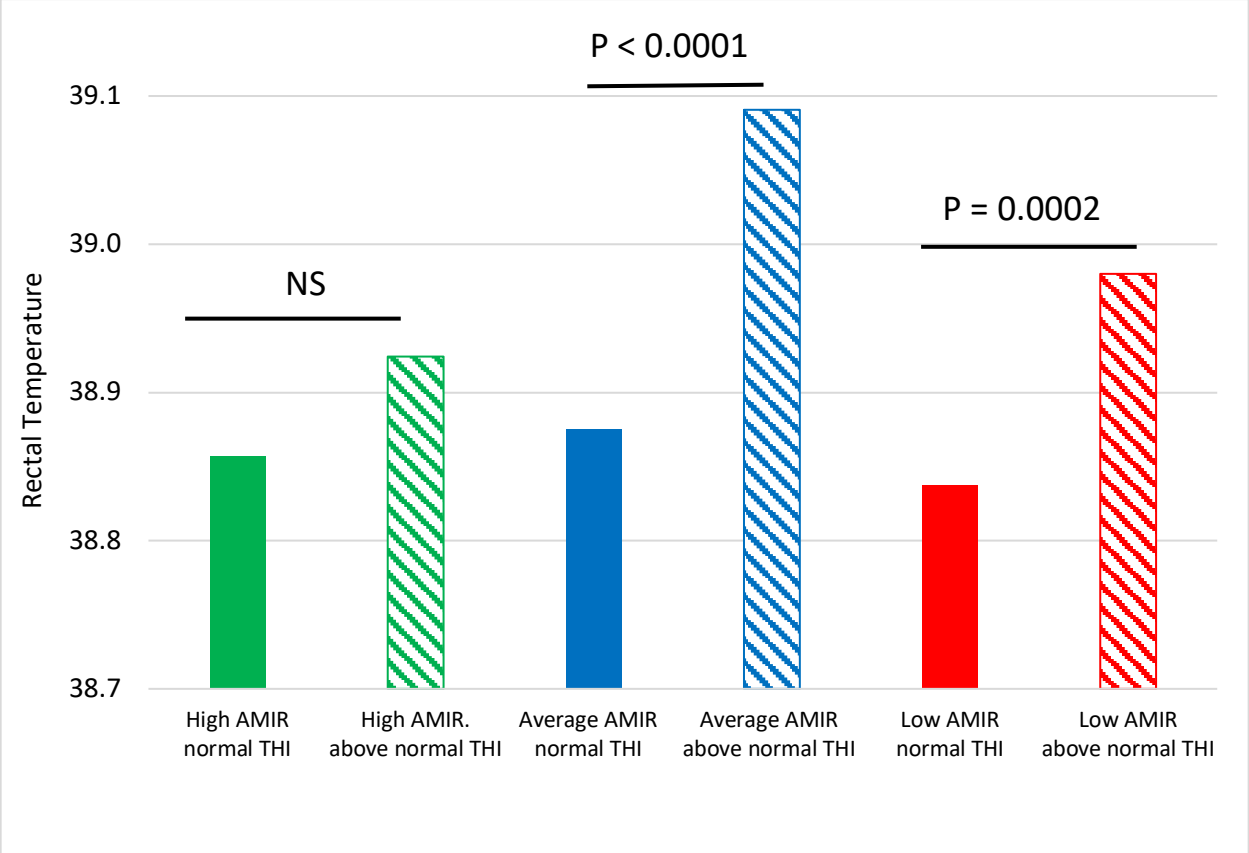
[1] columns with different letters differ significantly at p<0.05

[2] The mean body temperature of cattle in this study during normal THI was 38.8 +/- 0.23

Results showed no significant difference in CMIR phenotype (p = 0.18). A significant interaction between AMIR and THI category (p = 0.015) indicated that there was a significant difference in how H, A and L AMIR cows responded to changes in

environmental temperatures. The cows that ranked high for AMIR did not have significantly higher rectal temperatures when the THI was ≥ 74 than when THI was below 74. In contrast, A and L AMIR responder cows had significantly higher LSmean body temperatures during periods of high THI ($p < 0.0001$ and $p = 0.0002$, respectively, **Table 3.6, Figure 3.4).**

Figure 3.4: A comparison of least square means rectal temperature of 36 mixed breed beef cows with known antibody-mediated immune response (AMIR) phenotypes during normal temperature-humidity index (THI) and above normal THI with their standard errors from the mean



[1]No significant difference was observed in rectal temperature of high AMIR beef cows in THI < 74 and THI ≥ 74

[2] The mean body temperature of cattle in this study during normal THI was 38.8 +/- 0.23

In contrast to interaction AMIR*THI category, the interaction CMIR*THI was not significant.

3.5 Discussion

Increases in weather severity and average global temperatures make heat stress substantially more prevalent, particularly in countries such as Canada where cattle have not been previously adapted to a hot climate. This problem will affect animal well-being and productivity within the beef industry. For feedlot cattle, changes in management may help to resolve some of these challenges; however, heat stress could be more of a challenge for the cattle on pasture depending on the particular conditions. For example, in large feedlots with sometimes more than 30,000 head of cattle, the accessibility of shade and a cooling system maybe a concern.

Under hot and humid conditions, as often found during the summer in southwestern Ontario, cattle are often not able to dissipate heat effectively, particularly if they do not have access to cooling systems other than their own biological systems. Hamzaoui et al. (2018) reported that blood supply to the sweat glands of Ayrshire cattle is poor and they do not function as efficiently or in the same manner as human sweat glands in heat regulation. Since beef cattle do not appear to have sweat glands that are as efficient as

some other species, they tend to accumulate the heat (digestion and environment heat load) during the day and dissipate it at night when the temperature is lower. However, during heat waves with continuous hot days and nights, this process of heat dissipation is less feasible, and the beef cattle are not able to efficiently dissipate the accumulated heat back to the environment. According to Hamzaoui et al. (2018), 70-80% of heat loss happens via evaporation and through the skin.

Additionally, pastured beef cattle may have to walk a long distance to reach the water, and in many pastures, cattle do not have access to shelter. Heat stress also affects the quality of the pasture grass nutrition and these cattle might not receive optimal nutrition that could be an additional stress factor. When there are many stress factors involved, it is more challenging for the animal to handle the heat stress.

Studies of housed dairy cattle indicate that heat stress can still impact cows even in well-managed barns (West, 2003). Heat tolerance is among the heritable traits (Ravagnolo and Misztal 2000, 2002), with heritability estimates ranging from 0.11 to 0.68 (Burrow 2001; Da Silva et al, 2003; Dikme et al., 2012; MacKinnon et al., 1991) indicating that selection for heat tolerance is possible.

Cattle identified as HIR have been reported to have fewer incidents of disease, better colostrum and milk quality, as well as better performance compared to A and L responders (Thompson-Crispi et al., 2014; Fleming et al., 2016; Stear et al., 2017; Emam et al., 2018). The focus of the current study was to evaluate the hypothesis that high immune responders are able to regulate their body temperature better than A and

L responders in the face of global warming, specifically heat stress. It may also be possible to select cattle for both improved disease resistance and heat tolerance to obtain more resilient cattle in the future.

The results of the current study indicated that overall 27.3% of cows showed rectal temperatures above 39.1°C (1 SD above the mean) when THI was $\geq$ 74, indicating potential heat stress. This is in line with other studies that reported an increase in rectal temperature as the THI increased (Srikandakumar et al., 2004; Kaufman et al., 2018). The differences in rectal body temperatures found in this study were significant but relatively small in magnitude; however, relatively small changes in body temperature can have important biological consequences.

It is also important to consider that the maximum outdoor temperatures during the current study were not that high, and the durations of heat waves were relatively short. Therefore, these effects on body temperature may be greater during longer periods of heat stress.

Additionally, there was a circadian rhythm in rectal temperature of beef cattle with a significantly higher body temperature ($P < 0.0001$) in the afternoon. This was as expected and has been reported previously in the literature as cattle are known to accumulate the heat generated from digestion and the heat absorbed from the environment during the day; throughout the night as ambient temperature drops, they dissipate this accumulated heat back to the environment (Lewis and Newman, 1984).

Results from the current study also indicated that there was a positive and significant correlation between THI and rectal temperature; when the THI was above normal, (THI ≥74) animals had higher rectal temperatures. Previous studies reported similar findings with body temperature increasing as THI increased (Allen et al., 2015).

In Group 1, during the first observation period, cows had higher than normal rectal temperatures on a day with normal THI; this appeared to be as a result of induced estrus in the research station herd. Synchronization was used to help create a shorter calving season the following year, to use labour more efficiently during the breeding season, and also to permit efficient use of artificial insemination with the best beef bulls. The findings of Lewis and Newman (1984) confirm that rectal temperatures increase during estrus. They indicated that body temperature fluctuates during the estrous cycle with an animal having the lowest temperature prior to estrous and the highest temperature on the day of estrus. Synchronization of estrus, with 12 cows in one pen simultaneously in estrus will accentuate this effect. Higher activity during this time and the increase of vaginal blood flow is considered to be the reason for this increase (Suthar, 2011). The duration of this increase was reported as 3-6.5 hours (Suthar, 2011) and the amplitude of it was reported up to 0.9 -/+0.3 C (Kyle et al., 1998).

Results of this study indicated an association between AMIR*THI demonstrating that immune response phenotype, may influence body temperature during high THI. Even though the magnitude of the difference in rectal temperature is small, beef cows with the H-AMIR phenotype, ranked based on standardized residuals, were able to regulate their rectal temperature better than A or L immune responders in that H responders showed

no significant increase in rectal temperature when THI was ≥ 74. This is the first report of an association of immune response phenotype on heat stress, specifically rectal body temperature regulation. If cattle ranked H for AMIR are also better able to maintain their rectal body temperature during high THI it would offer an additional advantage to cattle with this phenotype. The model did not show any significant association of CMIR with THI. Critical to control response to pathogenic infection, particularly extracellular pathogens. Several studies have reported that dairy cows ranked as high antibody responders have fewer incidents of mastitis and other economically important diseases (Hernández et al., 2003; Thompson-Crispi et al., 2013; Larmer and Mallard

Since both immune response and heat stress are heritable traits it should be possible in the future to select for more resilient cattle that are both healthy and able to withstand increases in global warming.

3.6 Conclusion

Global warming with more frequent hot and humid summers not only will adversely affect animal health but livestock production and reproduction. As it has been reported in the literature previously, during hot days when THI is high, animals are not able to dissipate their body heat to the environment effectively and this will lead to an increase in their core body temperature. This is more important in the context of feedlot beef cattle that consume high-energy diets to gain weight in a shorter period, as well as beef cattle on pasture. During hot and humid days of summer, it will be more difficult for beef cows to dissipate body heat (the metabolic heat and the heat they absorb from the environment) back to the environment. Dairy cattle ranked as high immune responders

using HIR™ methodology have been shown previously to have fewer incidents of disease, as well as better quality colostrum and milk compared to average and low immune responders. However, cattle with these diverse immune response phenotypes have not been evaluated in the context of *in vivo* heat stress which was the focus of this study. Results based on checking the rectal temperature of 36 mixed breed beef cows of various immune phenotype indicated that H AMIR beef cows are able to regulate their body temperature better than A and L AMIR phenotypes. CMIR category, however, was not associated with rectal temperatures in the present study. This is the first time that genetic regulation of an immune response trait has been associated with *in vivo* heat stress thereby indicating that it may be possible to select cattle with both improved health and heat tolerance.

3.7 Acknowledgements

The field work would not have been possible without the help of Elora Beef Research Station staff. I would like to thank the Arrell Food Institute for their support. This project was funded by Arrell Food Scholarship, Food from Thought, and Ontario Veterinary College Scholarship. The research was support by Canadian First Research Excellence Fund (CFREF) and Food from Thought (FFT) funding to Dr. Mallard.

4 In Vitro Response of Bovine Blood Mononuclear Cells to Heat Stress as an Indicator of Resilience

4.1 Abstract

Climate change with accompanying increases in environmental temperatures not only decreases livestock production but impacts animal health. Nonetheless, animals have some physiological mechanisms to combat the negative effects of heat stress. Inducible heat shock protein70 (HSP70) production is one of the physiological mechanisms which has been conserved over generations and across species to respond to heat stress. Disease resistance and immune responses vary between individuals and across breeds. In fact, cattle can be selectively bred for improved immunity based on estimated breeding values of immune response traits. Cattle classified as high immune responders have about half the disease occurrence of their herd-mates. However, cattle classified based on their immune response phenotypes have not been evaluated for their cellular responses to heat stress; animals with both enhanced immunity and adaptation to heat stress would be an asset in the context of global warming and could be classified as resilient. Therefore, the objective of this study was to examine the *in vitro* response of blood mononuclear cells (BMCs) to heat stress from beef cattle classified as high (H), average (A) or low (L) immune responders. In this study, BMCs of 40 beef cows classified by phenotype as having H, A and L antibody-mediated immune responses (AMIR) or cell-mediated immune responses (CMIR) were compared for their response to heat stress. Cultured cells were exposed to 42ºC heat stress for 4-hour periods on two consecutive days and concentrations of HSP70 produced by these cells were assayed using an enzyme-linked immunoassay.

The BMCs were also challenged with lipopolysaccharide (LPS) to measure nitric oxide (NO) production prior to heat stress and after heat stress treatments. Results of this study indicate a significant increase in HSP70 production after the first heat stress treatment, but a decline after the second heat treatment. Pre-heat stress values of HSP70 and NO were predictive of subsequent responses following heat stress. No significant effect of AMIR or CMIR category was observed with respect to HSP70 responses to heat stress. The NO responses to LPS were attenuated following heat stress. Cells from heavy and medium weight beef cows had significantly higher concentrations of NO in response to LPS than cells from light weight cows. The NO response to LPS was significantly lower in H CMIR beef cows under thermoneutral conditions compared to L and A CMIR phenotypes; whereas, the AMIR phenotypes were not significantly associated with NO production in response to LPS.

Key words: Heat shock protein 70, Nitric oxide, Heat stress, Beef cattle, Immune response

4.2 Introduction

Increases in global atmospheric temperature (1.8 - 4.01°C) over the past 100 years are increasing the number of hot days (Noyes et al., 2009). Severe weather conditions such as high environmental temperatures, intense solar radiation and humidity all have negative influences on cattle performance making them susceptible to disease. Feed intake, growth efficiency and reproductive efficiency of animals exposed to heat stress (HS) are also compromised (Bagath et al., 2019). A range of adaptive physiological and cellular mechanisms are triggered by HS to prevent hyperthermia and cellular damage (Bouchama et al., 2017) with the primary response being heat dissipation (Cronje, 2007). As HS persists in animals, oxygen and nutrient supplies to the cells are reduced and this causes impairment in cell function (Hall et al., 2001; Cronje, 2007), and integrity of the intestinal barrier (Doklandy et al., 2006; Lambert, 2009, Naylor 2020 MSc thesis). The latter will lead to an increase in intestinal permeability, which leads to penetration of endotoxin and inflammation (Shapiro et al., 1986; Lambert, 2009). Cells in stress reprioritize their gene expression to enhance their chances of survival; growth-related genes are suppressed, and energy is redirected to stress-related functions to help cells survive (Bouchama et al., 2017). If the effects of stress cannot be mitigated by repair of misfolded or aggregated proteins, proteins will be transported to a site of degradation (Bouchama et al., 2017; Luders et al., 2000). Transcription and activation of HSP70 are prominent cellular responses recorded under HS (Bouchama et al., 2017). This protein was discovered accidentally in thermally stressed drosophila in 1962, and it is believed

to play a critical role in the HS response of cells (Bhanuprakash et al., 2016). HSP70 is a molecular chaperon involved in overall protein integrity and protection of cells (Beere et al., 2000; Pelham, 1986; Geething and Sambrook, 1992). There are several members in this family; HSP70 is expressed constitutively; and is also induced by hyperthermia, change in pH, and oxidative stress (Milarski et al., 1989; Bhanuprakash et al., 2016). The transcription factor heat shock factor 1 (HSF1) is responsible for inducing HSP70 during HS. (Pirkkala et al., 2001). HSF1 is conserved in yeast, fruit flies, vertebrates, and plants demonstrating its physiological importance (Pirkkala et al., 2001). This gene has been mapped to chromosome 14 in cattle (Winter et al., 2007). Under normal conditions, HSF1 is a monomer, however, during HS, monomers of HSF1 forms a trimer that can translocate into the nucleus and induce the transcription of HSF1 within minutes of temperature elevation (Page et al., 2006).

Hyperthermia affects immunity, and individuals exposed to HS have weaker immune responses making them more susceptible to numerous infectious diseases (Asres and Amha, 2014). Nitric oxide (NO) is one of the common noxious compounds expressed during host defence against infectious agents (Eisenstein, 2001). However, during hyperthermia its synthesis declines leaving the host susceptible to various infectious diseases (Evans et al., 2015; Bhanuprakash et al., 2016). Nitric oxide also has an important role in regulation of physiological, pathophysiological and immunological mechanisms (Aktan, 2003). In pathological processes such as inflammatory disorders, NO acts as a cytotoxic agent (Alderton et al., 2001; Bogdan, 2001; Dawn and Bolli, 2002; Moncada and Higgs, 1991), and inhibition of inducible nitric oxide synthase

(iNOS) may be beneficial for the treatment of inflammatory disease (Aktan, 2003; Bogdan, 2001; Kroncke et al., 1998).

Breeds of cattle vary in their response to HS (Bhanuprakash et al., 2016). For example, *Bos taurus* breeds are well known for their lack of adaptation to HS while *Bos indicus* breeds can perform quite well under tropical conditions (Bhanuprakash et al., 2016). Not only do cattle vary genetically in their adaptation to HS but they differ in their immune response genetics and disease resistance (Thompson-Crispi et al., 2014; Fleming et al., 2016; Stear et al., 2017; Emam et al., 2019a). In fact, Holstein cattle can be selectively bred for improved immunity based on estimated breeding values (EBVs) of immune response traits (Emam et al., 2019a; Thompson-Crispi et al., 2014; Fleming et al., 2016). Beef cattle of mixed breeds can also be classified based on their immune response phenotypes (Husseini et al., Thesis Chapter 2). Preliminary evidence suggests that beef cows with high antibody-mediated immune responses (AMIR) are better adapted, based on rectal temperature during summer heat waves in Ontario, Canada when the temperature-humidity index (THI) climbs above 74 (Husseini et al., Thesis Chapter 3). However, beef cattle with different AMIR and CMIR phenotypes have not been evaluated for their responses to HS at the level of their cellular immune responses. Individuals with both enhanced disease resistance and adaptation to heat stress would be an asset in the context of global warming and could be defined as resilient. Therefore, the objective of this study was to examine the *in vitro* response of BMCs to heat stress of beef cattle classified as high (H), average (A) or low(L) immune responders. It was hypothesized that H immune responder beef cattle, which have

robust and balanced immune responses compared to A and L immune responders, would be able to manage the effects of heat stress better than cattle with other immune response phenotypes at a cellular level based on HSP70 and NO production of BMCs.

4.3 Material and Methods

Bovine blood mononuclear cell isolation and cell culture

Forty mixed breed beef cows at the Elora Beef Research Station (University of Guelph) with identified immune response phenotypes were selected for this *in vitro* study. Previously these cattle were classified based on both their AMIR and CMIR using an antigen preparation containing both type 1 and type 2 antigens in adjuvant using the University of Guelph patented High Immune Response (HIR™) method (patent # US7258858B2) as described previously (Hernández et al., 2003). These beef cows were ranked based on standardized residuals generated in SAS (SAS®/STAT, 1999) using a General Linear Model (GLM). Beef cows selected for this study (H AMIR = 11, A AMIR = 20, L AMIR = 9; H CMIR = 8, A CMIR = 28, L CMIR = 4) were varied in age (2 – 16 years) and weight (500-950 kg). Blood samples were collected using 20-gauge, 1-inch multiple use drawing needles (Vacuette, Nipro Medical Industries Ltd, Japan) from the jugular vein into purple top EDTA sterile tubes (BDVacutainer, Becton, Dickinson and Company, UK). The tubes were inverted 10 times to mix the blood with the anticoagulant. Whole blood was centrifuged at 1200 G at room temperature for 20 minutes with brakes off. Then, the buffy coat layer was collected into a 15 ml conical

tube. Samples were then diluted with sterile Dulbecco's Phosphate Buffered Saline (DPBS) (Life Technologies, UK) in a 1:2 ratio and mixed gently. Afterwards, the buffy coat and DPBS mixture was layered carefully on the surface of Histopaque (1.077 g/ml, Sigma, USA) in a 1:1 ratio and was centrifuged again at 1200 G at room temperature for 10 minutes with brakes off. Next, the buffy coat layer was aspirated for a second time into a 50 mL conical tube and was topped up with sterile DPBS to approximately 25 mL; cells were gently resuspended and were centrifuged at 150 G for 10 minutes with brakes on. After centrifugation, the supernatant was removed to avoid disturbing the cell pellet. One ml of sterile deionized water (dH_2O) (Hyclone, Utah) was added to the cell pellet in order to remove red blood cells and cells quickly were resuspended in 10 mL of sterile DPBS and centrifuged at 150 G for another10 minutes with brakes on. On some occasions, this step was repeated twice to remove red blood cells completely. In the next step, supernatant was removed, and cells were resuspended in RPMI Medium 1640 (Gibco, UK). If cells appeared to be agglutinating, a sterile cell strainer was placed aseptically on top of a 15 mL falcon tube and the cell suspension was dispensed gently over the filter. Ultimately, BMCs were counted using an automated cell counter (ORFLO, USA). Cell numbers were adjusted to 1×10^6 cells/ml in RPMI media and were plated in flat bottom 24 well cell culture plates (Costar, USA) in triplicate for the HSP70 assay (3 wells for each time point with 4 time points ; preHS-1, postHS-1, preHS-2, and postHS-2). For the NO assay, BMCs were plated at 1×10^6 cells/ml in serum free medium (AIM-V +AlbuMAX, Gibco, USA) in flat bottom 24 well cell culture plates

(Costar, USA) in triplicate (cells were harvested on day 4 in each TN, 1-HS, and 2-HS conditions and plated in triplicate for control and triplicate for challenged with LPS).

Heat treatment protocol - HSP70

After plating the cells, plates were placed in a 37°C incubator overnight to rest. On the following day, plates were transferred to 42°C incubator for 4 hours and then transferred back to the 37°C incubator to rest overnight (1-HS). This is a procedure typically described in the literature to simulate *in vitro* HS (Dangi et al. 2014; Bhanuprakash et al., 2016). The following day, plates were transferred back to 42°C incubator for another 4 hours and then returned to 37°C incubator to rest overnight (2-HS). This procedure was done to simulate day and night heat cycles (Table 4.1).

To measure HSP70 production, the supernatant was collected from the wells at 4 time points. Cells were aseptically collected prior to 1-HS and immediately after 1-HS, prior to 2-HS and immediately after 2-HS. Afterward, cells were lysed using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, USA) following manufacturer's instructions.

Heat treatment protocol - Nitric Oxide

After the cells were plated, the plates were placed in a 37°C incubator overnight. The following day, cells were stimulated with 5μ g of lipopolysaccharide (LPS) endotoxin (after optimization) of LPS from *Escherichia coli* O127: B8 (Sigma, USA) in triplicate for measuring NO production; 3 wells were also dedicated to controls (with media only). Afterwards plates, except the thermoneutral control plates, were transferred to a 42°C incubator for 4 hours and then transferred back to 37°C incubator overnight (1-HS). The following day, plates were transferred back to the 42°C incubator for another 4 hours and then back to the 37°C incubator to rest overnight (2-HS). The supernatants from all NO plates were collected in micro tubes at a single time point on day 4 (24 hr post 2-HS) and stored at -80°C until the Griess assay was run to measure NO concentration.

Bovine HSP70 ELISA assay

The HSP70 ELISA kit (ABclonal, USA) used here was a quantitative competitive immunoassay. Plates were previously coated by the manufacture with bovine HSP70 specific antibody. Standards and samples were co-incubated with HSP70-horseradish peroxidase (HRP) conjugate in the wells of the ELISA plates. HSP70 in standards and samples competed with HSP70-HRP conjugates for binding to the antibody bound to the plate. Higher concentrations of HSP70 from the samples and standards led to lower binding of HSP70-HRP conjugate and therefore a reduced signal. The captured HSP70-

HRP conjugate was detected by incubation with HRP substrate; binding of the HSP70-HRP was visualized using colorimetric reaction products.

In this assay, the test kit and all its components, as well as test samples were brought to room temperature prior to use. The HSP70 ELISA kit provides a set of calibration standards that are assayed at the same time as the samples to measure the concentration of bovine HSP70. Wells were pre-blocked and 100 μL of test samples and standards (provided by the manufacturer) were added to the wells in duplicate, and 100 μL of dH_2O was added to the blank well. Afterwards, 50 μL of HSP70-HRP conjugate was added to each well (but not the blank well) and was mixed thoroughly. Plates were covered and incubated for 1 hour at 37°C in the dark. After 1 hour of incubation, plates were washed 5 times with 300 μL of wash solution per well. After the last wash, plates were blotted dry by tapping on absorbent paper. Subsequently, 50 μL substrate was added to each well. Plates were covered and incubated for 10-15 minutes at room temperature. After 10-15 minutes, 50 μL of stop solution was added to each well and mixed thoroughly. Plates were immediately read at 450 nm (BioTek, Winooski, VT, USA). On every plate there were two wells that received dH_2O ,but not sample and conjugate. The average value of these blank wells was subtracted from all samples or standards, and the average value for duplicate wells was calculated. The standard curve with a range of different concentrations (0, 2.5, 5, 25, and 50 ng/ml) was used to calculate the concentration of HSP70 in the samples.

Nitric oxide Griess assay

Nitric oxide rapidly oxidizes to nitrite and/or nitrate and can be estimated using either nitrite or nitrate concentration. In this assay, nitric oxide was measured using the Griess assay as follows: Two-fold dilutions of nitrite standard were prepared from 100 μM to 1.56 μM concentration. Three control wells received media only. Fifty μl of each experimental sample was added to wells in triplicate. Fifty μl of sulphanilamide solution was dispensed to all experimental samples and wells containing the standards; plates were thoroughly mixed and incubated for 8 minutes in the dark. Subsequently, 50 μl of N-1 naphthyl ethylenediamine dihydrochloride (NED) solution was dispensed to all wells and plates were mixed and incubated again in the dark for another 8 minutes. After 8 minutes plates were read at 535 nm and 670 nm wavelengths (BioTek, Winooski, VT, USA).

4.4 Statistical Analysis

HSP70 analysis

Competitive HSP70 ELISA results were calculated following the kit instructions: standard curves were generated and the HSP70 concentration was calculated based on those curves.

Proc Univariate (SAS®/STAT, 1999, USA) was used to check the normality of all data sets. Data sets were normally distributed and a SAS mixed model (Proc Mixed) was used to examine fixed effects influencing HSP70 produced by BMCs from beef cattle of varying ages after 1-HS and 2-HS as follows:

$$y_{ijklmnopqrs} = \mu + \alpha \times c_i + a_j + c_k + p_l + g_m + BA_n + RA_o + SI_p + PI_q + w_r + y_s + e_{ijklmnopqrs},$$

where $y_{ijklmnopqrs}$ = delta HSP70 produced after 1-HS and 2-HS (difference in concentrations of HSP70 before and after HS); μ = population mean; α is the regression coefficient; c_i= control (ctrl) pre-heat stress concentration of HSP70 (Pre-HS1 and Pre-HS2) as a covariate; a_j = effect of AMIR (categorical); c_k= effect of CMIR (categorical); p_l = plate (HSP70 ELISA plates, 1-3); g_m= group (sampling groups, 1 to 5) ; BA_n = effects of black Angus (number of Black Angus out of 32 possible progenitors (data from bioTract (Go 360 bioTrack, AgSights, agsights.com))

for the progenitors going back 5 generations); RA_o = effects of Red Angus (number of Red Angus out of 32 possible progenitors); SI_p = effects of Simmental (number of Simmental out of 32 possible progenitors); PI_q = effects of Piedmontese (number of Piedmontese out of 32 possible progenitors); w_r = weight as a categorical variable (heavy 801-950 kg, medium 651-800 kg, or light 500-650 kg), y_s = age as a continuous variable (2- 11 years old); and $e_{ijklmnopqrs}$ = residual error.

Nitric oxide analysis

SAS Proc Univariate was used to check the normality of all data sets. Almost half of BMCs isolated from beef cows and cultured in thermoneutral conditions in the absence of LPS produced NO at, or below, the limit of detection (1μM) and therefore the Kruskal-Wallis (non-parametric) test was used to analyze NO concentration data. At the end of 4 days, for each cow there were 6 numbered values for NO: control thermoneutral, control 1-HS, control 2-HS, delta TN (the concentration of NO produced in the presence of LPS

minus the concentration of NO produced by the TN control group), delta 1-HS (the concentration of NO produced in the presence of LPS after 1-HS minus the concentration of the 1-HS control group), and delta 2-HS (the concentration of NO produced in the presence of LPS after 2-HS minus the concentration of the 2-HS control group).

Since so many of the control cells (in the absence of LPS) had NO concentrations at or below the limit of detection, and this affected the normality of the data, the Kruskal-Wallis nonparametric test was used for some comparisons. Spearman's correlation test was also used to indicate the correlation in NO production between the control group and LPS challenged group. Fisher's exact test was used to compare the proportion of samples with NO concentration at or below the limit of detection among H, A, and L AMIR and CMIR immune phenotypes.

The limit of NO detection recommended by the manufacturer was 2.5 μM. However, many of the samples were lower than 2.5 μM. After consultation with a company technical consultant, the limit of detection was redefined as 3 standard deviations above the average optical density (OD) of the blank. For purposes of analysis, a concentration of 1 μM was arbitrarily assigned to samples that had NO concentrations at, or below the limit of detection. SAS Proc Mixed was used to examine fixed effects influencing NO concentration produced by BMCs from beef cattle of varying ages after 1-HS and 2-HS as follows:

Nitric oxide mixed effects model

$$y_{ijklmnopqrs} = \mu + \alpha \times c_i + t_j + a_k + c_l + BA_m + RA_n + SI_o + PI_p + w_q + o_r + g_s + e_{ijklmnopqrs},$$

where $y_{ijklmnopqrs}$ = delta NO (difference between NO concentration in presence of LPS, and NO concentration in absence of LPS),; μ = population mean; α is the regression coefficient; c_i= control (NO concentration in absence of LPS (ctrl)) as a covariate; t_j = treatment (the number of heat stresses, 0, 1, and 2); a_k = effect of AMIR (categorical); c_l= effect of CMIR (categorical); BA_m = effects of black Angus (number of Black Angus out of 32 possible progenitors (data from BioTract(Go 360 bioTrack, AgSights, agsights.com) for the progenitors going back 5 generations); RA_n = effects of Red Angus (number of Red Angus out of 32 possible progenitors); SI_o = effects of Simmental (number of Simmental out of 32 possible progenitors); PI_p = effects of Piedmontese (number of Piedmontese out of 32 possible progenitors); w_q = weight as a categorical variable (heavy (801-950 kg, medium 651-800 kg, or light 500-650 kg), o_r = age as a continuous variable (2- 11 years old); g_s = group (sampling groups, 1 to 5), and $e_{ijklmnopqrs}$ = residual error.

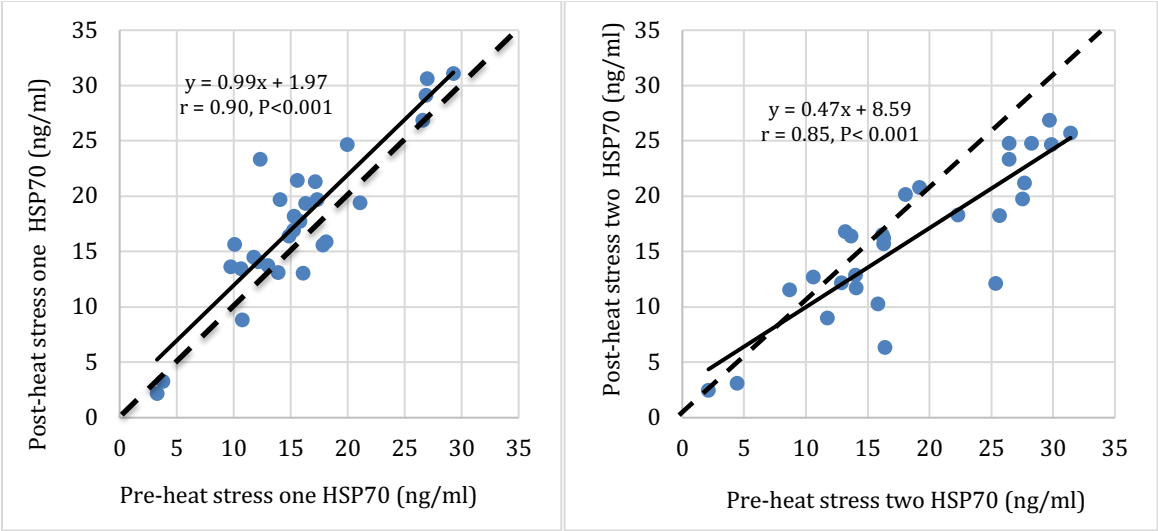
4.5 Results

Heat shock protein70 results

For this analysis, data were used from 30 beef cows; because of a technical problem in one of the HSP70 plates, the data from 10 beef cows were excluded from this analysis. Heat stress increased the HSP70 production significantly in BMCs cultured following 1-HS treatment ($p = 0.005$, mean increase of 1.9 ng/ml). Additionally, there was a high correlation ($r = 0.90$ Pearson correlation test, $P < 0.001$) between HSP70

concentrations before and after 1-HS. Thus, HSP70 production before 1-HS was highly predictive of HSP70 production after 1-HS (Figure 4.1, panel A).

Figure 4.1- Heat shock protein 70 produced by blood mononuclear cells isolated from 30 mixed breed beef cows after 1-heat stress (A), and after 2- heat stress (B) treatments



-Heat shock protein 70 was assayed for cells in all groups collected on day 2 and day 3 before heat stress and post heat stress

- Cells cultured with 1-heat stress were cultured at 42ºC for 4 hours on day 2; cells cultured with 2-heat stress treatments were cultured at 42ºC for 4 hours on day 2, and on day 3

-The solid line represents the regression line and the dashed line is the line of equivalency (data points above this line indicate higher heat shock protein 70 expression for cells after exposure to heat stress

-There was a high correlation (r= 0.90, Pearson's correlation test, p<0.001) between HSP70 production before 1-HS and after 1-HS; thus, HSP70 production prior to heat stress was highly predictive of HSP70 production after heat stress. The concentration of HSP70 increased significantly following 1-HS (Panel A: p = 0.005, lsmean increase of 1.9 ng/ml).

-There was a correlation (r = 0.85 Pearson's correlation test, p<0.001) between HSP70 production before heat stress two and after heat stress two; thus, HSP70 production prior to heat stress was highly predictive of HSP70 production after heat stress. The concentration of HSP70 decreased significantly following 1-HS (Panel B: p = 0.008, lsmean decrease of 2.5 ng/ml).

Heat stress decreased HSP70 production significantly in cultured BMCs over the second HS treatment ($p = 0.008$, Figure 4.1, panel B). Similar to 1-HS, there was a correlation ($r = 0.85$, Pearson correlation test, $p < 0.001$) between HSP70 produced before and after 2-HS (Figure 4.1, panel B), but a net decline in HSP70 production (mean decrease of 2.5 ng/ml) from pre2-HS concentrations.

No significant effect of AMIR or CMIR category was observed in HSP70 responses to heat stress.

Nitric oxide results

Comparing nitric oxide concentration produced by BMCs isolated from 40 beef cows with different AMIR and CMIR immune phenotypes after 1-HS and 2-HS *in vitro* treatments

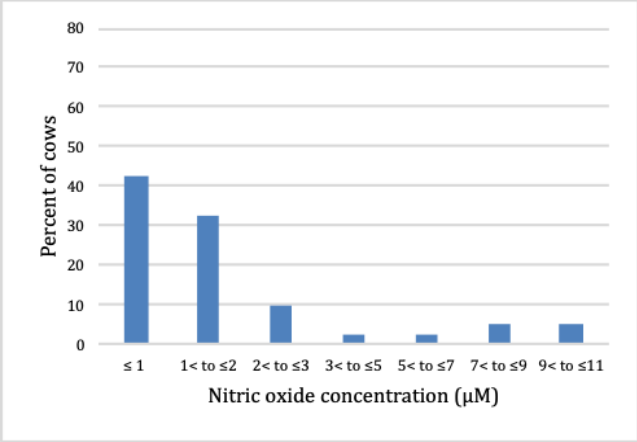
Nitric oxide production in the absence of LPS stimulation

Over 40 percent of control samples (not incubated with LPS), in each of TN, 1-HS and 2-HS groups had NO concentrations at or below the limit of detection at the end of day 4 (Figure 4.2, panel A). Nitric oxide concentrations in the absence of LPS stimulation did not differ significantly among TN, 1-HS, and 2-HS groups (Kruskal-Wallis non-parametric test, $P = 0.95$, data not shown). The proportion of cows with NO concentration at or below the limit of detection did not differ significantly by AMIR and CMIR phenotypes in absence of LPS and during the thermoneutral condition (Figure

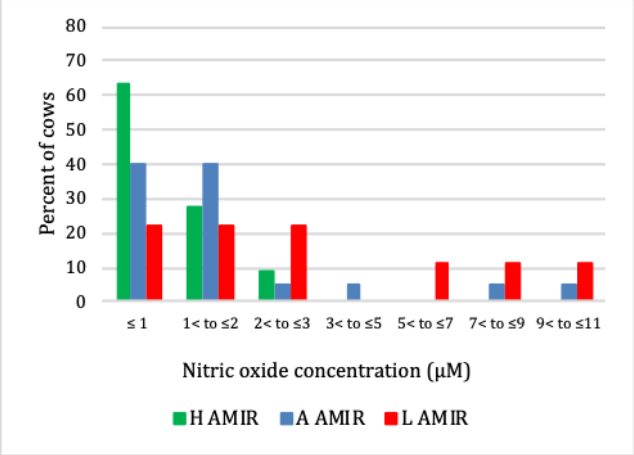
4.2, panels B and C, Fisher Exact test). There was a trend toward higher NO concentration in BMCs isolated from L AMIR beef cows during thermoneutral conditions and in the absence of LPS ($p = 0.07$, Kruskal-Wallis non-parametric test).

Figure 4.2- Histogram showing distribution of nitric oxide production during thermoneutral condition in absence of LPS: all beef cows (A), sorted by antibody-mediated immune response (AMIR) phenotype (B), sorted by cell-mediated immune response (CMIR) phenotype (C)

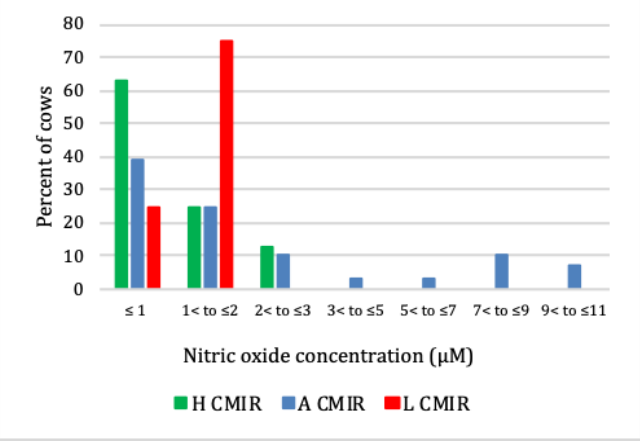
A*



B



C



* Note in panel A that over 40% of cows had nitric oxide concentrations at or below the limit of detection (≤1 μM) in the absence of LPS and during thermoneutral conditions The proportion of cows with nitric oxide concentration at or below the limit of detection did not differ significantly by AMIR and CMIR phenotypes in the absence of LPS and during thermoneutral condition (Fisher's exact test).
# Note in panel B there was a trend toward higher nitric oxide concentration in peripheral blood mononuclear cells (BMCs) isolated from L AMIR beef cows during thermoneutral conditions and in the absence of LPS (p = 0.07, Kruskal-Wallis non-parametric test). Nitric oxide concentrations of BMCs did not differ significantly among CMIR phenotypes during thermoneutral conditions and in the absence of LPS.

High AMIR = 11, Average AMIR = 20, Low AMIR = 9

High CMIR = 8, Average CMIR = 28, Low CMIR = 4

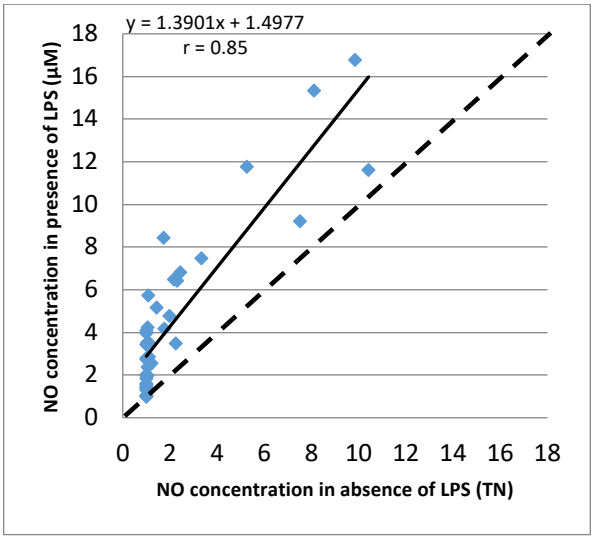
Nitric oxide production in response to LPS stimulation

After LPS treatment only, 5-10% of samples were below the limit of detection (data not shown). There was a high correlation ($r \geq 0.78$, Spearman's correlation test, $P<0.001$) between NO production in the absence of LPS and in the presence of LPS. Thus, NO production in the absence of LPS was highly predictive of NO production after LPS treatment (Figure 4.3).

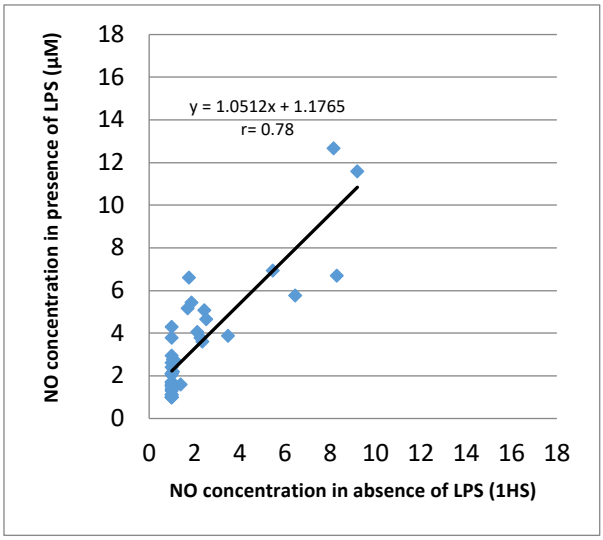
Treatment with LPS increased the NO production significantly in BMCs cultured under TN, 1-HS, and 2-HS conditions (Figure 4.3, panels A, B and C, $P< 0.001$, Kruskal-Wallis non-parametric test).

Figure 4.3- Nitric oxide produced by blood mononuclear cells isolated from 40 mixed breed beef cows in presence of lipopolysaccharide versus in absence of lipopolysaccharide in thermoneutral condition (A), after 1-heat stress (B), and after 2-heat stress treatments (C)

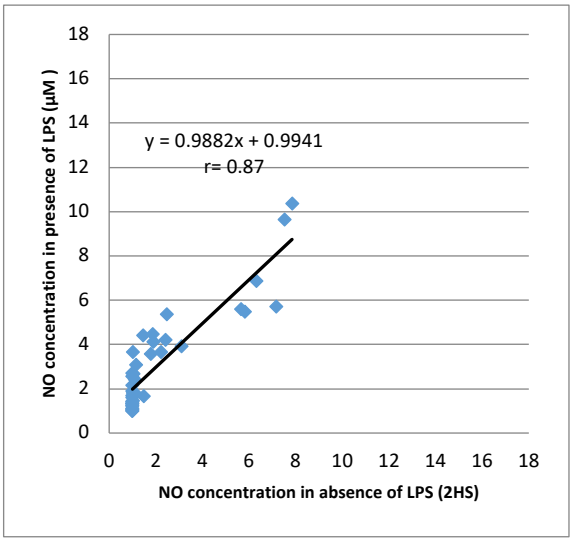
A. TN



B. 1HS



C. 2HS



- Treated cells were cultured in medium containing 5µg/ml LPS of *Escherichia coli* O127:B8 from day 2 to day 4.

All nitric oxide concentrations were assayed for cells in all groups collected on day 4 of culture

-The solid line represents the regression line and the dashed line is the line of equivalency (data points above this line indicate higher nitric oxide expression for cells cultured with LPS compared to cells cultured with medium)

- Nitric oxide concentrations at or below the limit of detection of the assay (1μM) were arbitrarily assigned a value of 1 μM for purposes of analysis

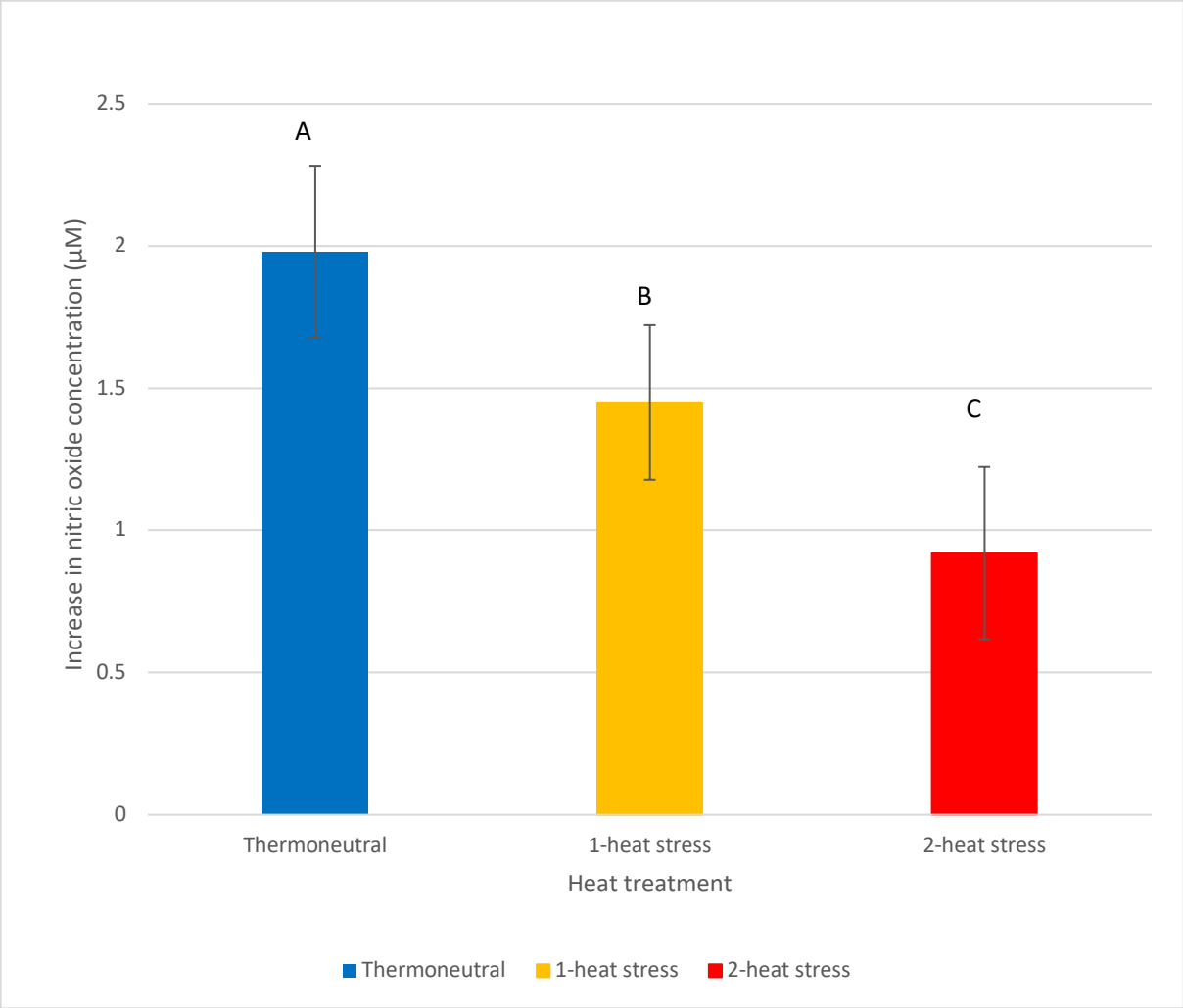
-Treatment with LPS increased the NO production significantly in BMCs cultured under thermoneutral, 1-heat stress, and 2- heat stress conditions ($p < 0.001$, Kruskal-Wallis non-parametric test).

-There was a high correlation ($r \geq 0.78$, Spearman's correlation test, $p<0.001$) between NO production in absence of LPS and in presence of LPS; thus, NO production in absence of LPS was highly predictive of NO production after LPS treatment.

- Cells cultured under thermoneutral conditions were cultured at 37ºC for 4 days; cells treated with 1-HS were cultured at 37ºC except for 4 hours at 42ºC on day 2; cells treated with 2-HS were cultured at 37ºC except for 4 hours at 42ºC on day 2, and 4 hours at 42 ºC on day 3.

The effect of HS on NO responses to LPS stimulation was further analyzed using a mixed effects model. The dependent variable was the difference in NO concentration between cells cultured with LPS and cells cultured in medium. The number of HS treatments was a fixed effect. Covariates considered in the model were age, weight, breed, AMIR, and CMIR phenotypes. Nitric oxide production in the absence of LPS was forced into the model, since there was a high correlation with NO production after culture with LPS. Nitric oxide responses to LPS after 1-HS and 2-HS were significantly lower than responses to LPS under thermoneutral condition (Figure 4.4, $p < 0.001$). Additionally, NO production after 2-HS was significantly lower than 1-HS (Figure 4.4, $p < 0.001$).

Figure 4.4- Least square means of nitric oxide response* to lipopolysaccharide (LPS) in thermoneutral condition, after 1-heat stress, and after 2- heat stress treatments with the standard errors from the mean



*Response = nitric oxide concentration produced by cells stimulated by LPS minus nitric oxide concentration produced by cells cultured in medium

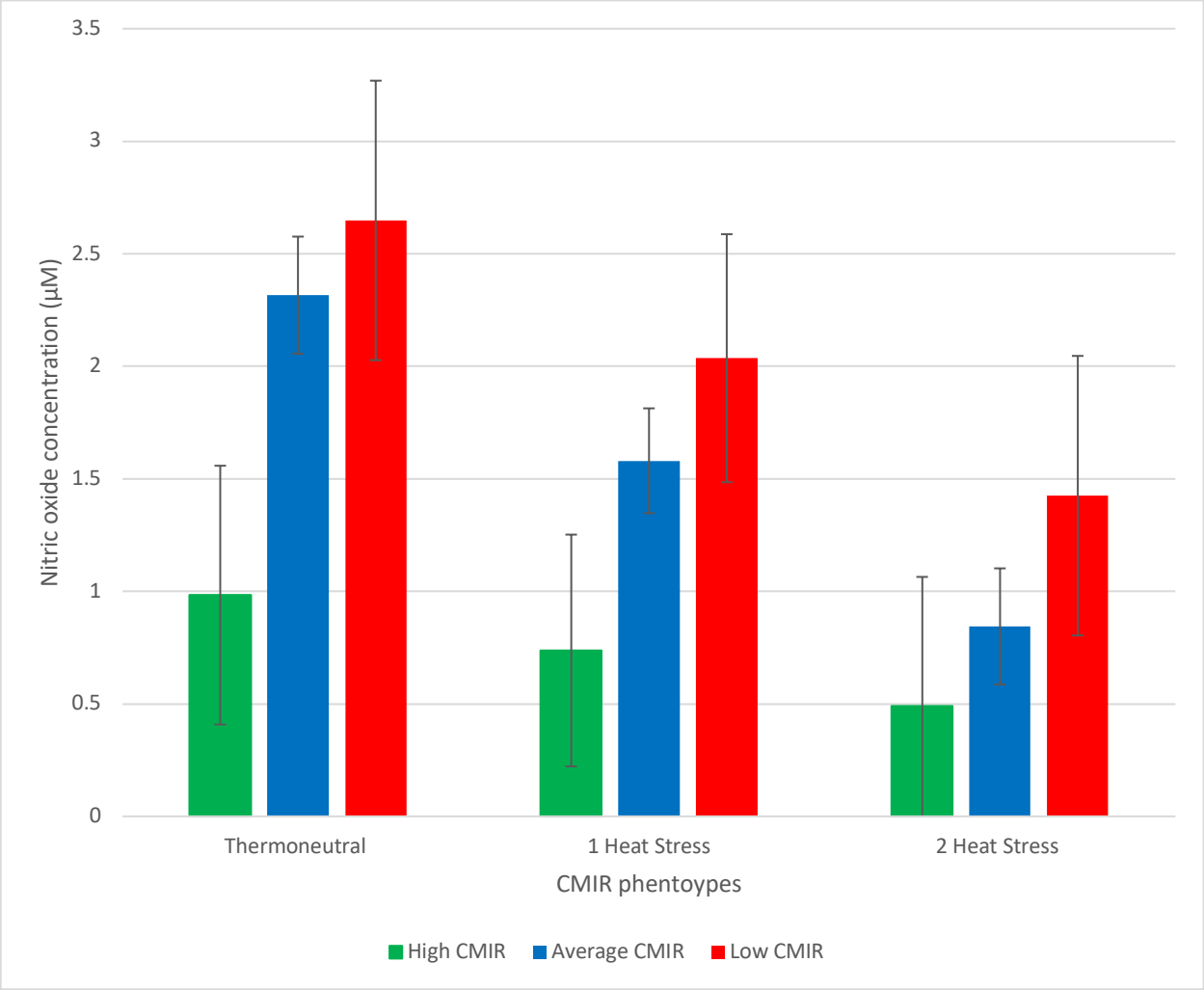
[1]Columns with the same letter do not differ significantly; columns with different letters differ significantly at $p<0.05$

Least square means are adjusted for covariates: nitric oxide production by cells cultured in medium alone, cell-mediated immune response phenotypes, and body weight categories

Nitric oxide response to LPS under TN conditions was significantly lower in H CMIR beef cows compared to L and A CMIR phenotypes (after adjusting for weight, Figure 4.5). However, in the analysis of NO responses to LPS stimulation, the interaction term CMIR*HS number was not significant ($p = 0.22$), suggesting that the suppressive effects of HS on responses to LPS were similar in cows of different CMIR phenotypes (Figure 4.5).

Nitric oxide response to LPS was significantly higher in heavy (801- 950 kg) and medium (500-650 kg), weight beef cows compared to light weight cows (500-650 kg), after adjusting for number of HS and CMIR (Figure 4.6). Breed, age, and AMIR were not significantly associated with NO production in response to LPS.

Figure 4.5- Least square means of blood mononuclear cell nitric oxide response* to lipopolysaccharide (LPS) in high (H), average (A), and low (L) cell-mediated immune response (CMIR) phenotype cows with the standard errors from the mean in all time points of treatment



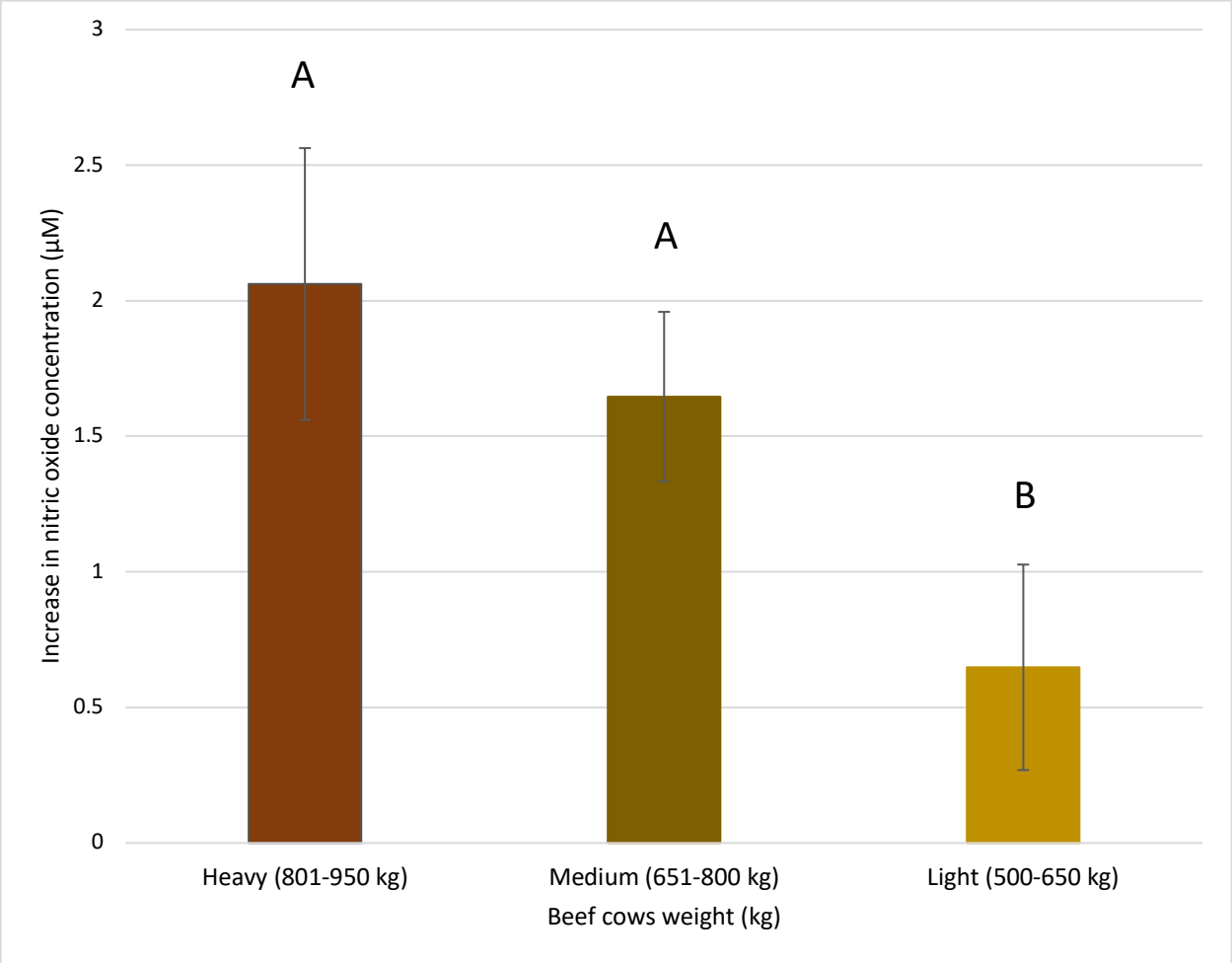
For thermoneutral treatment, NO response of H CMIR cows to LPS was significantly lower than responses of A CMIR and L CMIR cows

*Response = nitric oxide concentration produced by cells stimulated by LPS minus nitric oxide concentration produced by cells cultured in medium

[1]Columns with the same letter do not differ significantly; columns with different letters differ significantly

Least square means are adjusted for covariates: nitric oxide production by cells cultured in medium alone, and body weight categories

Figure 4.6- Least square means of blood mononuclear cell nitric oxide response* to lipopolysaccharide (LPS) in heavy (801-950 kg), medium (651-800 kg), and light (500-650 kg) weight cows with standard errors from the mean



*Response = nitric oxide concentration produced by cells stimulated by LPS minus nitric oxide concentration produced by cells cultured in medium

[1]Columns with the same letter do not differ significantly; columns with different letters differ significantly

Least square means are adjusted for covariates: nitric oxide production by cells cultured in medium alone, number of heat stresses, and cell-mediated immune response

4.6 Discussion

Heat stress triggers a range of different adaptive physiological and cellular mechanisms in animals in order to help them combat the effects of HS. Some of these mechanisms, such as HSP70 production, which was discovered in thermally stressed drosophila, are ancient and help cell survival and integrity. As the body temperature increases in mammals as a result of heat stress, HSP70 expression increases. If the HS persists, the animal will seek acclimation over time which will adjust the metabolism to minimize the detrimental effects of HS. Therefore, HSP70 is a relevant indicator of HS in cattle (Bhanuprakash et al., 2016).

Bhanuprakash et al. (2016) investigated the effect of heat and cold stress on BMCs Sahiwal breed and Sahiwal-Holstein crossbred cattle; they reported a breed difference in withstanding the effect of heat and cold stresses, with Sahiwal breed cattle withstanding the effects of heat and cold stresses significantly better than Sahiwal-Holstein crossbred cattle. Kamwanja et al. (1994) showed earlier that lymphocytes from *Bos indicus* (Brahman) had higher survivability under HS compared to lymphocytes from *Bos taurus*.

Previously, it has been reported that high immune responder dairy cattle and pigs have lower incidence of disease compared to average and low immune responders, as well as better quality milk and colostrum (Thompson-Crispi et al., 2014; Fleming et al., 2016; Stear et al., 2017; Emam et al,. 2019a). The literature also reports breed differences in combatting the effects of HS. Since there are individual differences in resistance to disease, we hypothesized that high immune responder beef cattle, may have a better

resistance to the effects of HS, particularly in light of the fact that some immune response phenotypes better regulate their core body temperature better when the temperature-humidity index is high (Husseini, Thesis Ch 3).

Studies detecting HSP70 in cultured BMCs have reported an increase in HSP70 concentration after HS (Bhanuprakash et al., 2016). Bhanuprakash et al. (2016) showed that BMCs isolated from Sahiwal and Sahiwal-Holstein crossbred breeds both had an increase in HSP70 concentration immediately after exposure *in vitro* to HS, with a peak after 6 hours. Lovell et al. (2007) have reported that, for human BMC, mean HSP70 concentrations did not differ for HS temperatures of 37, 38, 39, 40, and 41ºC. However, the time-profile HSP70 expression appeared temperature-dependent. During hyperthermic conditions (40- 41ºC), HSP70 was expressed immediately after heat treatment. However, in lower temperatures (38-39ºC) the mean HSP70 concentration increased 4 hours post heat stress. The results from the current study agree with literature showing a significantly higher HSP70 production after 1-HS. Heat shock protein 70 expression protects cells from stress; therefore, its expression is vital for survival of the cells, and in this experiment, BMCs increased the HSP70 production after 1-HS in order to cope better with the detrimental effects of HS and enhance their survival.

In future experiments, HSP70 production could also be measured 4 hours and 6 hours post HS to better establish the kinetics of this response. Additionally, these results indicated a high correlation between HSP70 production prior to HS and after HS both for 1-HS and 2-HS treatments. Thus, HSP70 production in absence of HS can be

predictive of HSP70 production after HS. No effect of AMIR and CMIR was observed in this analysis. This could be as a result of low numbers of animals and the unbalanced phenotypes available that were limitations of this study.

Bhanuprakash et al. (2016) in their study showed that baseline NO concentration was higher in Sahiwal (37μM) compared to Sahiwal-Holstein crossbred (21μM) under thermoneutral condition and assayed after 24 hours. The current study results showed that basal NO production in baseline was low in many animals (42% of cows at or below the limit of detection of the assay) in thermoneutral condition in the absence of LPS. It is not clear whether this difference is as a result of different assay reagents they used or as a result of a breed difference. Bhanuprakash et al. (2016) also reported a decrease in NO concentration after cells were exposed to HS. In the current study NO response of cultured BMCs to stimulation with LPS *in vitro* was significantly lower after heat stress. Macrophages differentiate from BMCs and in response to bacterial infection produce a number of molecules such as NO that are important in the inflammatory response. This could mean that in the event of a 1 or 2 degree increase in ambient temperature, NO production in animals will be decreased and this will make them more susceptible to different infectious agents. Optimum concentrations of NO induce peripheral vasodilation during hyperthermia. Nitric oxide also plays a major role in the circulatory and immune systems, specifically in BMCs of animals during heat stress (Bharati et al., 2017).

Delayed-type hypersensitivity is a method to measure cell-mediated immune response of beef cattle. Typically, the cells infiltrating into an affected area are a mixture of T-

lymphocytes and macrophages. As Emam et al. (2019b) showed in their research no association was observed between purified bovine macrophages with AMIR or CMIR suggesting that these are independent genetic traits. Macrophages are the key components of the innate immune system that recognize pathogens via their pathogen recognition receptors and then destroy the pathogens via phagocytosis and producing reactive oxygen and nitrogen species (Emam et al., 2019b). The current results showed that H CMIR beef cows had significantly lower NO production compared to A and L CMIR beef cows following exposure to LPS under thermoneutral conditions.

It may also be worth noting that during heat stress, a shift happens in the balance of type 1 to type 2 immune responses. T helper (Th) 1 cells tend to activate cellular immunity and inflammatory responses, whereas Th2 cells tend to regulate humoral immunity and anti-inflammatory responses (Sophia et al. 2016). Activation of HPA axis stimulation during heat stress results in the production of cortisol and suppression of the immune system (Grandin et al. 1997). Glucocorticoids affect the balance of Th1 and Th2 responses by inhibition of IL-12 and enhancing IL-10 shifting the response in a type 2 direction. Glucocorticoids also decrease the function of phagocytic cells and alter T cells function (Bagath et al. 2019). The results of the current study indicated that H CMIR beef cows had significantly lower NO production under thermoneutral conditions compared to A and L CMIR beef cows. Lsmean NO production by BMCs of H CMIR cows after 1 HS and 2 HS was lower than that of A CMIR and L CMIR cows, but the differences were not statistically significant. This might be as a result of a stronger shift in the Th1:Th2 ratio in these H CMIR beef cows in order to elicit an anti-inflammatory

and protective response to heat stress. Glucocorticoids reduce the activity of phagocytic cells and ultimately the T cell functions (Bagath et al. 2019). Since H CMIR beef cows elicit higher CMIR response and the macrophages in these H CMIR animals produce a greater amount of IL-12 compared to A and L CMIR beef cows (Emam et al 2019a), these macrophages may have been modulated to reduce Th1 response and increase Th2 or anti-inflammatory responses. This might be an explanation for why the NO production is lower in these H CMIR beef cows, but this hypothesis would need to be further tested.

Furthermore, due to cost, time and labour, the NO was measured only at the end of day 4 and not at intermediate time points. Therefore, the kinetics of NO production was not checked in these 4 days, and we do not know if BMCs from AMIR phenotyped beef cows had higher NO production on day 1, 2, or 3. Bharati et al., 2017 in their experiment showed that iNOS mRNA expression was significantly higher at 3 and 6 hours of incubation at 37ºC and at 1 hour incubation at 39-42ºC compared to the control group, and then it gradually declined. It has been also reported that HSP90 which also is induced at the time of stress is able to enhance the activity of iNOS (Bharati et al., 2017)

Beef cows with heavy and medium weight produced significantly more NO compared to those of lighter weight. Codoner-Franch et al. (2011) evaluated NO synthesis and metabolism in obese children to determine their relationship with oxidative stress and inflammation. Their results indicated that obese children had significantly higher concentrations of NO synthesis. They showed that NO synthesis and nitrosative stress

were increased in obese children and correlated with oxidative stress and inflammatory markers (Codoner-Franch et al., 2011). This warrants further investigation in cattle but in the current study even the heavier cattle were not obese by definition.

Overall, the current study showed that HSP70 production significantly increased after BMCs were exposed to 1-HS but decreased after the second HS treatment. Nitric oxide production significantly decreased after both 1- and 2-HS treatments. Beef cattle of heavy and medium weights also produced more NO than lighter weight animals. High CMIR beef cows produced significantly lower NO compare to A and L CMIR phenotypes following LPS stimulation under thermoneutral conditions. There was a limited ability to assemble groups of cows that were matched for age, and weight and represented all AMIR-CMIR combinations. The association of weight with NO suggests an interesting future direction for study.

4.7 Conclusion

Increase in environmental temperature not only affects animal production but impacts animal health and welfare. Animals in order to combat the negative effects of heat stress have developed some physiological mechanisms. One of these mechanisms in response to heat stress, which has been conserved over generations, is production of inducible HSP70. Disease resistance and immune response vary among individuals in herds and across breeds, and cattle can be bred for improved immunity based on estimated breeding values of immune responses traits. High immune responders have

about half the disease occurrence of their herd-mates. However, immune phenotyped cattle have not been evaluated for their cellular responses to heat stress. Individuals with both enhanced immunity and adaptation to heat stress would be an asset in the context of global warming. *In vivo* experiments previously indicated that beef cattle with H AMIR maintained their body temperature better than those of other phenotypes (Husseini N., Thesis Chapter 3).

Results of this study also indicated that heat stress significantly increased HSP70 production after 1-HS indicating that BMCs in order to survive and cope with HS increased the HSP70 production. However, a decline in HSP70 after 2-HS was noted. This may be indicative of some sort of an adaptive responses to repeated heat shock. Additionally, HSP70 production prior to HS can be predictive of HSP70 production after 1-HS. No effect of AMIR and CMIR was observed on HSP70 concentration. Further study with a more balanced number of immune phenotyped cows, may be required to check the effects of AMIR and CMIR on HSP70 responses of beef cattle.

Nitric oxide response to LPS decreased after HS treatments. Although NO was generally low prior to LPS challenge, NO production prior to challenge with LPS was predictive of NO production after challenge with LPS. Additionally, NO response to LPS was significantly lower in H CMIR beef cows compared to L and A CMIR phenotypes under thermoneutral conditions, but not follow HS. Finally, the cattle of high and medium weight had the highest amount of NO production which may be indicative of an inflammatory phenotype in cattle with heavier weights, but this needs further

assessment to determine if there is an actual connection to obese phenotypes as is the case with humans.

5 Overall conclusion

In chapter 2 this thesis evaluated the adaptation of HIR™ methodology in Canadian mixed breed beef cattle to determine the youngest age testing could be used to assess immune function of beef calves. Chapter 3 examined the ability of beef cows with various AMIR and CMIR phenotypes to regulate their body temperature during hot weather in Ontario when THI ≥ 74. Finally, *in vitro* HSP70 and NO production produced by BMCs isolated from H, A, and L immune responders after 1 and 2 heat treatments were evaluated in chapter 4 to determine any potential heat stress resilience among the immune responder phenotypes.

The results of the first study indicated that the HIR™ methodology can be used to classify mature and young beef calves. However, although immuno-phenotyping for AMIR can be performed as early as 2-3 weeks of age, evaluation of CMIR needs to be done in beef calves older than 3 weeks of age and if the goal is to immune-phenotype simultaneously for both of these traits, then it needs to be done in calves older than 3 weeks of age. In this study, 3-week-old and 9-month-old beef calves had significantly higher AMIR responses than mature beef cows. More study is required to evaluate the possibility of testing for CMIR in calves older than 3 weeks and younger than 9-months of age.

During hot and humid days of summer, it will be more difficult for beef cows to regulate their body temperature and dissipate heat (the metabolic heat and the heat they absorb from the environment) back to the environment. Results of chapter 3, based on monitoring the rectal temperature of 36 mixed breed beef cows of various immune phenotypes, indicated that high AMIR beef cows are able to regulate their body temperature better than average and low AMIR phenotypes. This is the first time that genetic regulation of an immune response trait has been associated with the response to *in vivo* heat stress indicating that it may be possible to select cattle with both improved health and heat tolerance. These animals may be termed resilient.

Results of chapter 4 indicated that heat stress significantly increased HSP70 production by BMC after 1-HS, but not after a second heat stress. Nitric oxide responses to LPS were reduced following heat stress. Results also indicated a significantly higher NO production in beef cows with heavy and medium weights compared to lighter weight beef cows. Additionally, beef cows with H CMIR phenotype had significantly lower NO production following LPS challenge compared to A and L CMIR under thermoneutral conditions.

6 Future Direction

Results in chapter 2 showed that mixed breed beef cattle can be tested for AMIR as young as 2-3 weeks of age, and for CMIR at 9 months of age. It would be worthwhile to check CMIR responses in beef calves older than 1 month of age and younger than 9 months of age to identify an age that both of these traits can be measured simultaneously.

In chapter 3, rectal temperatures of beef cows were recorded manually and during limited time points (once in the morning and once in the afternoon); it is recommended to use digital recording thermometers such as rumen boluses to have a better idea of beef cow body temperatures in other time points such as late evenings, midnights and early mornings. In this study, due to the limited number of phenotypes for both traits, AMIR and CMIR (High-High, Average-Average, and Low-Low), the rectal temperature was measured on AMIR phenotype (H, A, and L AMIR), and CMIR phenotype (H, A, and L CMIR) beef cows. It is suggested to study the rectal temperature of beef cows in individuals that are high, average and low for both of these traits and in beef cows with more balanced weight and age groups.

In chapter 4, HSP70 and NO production of BMCs isolated from beef cows were measured after 1-HS and 2-HS *in-vitro*. It is recommended to do the same experiments *in-vivo* by exposing the beef cows to heat stress and then collecting their blood and measuring expression of HSP70 and NO. To study further the effect to of HS on NO production *in vitro*, a larger number of beef cows of each IR phenotype with similar

weights is recommended. It would also be worthwhile to measure the BMC proliferation both *in vivo*, and *in vitro*.

7 Pitfalls and Limitations

Beef cattle body temperatures could be measured more frequently during the day or at least more often during hot days. However, due to limitations at the Elora Beef Research Station this was not possible. Also, the telemetry equipment required was not available for this study.

The Elora Beef Research Station only simulates on a small scale the actual management system used within the modern North American beef industry. Therefore, any relevant findings in this study will need to be validated under commercial conditions in the future.

There was a limited ability to assemble groups of cows that were matched for age, and weight and represented all AMIR-CMIR combinations. The association of weight with NO suggests an interesting future direction for study.

The *in vitro* experiments of heat stress on cells may not be a solid reflection of *in vivo* responses to climate change but provide a starting point for further *in vivo* studies

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APPENDICES

Chapter 8: Appendix

Chapter 2- Tables

Table 2.9 - Least squares means (bold) and p-value comparisons (values inside the table) of optical density of day 14 antibody-mediated immune response for age effect (1-week-, 2-weeks, 3- weeks, 9-months-old-calves with mature beef cows)

	1-week-old[1] beef calves n = 32 **0.076**[6]	2-week-old[2] beef calves n = 16 **0.271**	3-week-old[3] beef calves n = 49 **0.307**	9-month old[4] beef calves n = 47 **0.336**	Mixed breed beef cows[5] n = 170 **0.118**
1-week-old beef calves n = 32 **0.076**	NA	0.005	<0.0001	<0.0001	0.157
2-week-old beef calves n = 16 **0.271**	0.005	NA	0.757	0.601	0.050
3-week-old beef calves n = 49 **0.307**	<0.0001	0.757	NA	0.677	<0.0001
9-month old beef calves n = 47 **0.336**	<0.0001	0.601	0.677	NA	<0.0001
Mixed breed beef cows n = 170 **0.118**	0.157	0.050	<0.0001	<0.0001	NA

[1] The 15-day test protocol was begun at 2 to 7 days of age

[2]The 15-day test protocol was begun at 11 to 14 days of age

[3] The 15-day test protocol was begun at 21 to 27 days of age

[4] The 15-day test protocol was begun at a nominal age of 9 months

[5] Beef cows tested in October and November of 2017

[6]Least Square Mean of AMIR based on optical density from ELISA for age effect (1-week, 2-week, 3-week-, 9-month, mature beef, and mature Holsteins)

Table 2.10- Least squares means (bold) and p-values (values inside the table) of log_{10} ratio of DSFT[2] 24 hours to 0 hour indicating cell-mediated immune response for age effect (1-week-, 2-weeks, 3- weeks, 9-months-old-calves with mature beef cows)

	1-week-old[1] beef calves n = 32 **0.220[6]**	2-week-old[2] beef calves n = 16 **0.153**	3-week-old[3] beef calves n = 49 **0.227**	9-month old[4] beef calves n = 47 **0.332**	Mixed breed beef cows[5] n = 170 **0.322**
1-week-old[1] beef calves n = 32 **0.220**	NA	0.110	0.802	<0.0001	0.0001
2-week-old[2] beef calves n = 16 **0.153**	0.110	NA	0.042	<0.0001	<0.0001
3-week-old[3] beef calves n = 49 **0.227**	0.0802	0.042	NA	<0.0001	<0.0001
9-month old[4] beef calves n = 47 **0.332**	<0.0001	<0.0001	<0.0001	NA	0.543
Mixed breed beef cows[5] n = 170 **0.322**	<0.0001	<0.0001	<0.0001	0.543	NA

[1] The 15-day test protocol was begun at 2 to 7 days of age

[2]The 15-day test protocol was begun at 11 to 14 days of age

[3] The 15-day test protocol was begun at 21 to 27 days of age

[4] The 15-day test protocol was begun at a nominal age of 9 months

[5] Beef cows tested in October and November of 2017

[6]Least Square Mean of Log_{10} Ratio of DSFT 24 Hours to 0 Hours CMIR day 15 for age effect (1-week, 2-week, 3-week-, 9-month-old calves, mature beef cows)

Table 2.11- Least squares means (bold) and p-value comparisons (values inside the table) of optical density of day 14 antibody-mediated immune response for age effect (1-week-, 2-weeks, 3- weeks, 9-months-old-calves with mature beef cows and mature Holstein cows)

	1-week-old[1] beef calves n = 32 **0.072**[7]	2-week-old[2] beef calves n = 16 **0.296**	3-week-old[3] beef calves n = 49 **0.287**	9-month old[4] beef calves n = 47 **0.270**	Mixed breed beef cows[5] n = 170 **0.130**	Mature Holsteins[6] (Historical data) n= 3308 **0.250**
1-week-old[1] beef calves n = 32 **0.072**	NA	0.004	<0.0001	<0.0001	0.053	<0.0001
2-week-old[2] beef calves n = 16 **0.296**	0.004	NA	0.9645	0.835	0.051	0.675
3-week-old[3] beef calves n = 49 **0.287**	<0.0001	0.964	NA	0.732	<0.0001	0.307
9-month old[4] beef calves n = 47 **0.270**	<0.0001	0.835	0.732	NA	0.0001	0.592
Mixed breed beef cows[5] n = 170 **0.130**	0.053	0.051	<0.0001	0.0001	NA	<0.0001
Mature Holsteins[6] (Historical data) n= 3308 **0.250**	<0.0001	0.675	0.307	0.592	<0.0001	NA

[1] The 15-day test protocol was begun at 2 to 7 days of age

[2]The 15-day test protocol was begun at 11 to 14 days of age

[3] The 15-day test protocol was begun at 21 to 27 days of age

[4] The 15-day test protocol was begun at a nominal age of 9 months

[5] Beef cows tested in October and November of 2017

[6]Holstein cows tested in 71 herds in Prince Edward Island, Nova Scotia, New Brunswick, Quebec, Ontario, Alberta and British Columbia as part of previous study

[7]Least Square Mean of AMIR based on optical density from ELISA for age effect (1-week, 2-week, 3-week-, 9-month-calves, mature beef cows, and mature Holsteins cows)

Table 2.12- Least squares means (bold) and p-values (values inside the table) of log $_{10}$ ratio of DSFT[2] 24 hours to 0 hour indicating cell-mediated immune response for age effect (1-week-, 2-weeks, 3- weeks, 9-months-old-calves with mature beef cows and mature Holstein cows)

	1-week-old[1] beef calves n = 32 **0.216[7]**	2-week-old[2] beef calves n = 16 **0.148**	3-week-old[3] beef calves n = 49 **0.221**	9-month old[4] beef calves n = 47 **0.329**	Mixed breed beef cows[5] n = 170 **0.314**	Mature Holsteins[6] (Historical data) n= 3308 **0.228**
1-week-old beef calves n = 32 **0.216**	NA	0.0576	0.819	<0.0001	<0.0001	0.540
2-week-old beef calves n = 16 **0.148**	0.0576	NA	0.018	<0.0001	<0.0001	0.005
3-week-old beef calves n = 49 **0.221**	0.0819	0.018	NA	<0.0001	<0.0001	0.486
9-month old beef calves n = 47 **0.329**	<0.0001	<0.0001	<0.0001	NA	0.280	<0.0001
Mixed breed beef cows n = 170 **0.314**	<0.0001	<0.0001	<0.0001	0.280	NA	<0.0001
Mature Holsteins (Historical data) n= 3308 **0.228**	0.540	0.005	0.4866	<0.0001	<0.0001	NA

[1] The 15-day test protocol was begun at 2 to 7 days of age

[2]The 15-day test protocol was begun at 11 to 14 days of age

[3] The 15-day test protocol was begun at 21 to 27 days of age

[4] The 15-day test protocol was begun at a nominal age of 9 months

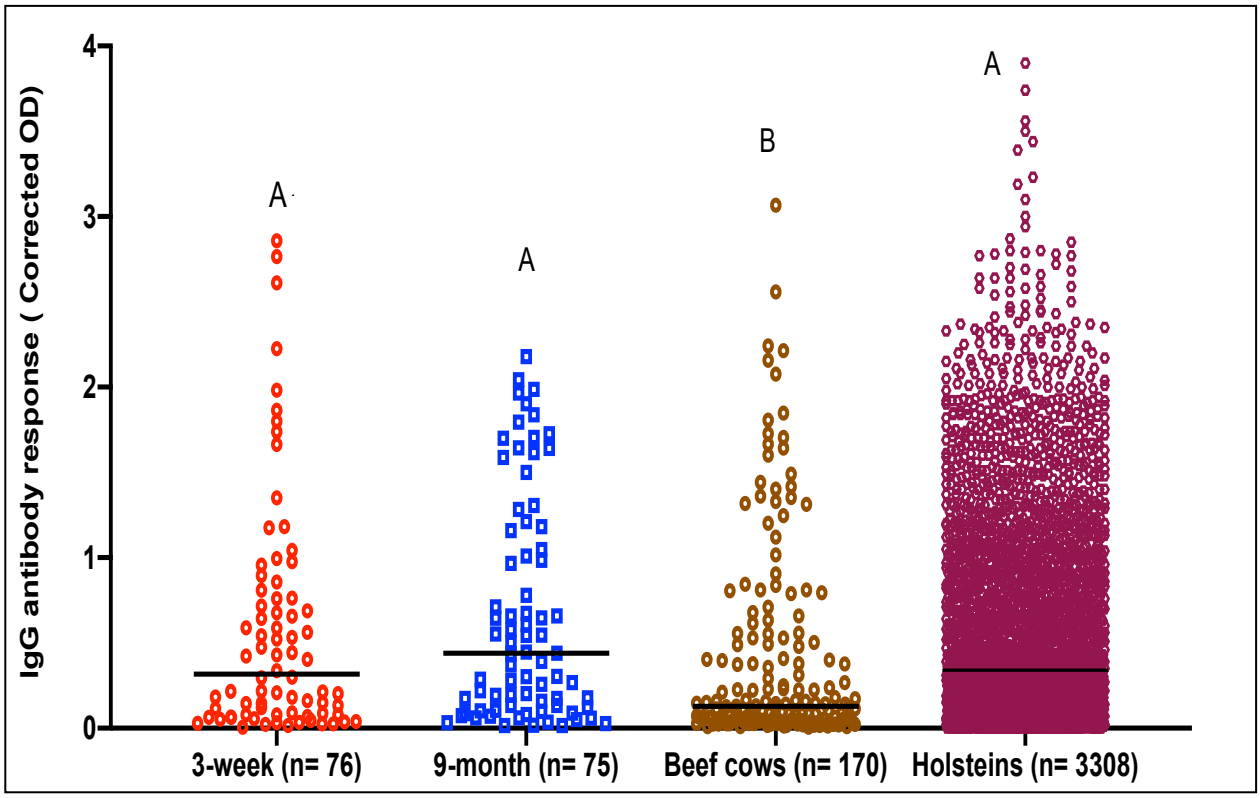
[5] Beef cows tested in October and November of 2017

[6]Holstein cows tested in 71 herds in Prince Edward Island, Nova Scotia, New Brunswick, Quebec, Ontario, Alberta and British Columbia.

[7]Least Square Mean of Log_{10} Ratio of DSFT 24 Hours to 0 Hours CMIR day 15 for age effect (1-week, 2-week, 3-week-, 9-month-calves, mature beef cows, and mature Holsteins cows). CMIR was measured based on cutaneous delayed-type hypersensitivity (DTH) to the type 1 test antigen with an intradermal injection (into skin of the tail fold) on day 14, and double skin fold thickness (DSFT) measured on day 15 (24 hours after intradermal injection).

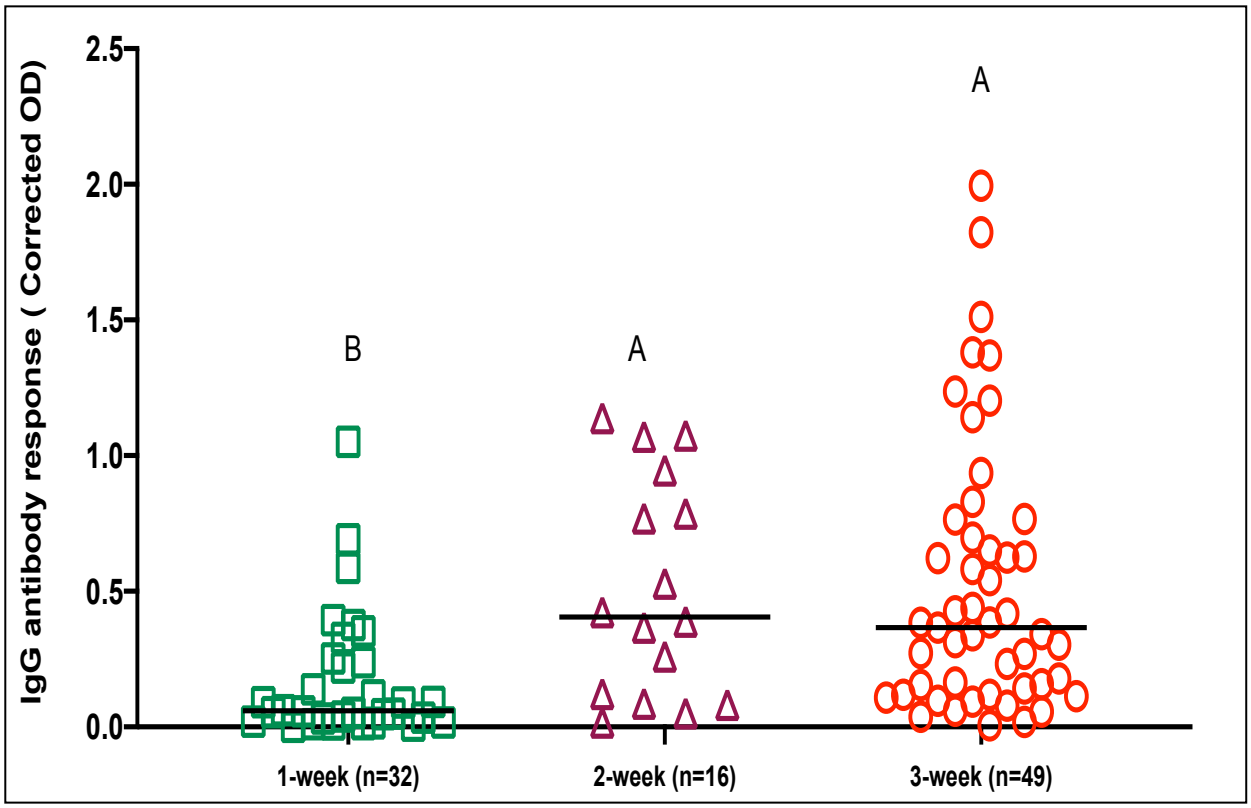
Chapter 2- Figures

Figure 2.8- Comparison of serum IgG antibody-mediated immune responses in 3-week- and 9-month-old mixed breed beef calves (born in 2016) with mature beef cows and Holsteins on day 14 following immunization with an HIR™ Type 2 antigen with their median



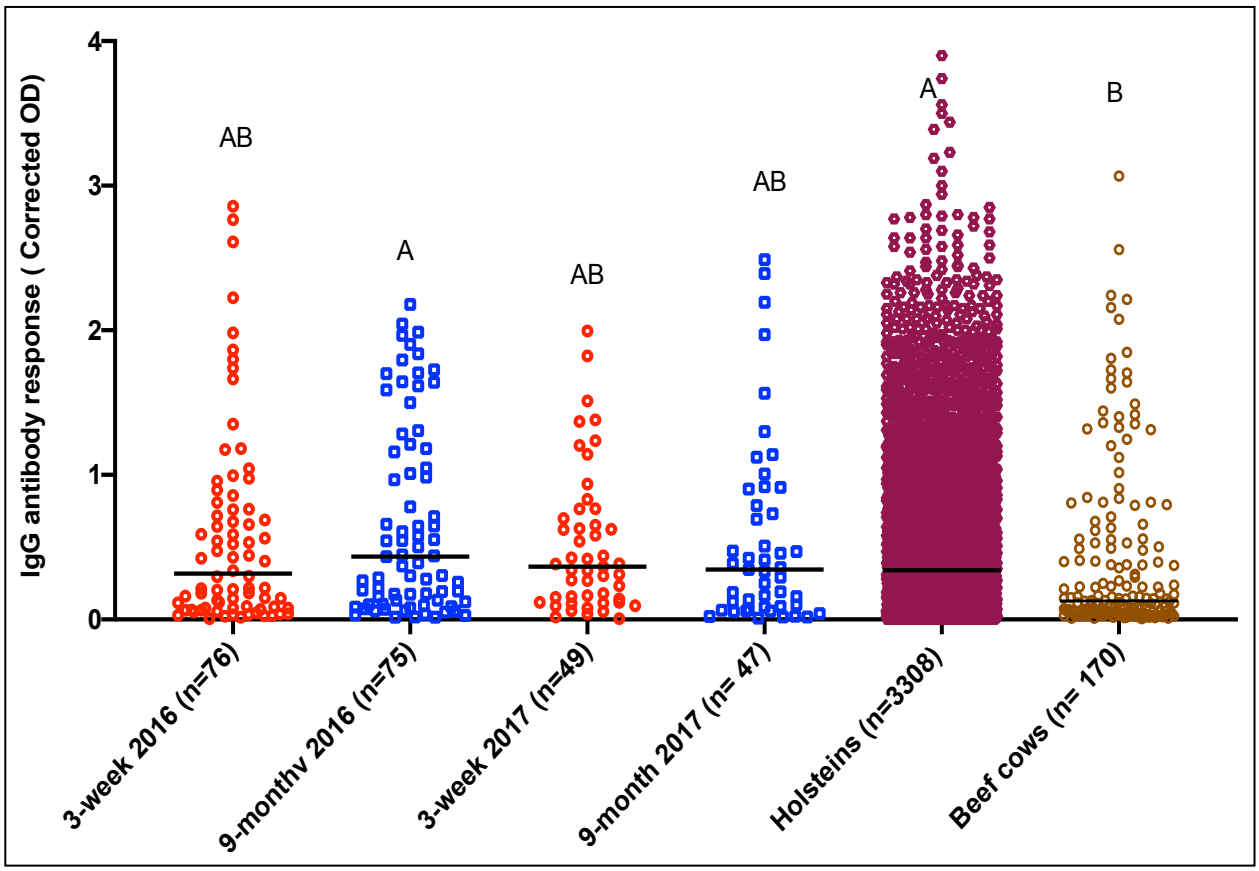
[1] Groups with the same letter do not differ significantly; groups with different letters differ significantly

Figure 2.9- Comparison of serum IgG antibody-mediated immune responses in 1, 2, and 3- week old mixed breed beef calves (born in 2017) on day 14 following immunization with an HIR™ type 2 antigen



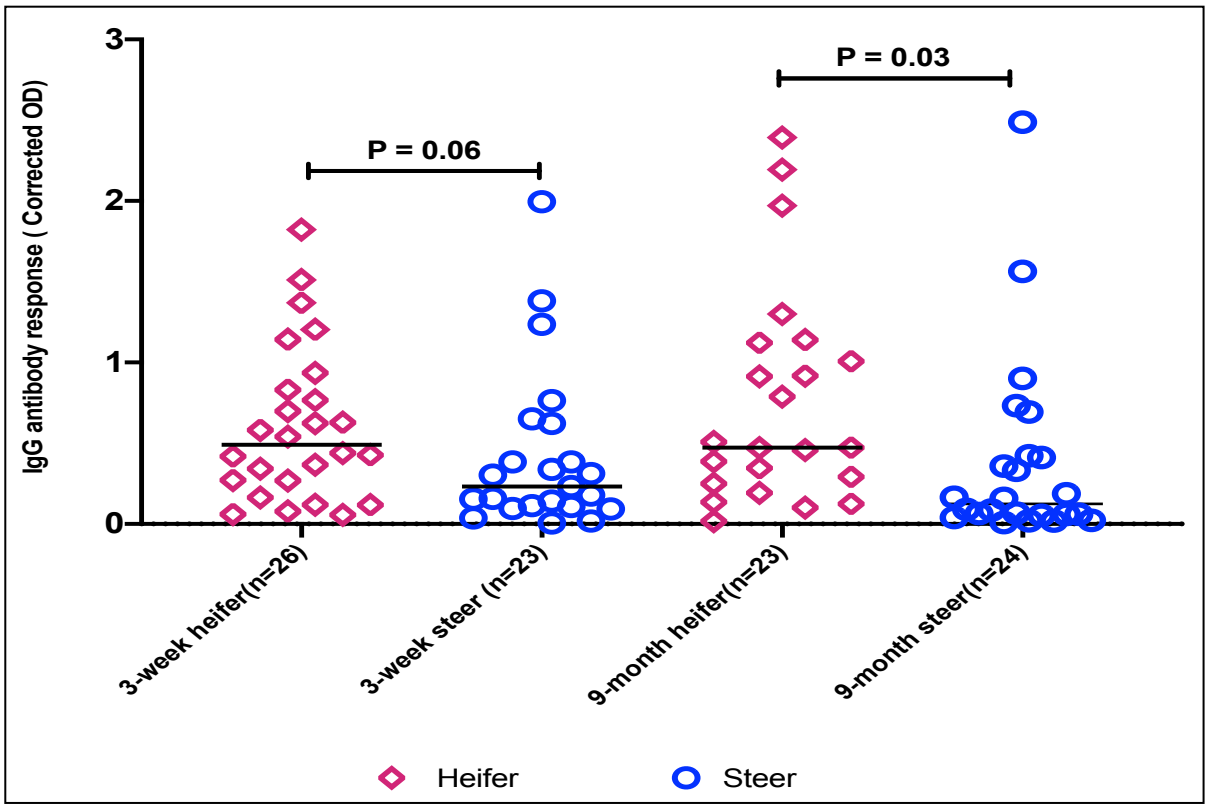
[1] Groups with the same letter do not differ significantly; groups with different letters differ significantly

Figure 2.10- Comparison of serum IgG antibody-mediated immune response sin 3 week and 9-month-old mixed beef calves (born in 2016 or 2017) with mature beef and Holstein cows on day 14 following immunization with an HIR™ Type 2 antigen with their medians



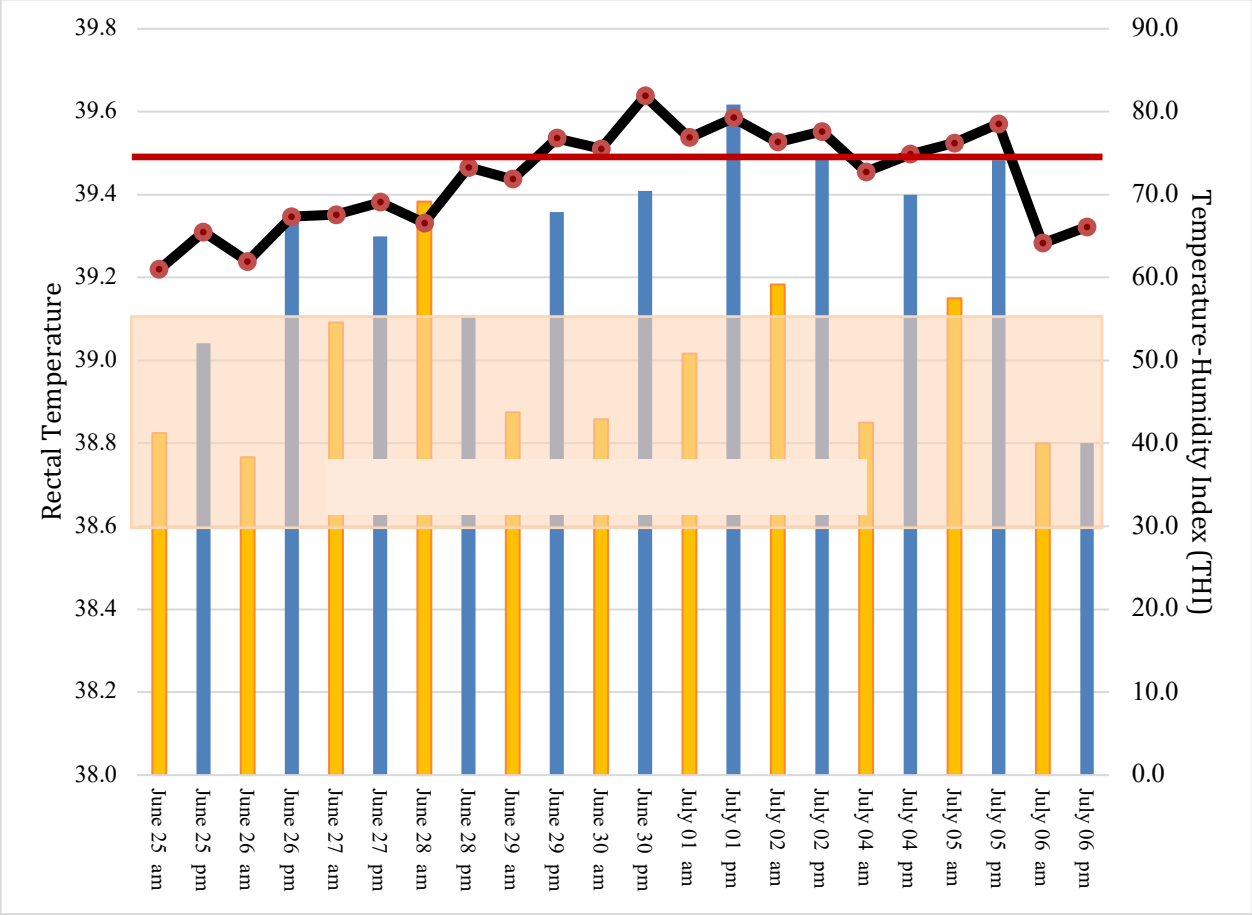
[1] Groups with a letter in common do not differ significantly; groups with different letters differ significantly

Figure 2.11- Comparison of serum IgG antibody-mediated immune responses in 3-week and 9-month-old mixed breed beef heifer and steer calves born in 2017 on day 14 following immunization with an HIR™ Type 2 antigen. No differences were seen between heifers and steers in calves born in 2016.



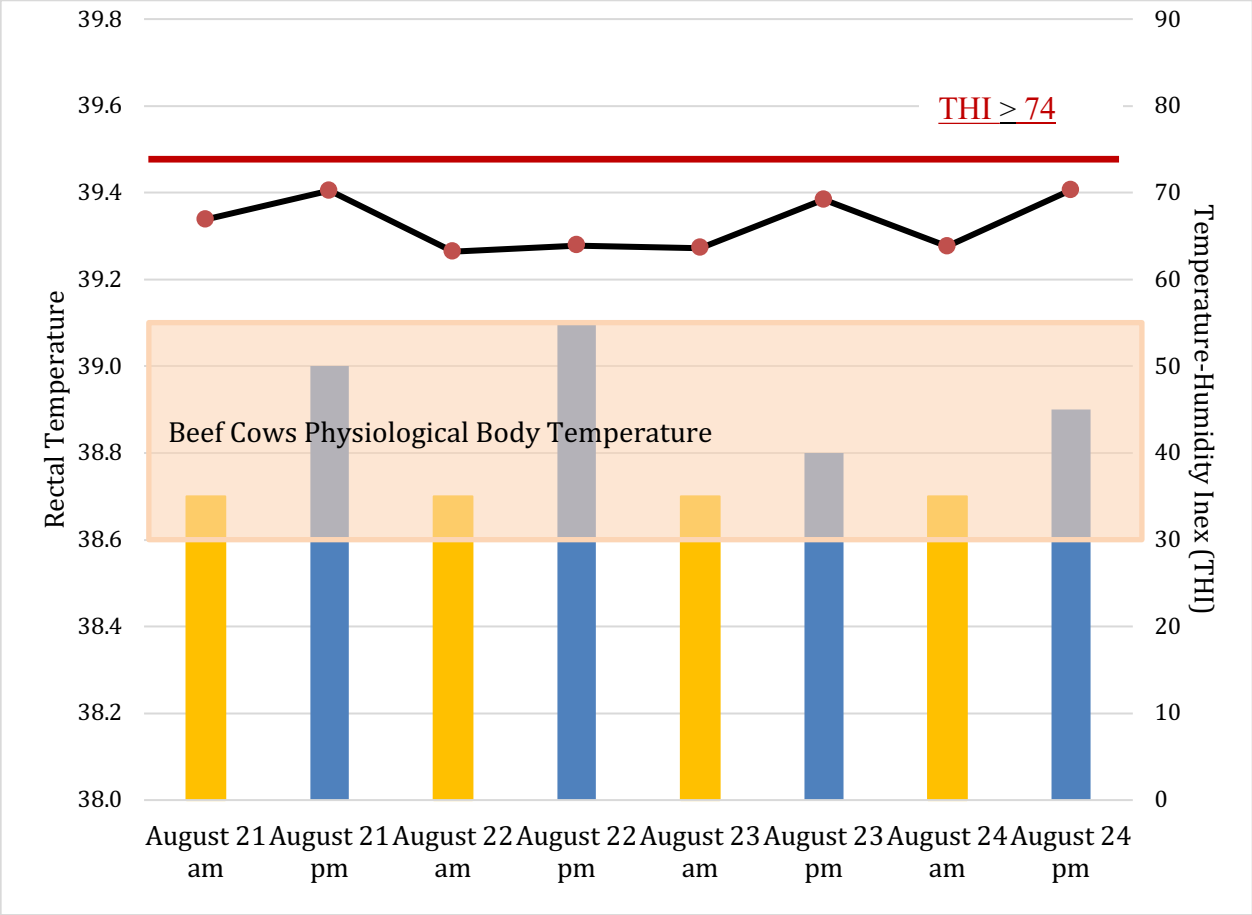
Chapter 3- Figures

Figure 3.5- Average rectal temperatures of group 1 (n=12) mixed breed beef cows with various immune phenotypes measured in morning and afternoon during normal THI and above normal THI from June 25 to July 6



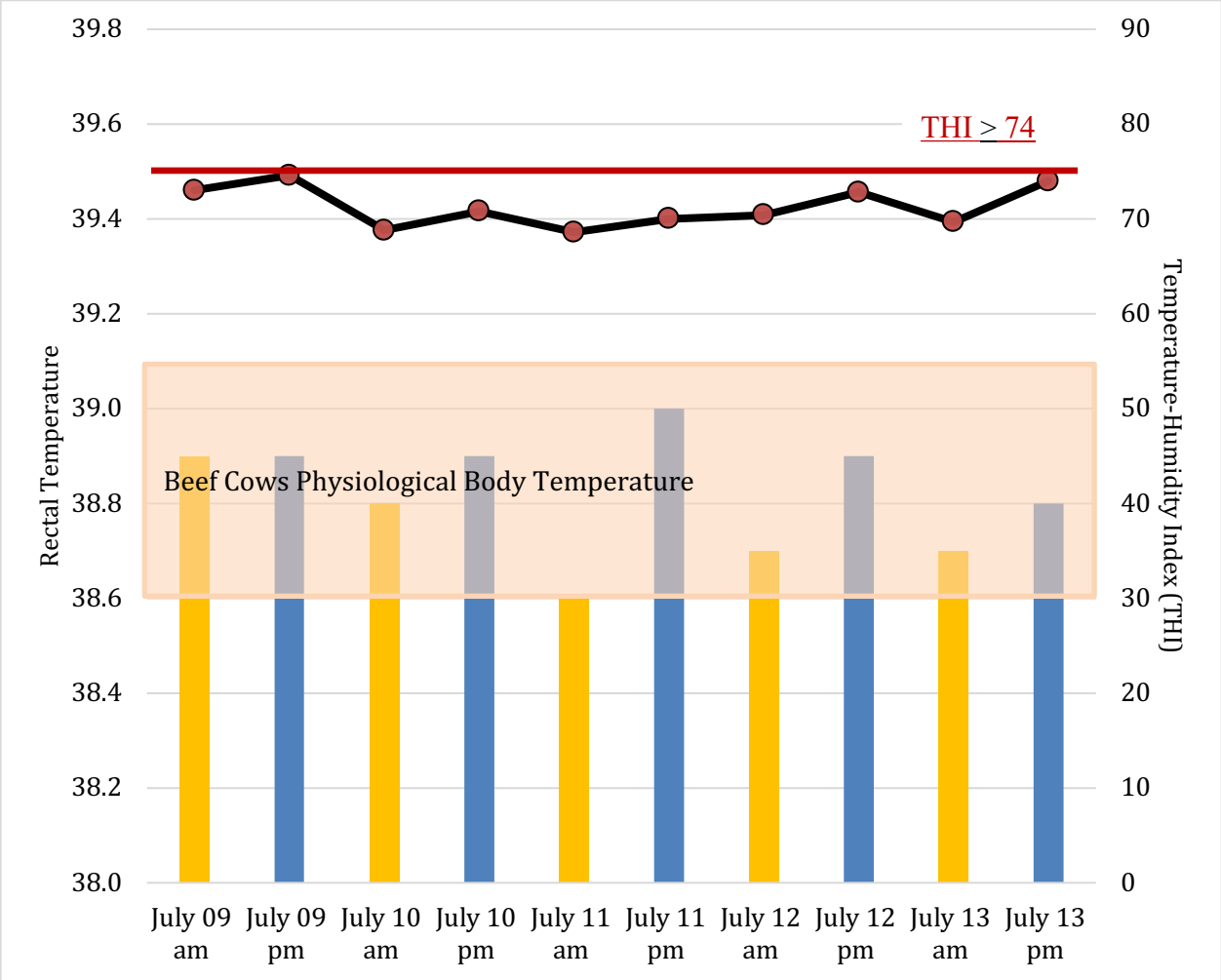
1. Orange bars indicate am rectal temperatures (8 am), and blue bars indicate pm rectal temperatures (3 pm)
2. Red horizontal line indicates the THI = 74
3. Thick black line indicates average daily rectal temperature in the morning and afternoon

Figure 3.6- Average rectal temperature of group 1 (n=12) mixed breed beef cows with various immune phentoypes measured in morning and afternoon during normal THI and above normal THI from August 21 to August 24



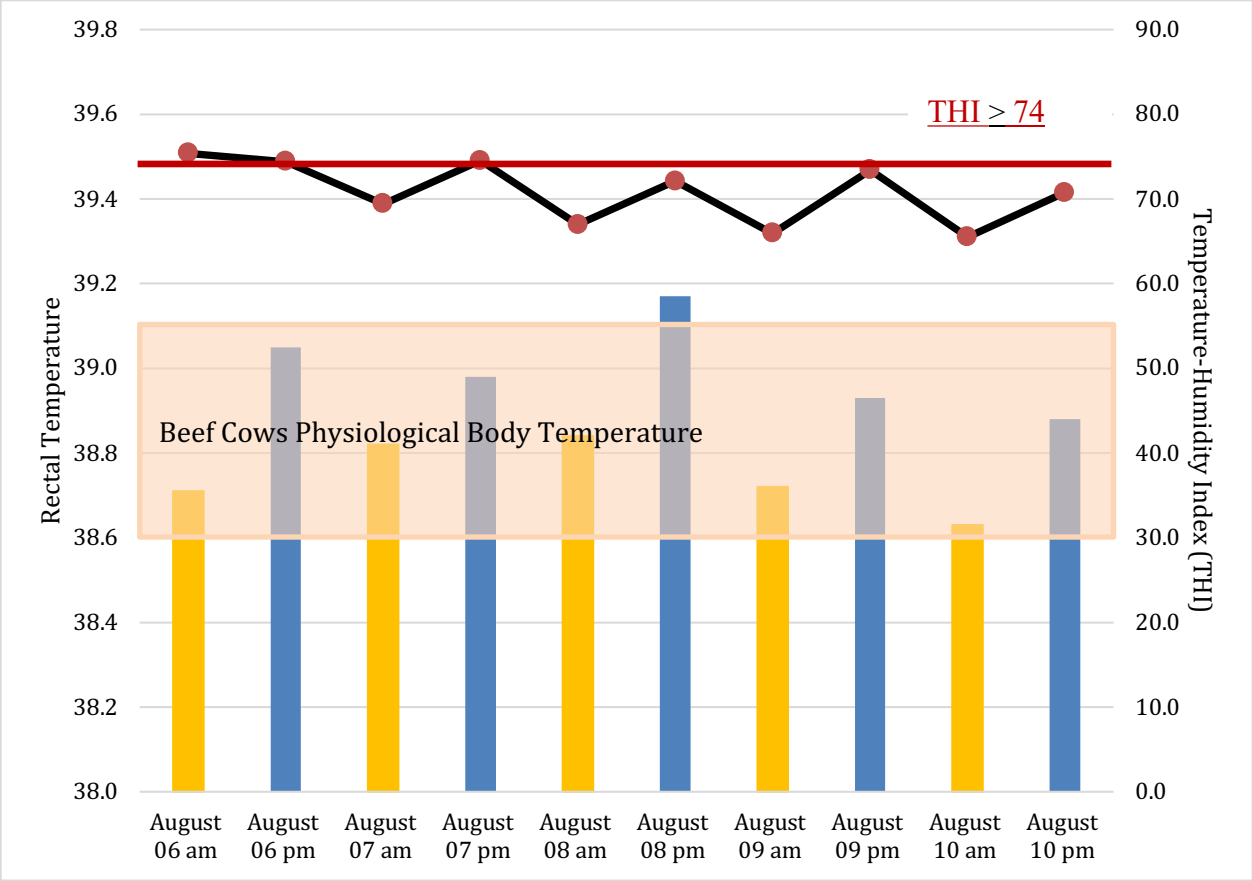
Orange bars indicate am (8 am) rectal temperatures, and blue bars indicate pm rectal temperatures (3 pm)
Red horizontal line indicates the THI = 74
Thick black line indicates average daily rectal temperature in the morning and afternoon

Figure 3.7- Average rectal temperature of group 2 (n=12) mixed breed beef cows with various immune phentoypes measured in morning and afternoon during normal THI and above normal THI from July 9 to July 13



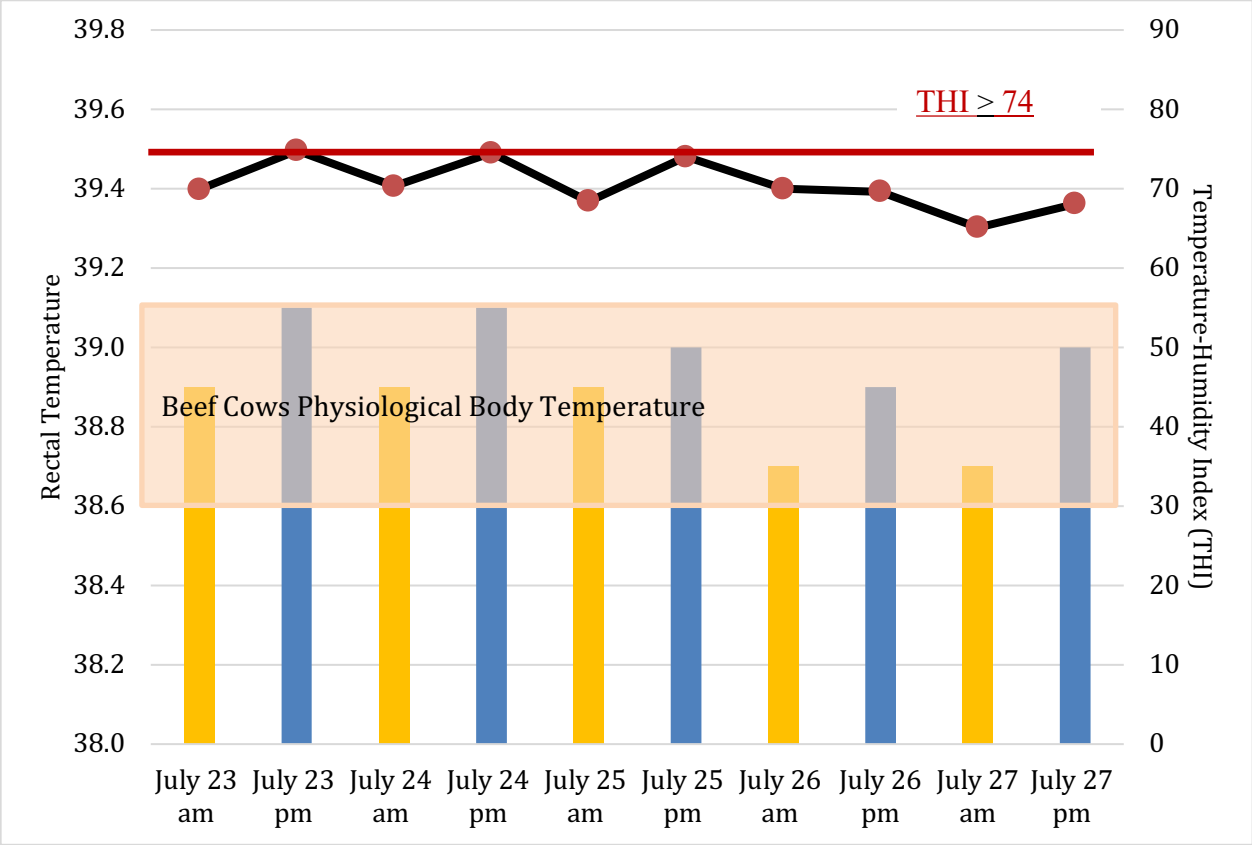
Orange bars indicate am (8 am) rectal temperatures, and blue bars indicate pm rectal temperatures (3 pm)
Red horizontal line indicates the THI = 74
Thick black line indicates average daily rectal temperature in the morning and afternoon

Figure 3. 8- Average rectal temperature of group 2 (n=12) mixed breed beef cows with various immune phentoypes measured in morning and afternoon during normal THI and above normal THI from August 6 to August 10



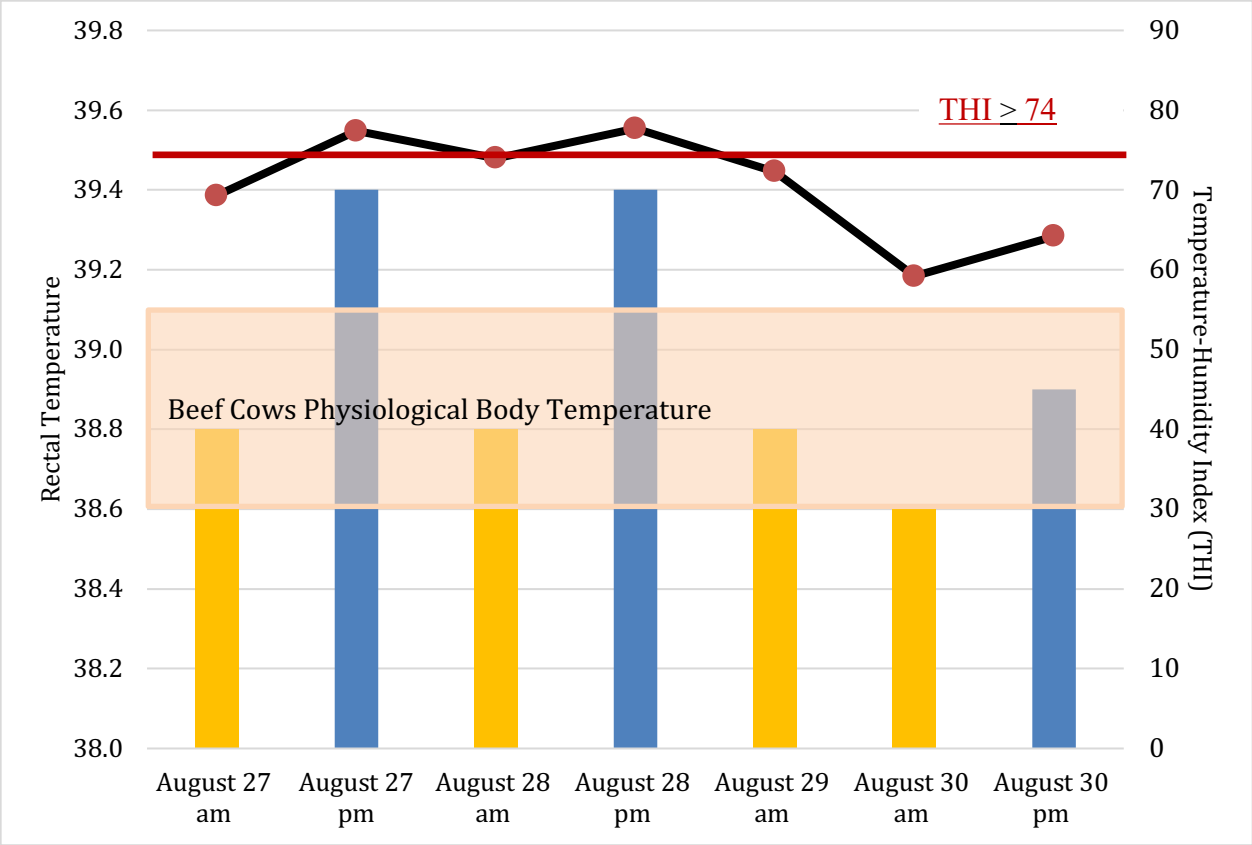
Orange bars indicate am (8 am) rectal temperatures, and blue bars indicate pm rectal temperatures (3 pm)
Red horizontal line indicates the THI = 74
Thick black line indicates average daily rectal temperature in the morning and afternoon

Figure 3.9- Average rectal temperature of group 3 (n=12) mixed breed beef cows with various immune phentoypes measured in morning and afternoon during normal THI and above normal THI from July 23 to July 27



Orange bars indicate am (8 am) rectal temperatures, and blue bars indicate pm rectal temperatures (3 pm)
Red horizontal line indicates the THI = 74
Thick black line indicates average daily rectal temperature in the morning and afternoon

Figure 3.10- Average rectal temperature of group 3 (n=12) mixed breed beef cows with various immune phentoypes measured in morning and afternoon during normal THI and above normal THI from August 27 to August 30



Orange bars indicate am (8 am) rectal temperatures, and blue bars indicate pm rectal temperatures (3 pm)

Red horizontal line indicates the THI = 74

Thick black line indicates average daily rectal temperature in the morning and afternoon