INFLUENCE OF HEAT STRESS ON BOVINE UTERINE HEALTH AND IMMUNE CELL FUNCTION

By

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To my mother, my father, and my grandmother, who are and will always be with me

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BEND	Bovine endometrial epithelial cells
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
CL	Cool
CXCL8	C-X-C Motif Chemokine Ligand 8
d	Day
DIM	Days in milk
DMEM	Dulbecco’s modified Eagle medium
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
DPBS	Dulbecco’s phosphate buffered saline
ECM	Energy corrected milk
ELISA	Enzyme-linked immunosorbent assay
EnPEC	Endometrial pathogenic *Escherichia coli*
FAWN	Florida Automated Weather Network
F	Forward
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HPA	Hypothalamic-pituitary-adrenal
HS	Heat stress
HSF1	Heat shock factor 1
HSP	Heat shock proteins
HSP70	Heat shock protein 70
HSPA1A	Heat shock protein family A member 1A

IFAS	Institute of Food and Agricultural Sciences
IL	Interleukin
LH	Luteinizing hormone
LPS	Lipopolysaccharide
m	Meters
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential media
MIQE	Minimum information for publication of quantitative real-time PCR experiments
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor kappa B
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PLO	Pyolysin
PPR	Pattern recognition receptor
PTA	Predicted transmitting ability
R	Reverse
ROS	Reactive oxygen species
RPL19	60S ribosomal protein L19
rRNA	Ribosomal ribonucleic acid
RT	Retained placenta
RT-PCR	Real-time polymerase chain reaction
siRNA	Small-interfering ribonucleic acid
spp.	Species pluralis
SPSS	Statistical package for the social sciences
THI	Temperature humidity index

TLR	Toll-like receptor
TMR	Total mixed ration
TN	Thermoneutral
TNF	Tumor necrosis factor

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Uterine diseases and heat stress are major challenges for the dairy cow. Though heat stress is known to modulate host immune responses, there is a lack of information on the impacts of heat stress on uterine health. The mechanisms by which heat stress can predispose cows to the development of uterine disease are unknown. Elevated temperatures facilitate proliferation of pathogenic bacteria, including those known to be causative of uterine disease, such as *Escherichia coli*. Bacterial components including lipopolysaccharide, trigger host resilience mechanisms to remediate the negative impacts of the pathogen. During heat stress there is an activation of heat shock proteins that act as chaperones that also contribute to immune function. Heat stress alters host resilience and compromises immunocompetence, which may make cows more prone to develop uterine disease.

To better understand the role of heat stress in the pathophysiology of uterine disease in dairy cows I performed a series of experiments to: 1) assess seasonal variation in the incidence of uterine disease and content of bacteria in the lower reproductive tract; 2) assess the effect of heat stress during the prepartum period on

content of bacteria in the lower reproductive tract and systemic immune responses in postpartum cows; and 3) determine the effect of heat stress on endometrial epithelial innate immunity and attempt to determine the molecular pathways involved in this heat stress mediated immune function.

This dissertation presents a series of studies that demonstrate that the incidence of uterine disease is increased in warmer months but is independent of bacterial content of the lower reproductive tract. I also demonstrate an enduring effect of dry period heat stress that exacerbates peripheral immune responses to bacterial components 3 weeks after heat stress is resolved. In addition, my cell culture model suggests that exacerbated inflammatory responses due to heat stress are also observed in endometrial epithelial cells which seem to be independent of heat shock proteins. I propose that increased uterine disease incidence following heat stress is likely associated with alterations to peripheral and uterine immune resilience mechanisms in the cow.

CHAPTER 1
LITERATURE REVIEW

Uterine diseases are characterized by pathogenic bacteria entering the uterus and causing tissue damage after parturition. During the postpartum period, most cows will have an influx of bacteria into the uterus. However, some animals are able to control the bacterial contamination and remain healthy while others develop uterine disease. Up to 40% of dairy cows will develop uterine disease in the postpartum period (Sheldon et al., 2009), including metritis, endometritis, or subclinical endometritis. Risk factors associated with an increased incidence of uterine diseases include dystocia, stillbirth, twin birth and retained fetal membranes. The impact of uterine disease goes beyond clinical symptoms and lasts long after resolution of disease. Cows that have uterine disease have reduced fertility in that lactation, with more days open, lower conception rates, increased chance of abortion, and increased chance of being culled by reproductive failure (LeBlanc et al., 2002, Fourichon et al. 2000). Moreover, cows with uterine disease produce less milk and are more likely to be culled from the herd (Gröhn & Rajala-Schultz, 2000).

Dairy cows experience reduced fertility during hotter months of the year, with up to a 30% reduction in conception rates compared with cooler months. Heat stress alters ovarian function and gonadotropin secretion resulting in reduced estrus duration, leading to reduced estrus detection and number of inseminations (De Rensis & Scaramuzzi, 2003). Exposure to elevated environmental temperatures triggers a stress response that can modulate the immune system and therefore influence how the cow responds to pathogens and predispose the animals to the occurrence of disease (Bagath et al., 2019). Here we will discuss how heat stress can predispose occurrence

of uterine disease in the cow, focusing on effects on systemic and uterine immune defense to pathogens.

Uterine Disease in Dairy Cows

Definitions of Uterine Disease

High-yielding dairy cows face major challenges when it comes to reproduction, having a high prevalence of uterine disorders and low fertility indices (Lucy 2001, Pryce et al., 2004, Walsh et al., 2011). The intensification of the dairy industry and genetic selection for production increased milk yield by five-fold over the last 5 decades. The increase in milk production observed since the 1960s is associated with increased periparturient metabolic disorders. During the transition period high-yielding dairy cows struggle to ingest enough food to meet the energy required for lactation, entering a period of negative energy balance (Roche et al., 2009). This metabolic stress compromises the immune system and predisposes animals to uterine and other diseases. Specifically, metabolic stress impairs neutrophil function by reducing reactive oxygen species (ROS) production and chemotaxis (Cai et al., 1994). It is estimated that around 40% of postpartum cows will develop some sort of uterine disease, with a mean cost of $513 per case of disease, ranging from $240 to $884, depending on milk price, treatment applied, replacement cost and feed price (Pérez-Báez et al., 2021).

Uterine disease in the postpartum period is classified according to symptoms and the period when symptoms occur. Metritis is an acute inflammation of the uterus that occurs within 21 days of calving, mostly diagnosed within 10 days of calving (Sheldon et al., 2009). It is characterized by an enlarged uterus with a watery brown, often fetid, uterine discharge. Cows with metritis can present systemic clinical symptoms associated with the severity of disease including lethargy, fever, reduced appetite or

even toxemia (Sheldon et al., 2006). According to a survey of 97,318 records from dairy cows in the US, the average incidence of metritis is 21% (Zwald et al., 2004) and risk factors for metritis include retained placenta, dystocia, stillbirth, and twinning.

Endometritis is the inflammation of the endometrial layer of the uterus and occurs after 21 days from parturition and can last until up to 50 days after calving. Clinical endometritis is characterized by mucopurulent vaginal discharge and often lacks other systemic symptoms. Ultrasonography to detected fluid in the uterus and uterine cytology for neutrophil count are often used to differentiate endometritis from vaginitis. Vaginal mucus discharge can be graded according to the amount of pus present to indicate severity of clinical endometritis (Williams et al., 2005). Clinical endometritis incidence ranges from 10 to 20% depending on the herd (LeBlanc et al., 2002). A predisposing factor for the development of clinical endometritis is the previous occurrence of metritis.

Subclinical endometritis occurs when there is inflammation of the endometrial layer with no clinical symptoms or vaginal discharge. Subclinical endometritis diagnosis requires sampling of the endometrium to detect cellular inflammation and is not commonly diagnosed on commercial farms. Subclinical endometritis is somewhat of a silent disease that can persist in the herd for long periods without being perceived, affecting 37% to 74% of cows in the postpartum period (Gilbert et al., 2005). However, cows with subclinical endometritis show decreased reproductive performance due to lower pregnancy rates and delayed conception and are more likely to be culled from the herd compared with healthy herd mates (Gilbert et al., 2005). The cause of subclinical endometritis may be associated with resolution of a previous bacterial infection or postpartum tissue repair.

Pathophysiology of Uterine Disease

During parturition there is a disruption of the endometrial epithelial barrier and a window of opportunity for the entrance of pathogenic bacteria into the uterus from the lower reproductive tract. Pathogen associated molecular patterns (PAMPs) bind to pattern recognition receptor (PRR) located on or in cells of the innate immune system. Of the PRRs, the Toll-like receptor (TLR) family in the cow consists of 10 different receptors that recognize specific PAMPs; TLR1, TLR2, TLR4 and TLR6 recognize bacterial lipids such as lipopolysaccharide (LPS) which is recognized by TLR4, while TLR3, TLR7, TLR8 and TLR9 recognize viral nucleic acids, TLR5 recognizes flagellin and TLR9 recognizes bacterial DNA (Takeuchi & Akira, 2010). Bovine endometrial epithelial and stromal cells express all 10 TLRs that recognize PAMPs and elicit an innate immune response (Davies et al., 2008, Turner et al., 2014, Herath et al., 2006). Activation of TLRs in endometrial cells triggers a signaling cascade via NF-κB and MAPK pathways, resulting in an increased expression of inflammatory mediators and an influx of immune cells to the uterus (Akira et al., 2006, Gilbert & Santos 2016). Recognition of LPS by TLR4 in the bovine endometrium *in vitro* increases expression of genes related to the innate immune function such as cytokines IL1A, IL1B, IL6, TNF and the chemokine CXCL8 (Herath et al., 2009, Cronin et al., 2012). Furthermore, uterine fluid of cows with clinical endometritis has higher concentrations of tumor necrosis factor-alpha (TNFα), interleukin (IL)-1β, IL-6 and IL-8 compared with uterine fluid collected from healthy cows (Kim et al., 2014). Inflammatory cytokines bind to their respective receptors and stimulate production of antimicrobial peptides and reactive oxygen species and cause further inflammation. Chemokines are important to further attract leukocytes to the site of infection to phagocytize and kill pathogenic bacteria.

When augmented in the peripheral blood, cytokines trigger systemic inflammation, causing fever, lethargy and decreased feed intake, signs commonly seen in cows with clinical uterine diseases.

Pathogens Associated with Uterine Disease

The uterus was previously considered a sterile environment, protected by the physical barrier of the cervix. However, the bovine uterus is physiologically a non-sterile environment. Bacterial 16S rRNA is detected in the uterus of virgin heifers and pregnant cows, indicating that the presence of bacteria in the uterus does not prevent establishment and maintenance of pregnancy (Moore at al., 2017). Among bacteria detected in the uterus of pregnant cows, some species associated with the development of uterine disease have been identified, including *Fusobacterium necrophorum* and *Trueperella pyogenes* (Karstrup et al., 2017). However, the development of uterine disease relies on a disbalance between the cow's resilience mechanisms and the pathogenicity of the bacteria. Risk factors such as retained placenta, metabolic stress and impaired immunity can affect this balance and predispose pathogen proliferation and occurrence of disease.

Using culture-based methods, pathogens isolated from cows with uterine disease include *Escherichia coli*, *T. pyogenes*, *F. necrophorum*, and Prevotella spp. (Williams et al., 2005, Griffin et al., 1974, Bonnett et al., 1991, Huszenicza et al., 1991). Recently, the use of metagenomic techniques has allowed for the identification of other bacteria phyla in cows with uterine disease, such as Bacteroidetes and Firmicutes, that could not be previously identified using culture-base methods and were not previously associated with uterine disease (Machado et al., 2012, Peng et al., 2013).

Nonpathogenic Gram-negative *E. coli* are normal commensal organisms of the digestive tract in humans and cattle that are shed in feces of healthy individuals (Houser et al., 2008, Bettelheim et al., 2004). Different strains of *E. coli* express various virulence factors that can lead to disease. The presence of *E. coli* in the uterus has been strongly associated with the development of uterine disease in dairy cows for decades (Sheldon et al. 2002, Williams et al. 2005). Endometrial pathogenic *E. coli* (EnPEC) isolated from the uterus of cows with uterine disease during the postpartum period are genotypically different from *E. coli* strains found in the gut or mammary gland, are more invasive to endometrial cells *in vitro*, and result in an exacerbated immune response in the uterus (Sheldon et al., 2010).

T. pyogenes is a Gram-positive bacterium that can be found in the mammary gland, gut, and respiratory tract of livestock. When present in the uterus of the dairy cow, *T. pyogenes* has been associated with severity of uterine disease (Bonnet et al., 1991). The virulence factor of *T. pyogenes*, pyolysin (PLO), is a cholesterol dependent cytolysin that forms pores in the host cell membrane leading to osmotic cell death (Jost & Billington, 2005). Bovine endometrial stromal cells have a high cholesterol content, making them very susceptible to pyolysin induced damage (Amos et al., 2014). The disruption of the protective epithelial layer during parturition exposes stromal cells to *T. pyogenes* and pyolysin and predisposes the underlying endometrial layers to tissue damage in the postpartum uterus.

F. necrophorum is an obligatory anaerobic Gram-negative bacterium commonly present in the digestive tract of mammals. The presence of *F. necrophorum* in the bovine reproductive tract between 8 and 10 days postpartum is associated with a two-

fold increase in the prevalence of metritis (Bicalho et al., 2012). There is also evidence that *F. necrophorum* may also have a synergistic effect with *Prevotella melaninogenica* and T. pyogenenes, increasing the severity of uterine disease (Olson et al., 1984, Ruder et al., 1981).

Gram-negative *P. melaninogenica* is an important pathogen in infections of the upper respiratory tract. *P. melaninogenica* belongs to the Bacteroidetes phyla, and it was previously named Bacterioides melaninogenicus. *P. melaninogenica* is commonly isolated from cows with uterine disease and often correlated with increased vaginal discharge (Williams et al., 2005, Dohmen et al., 1994, Cunha et al., 2018). Cunha et al. (2018) observed that while the absolute abundance of *P. melaninogenica* is 3 times lower than other uterine pathogens, it is increased in cows with metritis, suggesting that low abundance of *P. melaninogenica* is enough to trigger damage or promote disease development by synergistic action with more abundant pathogens.

Reproductive Consequences of Uterine Disease

After parturition, uterine involution, endometrial tissue regeneration and resumption of ovarian cycle take place to prepare the reproductive tract for another pregnancy. Approximately 50 days after calving, a dairy cow may be bred again to optimize milk production in the following lactation. However, cows that have purulent vaginal discharge are more likely to have a delayed return to cyclicity, and increased days open compared to healthy cows (Opsomer et al., 1999). Cows with uterine disease have lower conception rates, increased pregnancy loss and are more likely to be culled from the herd than cows that did not have disease in the postpartum period (LeBlanc et al., 2002, Ribeiro et al., 2016). The impact of uterine disease on reproduction persists even after antimicrobial treatment and resolution of clinical signs. Intrauterine infusion of

ceftiofur 44 days after parturition decreased bacterial prevalence in the uterus but did not rescue reproductive performance of cows that had metritis or endometritis (Galvão et al., 2009). It is hypothesized that uterine disease negatively impacts subsequent reproductive performance by perturbing mechanisms involved in endometrial function, ovarian function, oocyte developmental competence and hypothalamic-pituitary function – all of which could affect fertility.

Cows that had prior uterine disease have an accumulation of LPS in follicular fluid even after resolution of disease, which could be associated with a delayed return to cyclicity and subfertility in these animals (Herath et al., 2007, Cheong et al., 2017, Piersanti et al., 2019). Piersanti et al. (2020) and Dickson et al. (2020) demonstrate that oocytes collected from cows that had uterine disease have an altered transcriptome and impaired developmental capacity compared to oocytes from healthy cows. Ribeiro et al. (2016) showed that embryo transfer was not able to rescue pregnancy rates in cows that previously had uterine disease, indicating an impaired uterine capacity to sustain a pregnancy. Uterine disease is also associated with delayed uterine involution (Sheldon et al., 2004). Cytological examination of the endometrium showed that around 40% of cows did not resolve inflammation by 46 days in milk (DIM) (Lima et al., 2013). If chronic inflammation is established, pro-inflammatory cytokines released by the endometrium in response to bacterial infections can cause further tissue damage and interfere with implantation and embryonic biology.

Heat Stress

Definition and Consequences of Heat Stress for the Dairy Cow

Heat is essential to life as it provides energy to execute chemical reactions that sustain physiological functions. Endothermic homeotherms, such as cattle, can

generate their own heat and regulate body temperature to sustain physiological functions in a wide range of thermal environments like extreme heat or cold. This characteristic requires several physiological adaptations that occur depending on climate such as thickness of fur, changes in posture, body conformation and surface contact with the environment.

Environmental stress can be defined using a stress-strain analogy, where stress is a force applied to an object and strain is the amount of deformation caused in the object by this force. In animal physiology, stress is a force displacing the animal from its baseline and strain is the animal's adaptation trying to restore homeostasis. Any threat to the homeostasis of an organism will activate systems to restore balance, called the adaptive response. When a cow is exposed to temperatures higher than its thermoneutral zone it activates heat loss mechanisms such as sweating and limits generation of heat by decreasing movement and feed intake. When those mechanisms are not enough to maintain normal body temperature, the cow fails to adapt and heat stress occurs.

Dairy cows exposed to heat stress have increased body temperature, respiration rate and sweating, and decreased dry matter intake (DMI) (Collier et al., 2006, Collier et al., 2017). Heat-stress accentuates negative energy balance in the postpartum period and decreases milk yield even further. Interestingly, the impact of heat stress on milk production is not completely due to decreased dietary intake, as Wheelock et al. (2010) showed in study using a pair fed model, wherein reduced DMI accounted for 50% of the decreased milk production in heat stress cows, while 50% of heat stress induced milk loss is related to other mechanisms not yet elucidated. Reproductive performance is

also impaired during heat stress, resulting in reduced estrus expression, lower conception rates, and lower pregnancy rates compared to thermoneutral cows. Economic losses due to heat stress-induced reduction in productivity and performance have been estimated around $1 billion per year for the US dairy industry (St-Pierre et al., 2003).

To measure heat stress, one can use the temperature humidity index (THI) that accounts for environmental temperature and relative humidity, as it is easily calculated with available meteorological data. For dairy cattle, when the average THI is greater than 68 physiological signs of mild heat stress and decreased milk production are observed (Zimbelman et al., 2009).

Impact of Heat Stress on Reproduction

Cows exposed to heat stress have lower reproductive efficiency. Heat stress decreases estrus duration and expression in dairy cows, making it more difficult to detect cows that can be bred. Some studies have shown that cows exposed to heat stress have a reduced circulating LH and a decrease in LH pulse frequency and amplitude, in addition to reduced plasma progesterone and estradiol concentrations compared to thermoneutral cows (Ronchi et al., 2001, Rosenberg et al., 1977, Madan & Johnson, 1973, Wise et al., 1988, Gilad et al., 1993, Wolfenson et al., 1997). The heat-stress induced imbalance in reproductive hormones can compromise follicular development, resulting in implantation failure and early embryonic death (Wolfenson et al., 1995, Wilson et al., 1998, Lamming & Royal, 2018, Hansen 2019).

Beyond the effects in the ovary, elevated environmental temperatures increase uterine temperature and decrease uterine blood flow (Gwazdauskas et al., 1973, Roman-Ponce et al., 1978). Fertilization of oocytes and maturation of zygotes at 41°C

decreased cleavage rate and blastocyst rate when compared to oocytes and zygotes cultured at 38.5°C (Rivera & Hansen, 2001). Maternal hyperthermia during fetal development has lifelong consequences for the offspring. Calves from dams exposed to heat stress during late gestation have lower birth weight and compromised passive immune transfer compared with calves born to cooled dams (Tao et al., 2012). Moreover, daughters of heat stressed cows produce 5.1 kg/d less milk during their first lactation relative to heifers born to cooled cows (Monteiro et al., 2016). Thus, the consequences of heat stress exposure can persist for a long time in a herd.

Reproductive technologies such as fixed timed artificial insemination and embryo transfer can be used to avoid some of the negative impacts of heat stress on reproductive performance. Indeed, Hansen (2007) reviewed different strategies to bypass pregnancy loss due to heat stress, and embryo transfer effectiveness in rescuing pregnancy rates during heat stress has been well stablished in studies performed in Brazil and Florida (Al-Katanani et al., 2002, Rodrigues et al., 2004, Putney et al., 1989). However, these approaches of achieving a pregnancy do not overcome the previously mentioned impacts of *in utero* heat stress that compromise postnatal performance of offspring. An efficient management approach to avoid the effects of heat stress is to minimize exposure of cows to elevated temperatures. Recent analyses evaluated the economic feasibility of implementing cooling systems into barns and concluded that investing in heat abatement strategies alleviates the milk yield loss during heat stress across multiple stages of the lactation cycle and generates economic profit for producers in the long term (Gunn et al., 2019, Ferreira et al., 2016).

Heat Stress and Immune Function

Heat stress is known for its plurality of negative effects on dairy cattle production. Besides its effects on milk yield and reproduction, there is strong evidence that exposure of cows to heat stress causes remarkable alterations in the immune system throughout the life cycle of the animal. A large-scale study with over 2,600 calving events analyzed the effect of seasonality on the occurrence of postpartum disorders up to 60 DIM and found that cows that were dried-off during warmer months had a higher incidence of mastitis, respiratory problems, and retained fetal membranes compared to cows dried-off during cooler months (Thompson & Dahl, 2012). In accordance, Gernand et al. (2019) analyzed 22,212 Holsteins cows and found a positive association between increased peripartum THI and incidence of mastitis, reproductive disorders, and retained fetal membranes up to 10 days postpartum.

When there is an imbalance between pathogenic and host factors disease can occur. Environmental factors such as heat stress can influence this balance and predispose animals to disease. Changes in temperature during different seasons of the year can favor disease outbreaks by providing optimal growth condition for different pathogens (Altizer et al., 2006, Fisman et al., 2007). In humans, Gram-negative bacteria blood infections are increased by 50% in the summer compared with winter depending on the pathogen (Eber et al., 2011). Rowbotham and Ruegg (2016) reported higher counts of total Gram-negative bacteria, coliforms and *Kleibsiella spp*. in cow bedding samples collected during summer compared to other seasons, suggesting warmer environmental temperatures increase pathogen abundance.

For dairy cows, heat stress in the dry period results in decreased neutrophil phagocytosis and oxidative burst in early lactation (do Amaral et al., 2011). Relative to

cows that calved in the spring, where average THI was below 72, cows that calved during summer, where average daily THI was 79.5, had reduced peripheral blood mononuclear cells proliferation when exposed to extreme heat stress (Lacetera et al., 2005). Humoral responses to ovalbumin challenge are impaired in heat stressed dry cows, which produce less IgG relative to cooled cows (do Amaral et al., 2011). In contrast, Lacetera et al. (2005) reported an increased IgM secretion during the periparturient period in response to antigen in PBMCs from heat stressed cows. Contradictory effects observed during heat stress may be due to different definitions of season, length and intensity of heat stress exposure and physiological status of the animals when exposure to heat stress occurs.

Short-term stress induces a physiological response fundamental to survival. As such, short-term stress experienced during immune activation induces an enhancement of the immune response. Conversely, long-term stress suppresses and dysregulates the immune system, exacerbating and predisposing disease incidence (Dhabhar, 2014). Systemic effects of heat stress are mediated by activation of the hypothalamic-pituitary-axis that results in the release of stress hormones that have receptors in a variety of tissues and cells. In cattle, the main stress hormones, cortisol and prolactin, are increased after exposure to elevated temperatures (Ronchi et al., 2001, do Amaral et al., 2010). Cortisol and prolactin exert effects on the immune system and modulate the heat shock response by inducing heat shock proteins (HSPs) (Collier et al., 2008).

Heat shock proteins are a family of proteins activated in response to stressors. Many members of this family are chaperone proteins, responsible for preventing cellular damage and maintaining correct protein folding. Some members of the HSP family,

including HSP70 (encoded by HSP1A1), are involved in antigen presentation and induction of pro-inflammatory cytokines (Binder et al., 2014). In pigs and humans, chronic heat stress exacerbates the inflammatory response via upregulation of TLR2 and TLR4 (Ju et al., 2014, Zhou at al., 2005). Recently, Marins et al. (2021) reported that peripheral blood mononuclear cells from heat stressed cows increased production of IL-10 and TNFα in response to LPS. *In vivo* assessment of the bovine endometrium showed an increased expression of inflammatory cytokines during summer compared to samples collected in the winter (Bai et al., 2020). Moreover, endometrial epithelial cells exposed to heat stress *in vitro* increase IL-6 secretion in response to LPS compared to thermoneutral cells whereas endometrial stromal cells decrease IL-6 secretion (Sakai et al., 2021). Collectively, this suggests that heat stress modulates the systemic and endometrial defense to pathogens by exacerbating or prolonging the immune response.

Objectives for this Program of Study

Although heat stress has been shown to negatively impact dairy cattle reproduction and disease incidence, there is lack of information on how elevated environmental temperature impacts uterine resilience mechanisms to pathogens. The overall objective of the studies reported in this dissertation was to determine the impact of heat stress on uterine disease incidence and resilience mechanisms in dairy cattle. Using retrospective data, the seasonal variability in uterine disease incidence was evaluated followed by assessment of vaginal bacterial content during winter and summer (Chapter 2). Due to seasonal variations beyond environmental temperature, additional experiments to evaluate systemic immune response to pathogens and vaginal bacterial content was assessed in a controlled setting of heat stress during the prepartum period (Chapter 3). Finally, the effect of acute heat stress on the immune

function of endometrial epithelial cells was evaluated *in vitro* (Chapter 4). Unraveling the role of heat stress and the molecular pathways by which elevated temperatures predispose cows to uterine disease will allow for the development of interventions to improve dairy cattle resilience to uterine disease.

CHAPTER 2
EFFECT OF CALVING SEASON ON UTERINE DISEASE INCIDENCE AND BACTERIAL CONTENT OF THE VAGINA IN DAIRY COWS

Postpartum uterine disease is a common problem for the dairy industry. Uterine disease is associated with decreased milk yield and reduced reproductive performance. It is estimated that 40% of cows will develop uterine disease in the postpartum period, including metritis and endometritis (Sheldon et al., 2019). Even after the resolution of clinical signs, cows with metritis produce less milk in the subsequent lactation, take longer to become pregnant and have higher abortion rates compared with healthy counterparts (Mahnani et al., 2015, Sheldon et at., 2009). Cows with endometritis have lower conception rates, increased days open and higher culling due to reproductive failure after successful treatment (LeBlanc 2008, Sheldon et al., 2009). Combining treatment cost, milk loss, decreased fertility, culling and animal replacement, uterine disease costs the United States dairy industry between $650 million and $900 million per year (Sheldon et al., 2009, Lima et al., 2019).

Uterine diseases are characterized by the presence of pathogenic bacteria in the uterus following calving that cause localized tissue damage and inflammation. Bacteria routinely cultured from cows with uterine disease include *Escherichia coli*, Truperella pyogenes, *Fusobacterium necrophorum,* and *Prevotella melaninogenica* (Griffin et al., 1974, Bonnett et al., 1991, Huszenicza et al., 1991, Williams et al., 2005). Nonetheless, metagenomic techniques have revealed associations between uterine disease and other bacteria phyla that are challenging to culture using standard techniques or are not conventionally associated with uterine disease, such as Bacteroidetes and Firmicutes (Machado et al., 2012, Peng et al., 2013). Epithelial and stromal cells of the endometrium detect bacteria using Toll-like receptors that result in an innate

inflammatory response, characterized by increased production of proinflammatory cytokines and chemokines including interleukin (IL)-1β, IL-6 and IL-8 (Cronin et al., 2012).

In addition to the negative consequences of uterine disease on dairy production, heat stress decreases fertility and imparts negative consequences on offspring that were exposed to heat stress *in utero* (Negrón-Pérez et al., 2019, Ouellet et al., 2020). Heat stress of dairy cows is defined as environmental temperatures that exceed the thermoneutral zone of the cow, making it difficult for the animal to effectively dissipate heat to the environment and maintain normal body temperature.

The role of environmental temperatures on the pathogenesis of uterine disease in dairy cows is unknown. There is evidence that elevated temperatures increase pathogen proliferation and predispose infectious disease incidence. For example, during summer months bacterial loads are higher in cow bedding and on teat skin compared to winter months, which is associated with increased occurrence of mastitis (Rowbothan & Ruegg, 2016, Hogan et al., 1989). While these data suggest that elevated environmental temperatures favor pathogen loading in the environment, it is unclear if the prevalence of uterine disease is affected by environmental season. Increased environmental temperature could increase pathogenic bacterial prevalence or alter host immune function, both of which could impact the prevalence of uterine disease in the dairy cow.

Here, we hypothesized that seasonal variation in environmental temperature influences uterine disease incidence and severity in the dairy cow. To address this hypothesis, we evaluated uterine disease incidence in lactating dairy cows at a single

location in Florida during the winter and summer, while simultaneously evaluating bacterial content of the lower reproductive tract. This study determined the impact of season on uterine disease incidence, while evaluating if uterine disease was associated with seasonal variations of vaginal bacterial content in the dairy cow.

Materials and Methods

Influence of Season on Metritis Incidence

Data for retrospective analysis was obtained from the University of Florida Dairy Research Unit management software Afifarm database (SAE Afikim, Israel). The data included all calving events from January 1st, 2012, through December 31st, 2017 (n = 3,507). For each calving, cow ID, calving date, estimated 305-milk production, occurrence of clinical metritis and cow's lactation number were recorded. Metritis was characterized by a watery red-brown uterine discharge with foul smell and an enlarged uterus diagnosed between d 4 and 13 after calving. Cows that aborted were excluded from the analysis. Metritis incidence was calculated as the proportion of cows that developed clinical metritis divided by the total cows that calved during that month.

Retrospective weather data was collected from the Alachua Station of the Florida Automated Weather Network (FAWN, IFAS-University of Florida), located approximately 1.8 miles from the University of Florida Dairy Research Unit. Daily observation of air temperature (2 m) and relative humidity from the analyzed period were used to calculate the temperature-humidity index (THI) based on the equation developed by the National Research Council (1971) and recommended by Dikmen and Hansen (2009):

$$THI = (1.8 \times \mathrm{T} + 32) - [(0.55 - 0.0055 \times RH)] \times (1.8 \times T - 26) \quad (2\text{-}1)$$

where T = air temperature (°C) and RH = relative humidity (%). Calving between April 1st and September 30th was defined as the warm period and calving between October 1st and March 31st was defined as the cool period.

Animal Enrollment During Winter and Summer

All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee. To observe the development of uterine disease and assess bacterial content in the lower reproductive tract, pregnant Holstein cows or heifers were sequentially enrolled at the University of Florida Dairy Research Unit during the summer (September 2017; n = 51) or winter (February to March 2018; n = 51). All animals were housed on pasture without heat abatement until approximately 3 weeks before their expected calving date at which time animals were moved to a free stall barn with fans and water soakers, receiving TMR twice a day and *ad libitum* water (Fig. 2-1A). Animal health and uterine disease were assessed on d 7 and d 21 postpartum. Milk production data and health events until 60 DIM were collected using farm management software (Afifarm). The average 5-day prepartum and 5-day postpartum THI were calculated for each animal using the daily reports of air temperature and relative humidity obtained from the FAWN Alachua Station.

Collection and Grading of Vaginal Mucus

Vaginal mucus was sampled on d 7 and 21 after parturition from each cow. Briefly, the vulva was thoroughly cleaned using 70% ethanol and paper towel prior to the insertion of a sterile Metricheck tool (Simcro, Hamilton, New Zealand). The Metricheck tool consists of a stainless-steel rod with a rubber collector cup on the tip. Once inserted in the vagina the tool was gently moved in a consistent manner to sample the whole vaginal canal, collecting mucus from the ventral, dorsal and lateral portions.

After careful removal of the tool, the sampled content was examined and placed in a sterile bijou tube (Thermo Fisher Scientific, Waltham, MA). The mucus was graded based on the proportion of pus and scored from 0 to 4. Score 0 = clear vaginal mucus; score 1 = mucus with flecks of white pus; 2 = mucus with less than 50% white pus; score 3 = mucus with more than 50% white pus; score 4 = sanguinopurulent mucus (adapted from Sheldon et al., 2009; Fig. 2-1B). Cows were classified as having uterine disease if d 7 vaginal mucus grade ≥ 3 or d 21 grade ≥ 2. Samples were maintained on ice and transported to the laboratory and stored at -80°C for further analysis.

Bacterial DNA Isolation and Quantification of Bacterial Content

Total bacterial DNA was isolated from vaginal mucus samples using the DNeasy Power Soil kit (Qiagen, Germany) as previously described (Piersanti et al., 2019). Briefly, mucus samples were thawed on iced and then homogenized by vortexing for 5 min. A total of 250 mg of each sample was added to a tube containing garnet particles and guanidine thiocyanate and placed in a tissue homogenizer (Precellys 24, Bertin Technologies SAS, France). Samples were homogenized using 3 cycles (30 s at 6,000 × *g*, 60 s pause, 30 s at 6,000 × *g*) with a 5 min incubation on ice in between each cycle. Supernatants were then collected and added to the DNeasy Power Soil spin columns for purification of DNA following the manufacturer's instructions. Purified DNA was used to quantify total bacterial content using the Femto Bacterial DNA Quantification Kit (Zymo Research, CA) that targets 16S rRNA. Briefly, real time quantitative PCR was performed using the primer mix from the commercial kit containing SYTO 9 fluorescent dye and primers targeting 16s rRNA in a 20 µL reaction containing 2 µl of total extracted DNA in each well. A CFX Connect Real-Time PCR System (Bio-Rad Laboratories) was used for a 3-step protocol of initial denaturation at

95°C for 10 min, amplification consisting of 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. Quantification of 16S rRNA was based on the standard curve provided with the kit performed in parallel with the samples. All reactions were performed in duplicate and no-template controls were included. Results are described as nanograms of 16S rRNA per milligram of vaginal mucus. The extraction of 16S rRNA from mucus samples was validated by a spike-in/recovery method using known amounts of purified bacteria prior to the analysis of the samples. Intra-assay and inter-assay coefficient of variation were 0.3% and 2.2% respectively, with a recovery of 100.5% of expected 16S rRNA after extraction of spike-in mucus.

Detection of Specific Bacteria in Vaginal Mucus

Vaginal content of pathogenic bacteria associated with uterine disease was performed using DNA isolated from mucus samples above. Specific primers for pathogens associated with uterine disease, *E. coli, T. pyogenes, F. necrophorum* and *P. melaninogenica* (Williams, 2005), were designed using the NCBI primer-design tool or previously published and verified by BLAST (Malinen et al., 2003, Belser et al., 2015, Cunha et al., 2018) (Table 2-1). All primers were validated for amplification efficiency prior to sample analysis and conformed to MIQE guidelines (Pearson correlation coefficient $R^2 > 0.98$ and efficiency between 90 and 110%) (Bustin et al., 2009). Quantitative real time PCR was performed in 20 µL reactions containing 18 µL of SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA) with 300 nM of each forward and reverse primer and 2 µL of template DNA. PCR was performed using a CFX Connect Real-Time PCR System (Bio-Rad Laboratories) with a 3-step protocol with enzyme activation at 95°C for 3 min followed by amplification with 40 cycles of

denaturation at 95°C for 10 s, annealing between 53.5 and 64.5°C for 10s depending on the primer (Table 2-1), and extension at 72°C for 30 s. A melt curve was included for each reaction to ensure amplification of a single PCR product. Every reaction was performed in duplicate, and a no-template control was included. Quantification of specific pathogens was based on a standard curve with purified DNA from each pathogen. Total DNA of *E. coli* MS499, *T. pyogenes* MS249 and *F. necrophorum* (supplied by Dr. Klibs Galvão, University of Florida) was extracted using the DNeasy Power Soil kit from live cultures (preparation described in Piersanti et al., 2019). Purified DNA of *P. melaninogenica* was purchased from ATCC (Manassas, VA; #25845). Results for each pathogen were normalized by weight of mucus and are expressed as CFU/µL/mg for *E. coli* and *T. pyogenes* and pg/uL/mg for *F. necrophorum* and *P. melaninogenica*.

Statistical Analysis

All data were analyzed using SPSS Statistics v26 (IBM Corporation, Armonk, NY).

Estimated 305 d milk production, and THI data pertaining to the retrospective analysis of 3,507 animals utilized a generalized linear mixed model (GENLINMIXED) with pairwise comparisons between fixed effects of season, parity and the interaction between season and parity with season as a contrast field. Cow was added as a random factor. Records missing estimated 305-milk production were excluded from this analysis. Metritis incidence was analyzed using a linear mixed model (MIXED) for binomial distribution with fixed effects of season, parity, and the interaction of season by parity with pairwise comparisons and season as the contrast field. The effect of THI on metritis incidence was also evaluated using the same model by replacing the fixed

effects of season with either the 5-day average prepartum THI or the 5-day average postpartum THI.

Continuous variables presented in Table 2-2, estimated 305 d milk production and vaginal bacterial content of the 102 animals that calved in the summer, or the winter were analyzed using generalized linear mixed model with fixed effects of season, parity and the interaction of season and parity with pairwise comparisons with season as the contrast field for the interaction. Cow was added as a random factor. Milk yield, ECM, and milk components were analyzed using generalized linear mixed model with repeated measurements for daily milk production up to 60 DIM with fixed effects of season, parity and the interaction of season by parity, and cow was included as a random effect. Categorical variables in Table 2-2 were analyzed using a generalized linear mixed model for multinomial distribution with fixed effects of season, parity and the interaction of season and parity with pairwise comparisons and season as contrast field for the interaction. For Table 2-3, data were analyzed in a similar manner as Table 2-2 with fixed effect of season and cow as a random effect. The impact of metritis on daily milk yield (Fig. 2-4) was analyzed using a generalized linear mixed model with fixed effects of season, parity, metritis, the interaction of season and parity with season as contrast field, and the interaction of parity and metritis with parity as contrast field. Bacterial content of the vagina was analyzed using a generalized linear mixed model with fixed effects of season, parity, health status (cows were classified as having uterine disease if d 7 vaginal mucus grade ≥ 3 or d 21 grade ≥ 2) and the interaction of health status with season with pairwise comparisons. Statistical significance was set at $P \leq 0.05$ and tendency at $P \leq 0.08$.

Results

The Incidence of Metritis is Increased, and Milk Production is Decreased During Warmer Months in Florida

Retrospective analysis of 3,507 calving events between 2012 and 2017 (1,364 primiparous and 2,143 multiparous) was performed to determine the effect of season (warm versus cool) on milk production and the incidence of clinical metritis. Average monthly THI was calculated using daily averages for air temperature and relative humidity from 2012 to 2018. Calving between April 1st and September 30th was defined as the warm period and calving between October 1st and March 31st was defined as the cool period. The average daily THI for the warm period (74.3 ± 0.1) was higher (P < 0.05) than the average daily THI for the cool period (60.8 ± 0.3). Of the 3,507 calving events analyzed, 2,204 cows had recorded 305 d milk production estimates available for analysis. Multiparous cows produced more milk than primiparous cows (9,424 ± 78.18 *vs* 8,613 ± 91.72, P < 0.01). Average 305 d milk production for the warm period per cow (8,819 ± 89.51 kg) was lower (P < 0.01) than the 305 d milk production during the cool period (9,219 ± 85.15 kg; Fig. 2-2). However, there was a significant interaction of season by parity (P < 0.01) on 305 d milk production with the effect of season restricted to multiparous cows, that exhibited an 894 kg decrease in 305 d milk production in the warm period (9,871 ± 102.66 *vs* 8,977 ± 13.58, P < 0.01, Fig. 2-2). Average 305 d milk production in the warm period (8,661 ± 130.23 kg) and cool period (8,566 ± 128.68 kg) for primiparous cows was not affected by season (Fig. 2-2).

The overall incidence of metritis from 2012 to 2017 at the University of Florida Dairy Research Unit was 21.4%. The overall incidence of metritis during the warm period increased (24.1%; P = 0.035) compared with the cool period (21.1%; Fig. 2-3).

Overall, primiparous cows had a higher incidence of metritis (27.7%) relative to multiparous cows (17.5%, $P < 0.01$). Calving during the warm period increased the relative risk of developing metritis by 18% compared with calving in the cool period (RR = 1.18, 95% CI = 1.02 to 1.31). The incidence of metritis in primiparous cows was 29.5% during the warm period and 26% during the cool period ($P = 0.15$). The incidence of metritis in multiparous cows was 18.7% in the warm period and 16.2% in the cool period ($P = 0.13$). There was no effect of the 5-day average prepartum THI or 5-day average postpartum THI on metritis incidence, regardless of parity.

The Effect of Calving Season on Milk Production, Cow Performance and Uterine Disease Incidence

A total of 51 cows calving in the summer (September 2017; 5-day postpartum average THI = 78.6 ± 0.5) and 51 cows calving in the winter (February to March 2018; 5-day postpartum average THI = 64.4 ± 0.6) were enrolled to monitor daily milk production, evaluate cow performance, observe the development of uterine disease, and assess bacterial content of the lower reproductive tract (Table 2-2). As expected, the 5-day average prepartum and postpartum THI were increased ($P < 0.01$) during the summer relative to winter. Overall, milk production and energy corrected milk (ECM) were increased in multiparous cows relative to primiparous ($P < 0.01$) and milk production tended to be reduced during summer compared to winter ($P = 0.06$) while ECM was similar between seasons (Table 2-3). For multiparous cows, milk production was decreased in cows calving in the summer compared to winter, while for primiparous cows the production was the same between seasons. Overall, the percent milk protein was not affected by parity, and it was increased ($P < 0.01$) in cows calving in the summer compared with those calving in the winter; conversely, absolute milk protein

yield was increased ($P < 0.01$) in multiparous versus primiparous cows but did not differ between seasons. Overall, percent milk fat was increased ($P < 0.01$) in cows that calved during the winter compared with summer. Absolute milk fat yield was increased ($P = 0.01$) in cows that calved in the winter due to the increase in fat yield in milk produced by multiparous cows ($P = 0.02$). Milk produced by multiparous cows had a higher ($P < 0.01$) absolute lactose yield compared with milk produced by primiparous cows but there was no effect of calving season. Percent milk lactose was higher for multiparous cows ($P < 0.01$), and it was increased in cows calving in the summer ($P < 0.01$).

Overall, gestation length of cows calving in the winter and summer was the same, as was the gestation length of primiparous similar to the gestation length of multiparous cows. Intriguingly, primiparous cows had a longer gestation ($P < 0.01$) during the summer (275.92 ± 3.82 days) compared to winter (269.15 ± 5.05 days). The incidence of twin pregnancy, retained placenta, ketosis, and other diseases in the first 60 DIM was not affected by calving season. There was a tendency ($P = 0.06$) for an increased culling of cows that calved during the winter compared to cows that calved during the summer, due to an increase in culling of primiparous cows that calved in the winter ($P = 0.05$). Overall, the interval from calving to first insemination was increased ($P < 0.01$) by > 8 days in cows that calved in the summer, while the number of inseminations to achieve a pregnancy was not affected by season.

The overall incidence of clinical metritis during the summer (19.6%; 10/51) and winter (25.5%; 13/51) was the same (Table 1, $P > 0.05$). When considering the effects of clinical metritis on milk production, daily milk yield was affected ($P < 0.05$) by parity, clinical metritis, season and by the interaction between parity and clinical metritis (Fig.

2-4). There was no effect of the interaction between season and clinical metritis on daily milk production for the first 60 DIM. Cows that calved in the winter with no clinical metritis producing the highest yield and cows calving in the summer with clinical metritis producing the lowest yields.

Based on vaginal mucus grade, cows were categorized as having uterine disease if d 7 vaginal mucus was ≥ grade 3, or if d 21 mucus was ≥ grade 2 (Fig. 2-1), resulting in four groups of animals: 1) cows that were free of uterine disease on d 7 and d 21; 2) cows with uterine disease on d 7 which was resolved by d 21, 3) cows that were free of uterine disease on day 7 and developed uterine disease by d 21; and 4) cows that had uterine disease on both d 7 and d 21 (Fig. 2-5). The proportion of cows with no uterine disease on both d 7 and d 21 was equivalent for cows calving in the winter (27.5%) and the summer (19.6%); however, the proportion of cows with uterine disease on both d 7 and d 21 was greater ($P < 0.05$) in cows that calved in the summer (58.0%) compared with those that calved in the winter (29.4%), suggesting recovery from uterine disease on d 7 was reduced in cows that calved in the summer (Fig. 2-5).

The Effect of Calving Season on Bacterial Content in the Lower Reproductive Tract

Total bacterial 16S rRNA in vaginal mucus at d 7 postpartum was greater in abundance relative to d 21 (5.64 ± 1.33 *vs* 1.26 ± 0.50 ng/mg mucus, Fig 2-6A, $P < 0.05$). There was no effect of calving season on total bacterial 16S rRNA at d 7 or d 21 ($P = 0.65$ and $P = 0.11$, respectively). Total bacterial 16S rRNA was affected by vaginal mucus grade collected on d 7 or d 21 ($P < 0.05$), with total bacterial 16S rRNA increasing with vaginal mucus grade (Fig. 2-6B-C, $P < 0.05$). On d 21 there was an effect of calving season on total bacterial 16S rRNA ($P < 0.05$); however, this was

primarily driven by a single cow with grade 4 mucus that calved in the winter with an exceptionally high 16S rRNA content.

Known pathogens associated with uterine disease were quantified in vaginal mucus using real time RT-PCR with primers designed for *E. coli*, *T. pyogenes*, *F. necrophorum* and *P. melaninogenica* (Fig. 2-7 and 2-8). The content of *E. coli*, *T. pyogenes*, *F. necrophorum* or *P. melaninogenica* in vaginal mucus was the same at d 7 and d 21 regardless of calving season (Fig. 2-7). However, the content of *T. pyogenes* in vaginal mucus at d 7 was reduced ($P = 0.04$) in cows that calved in the summer compared to those that calved in the winter and tended ($P = 0.07$) to be reduced at d 21 in cows calving in the summer compared with the winter (Fig. 2-7B).

Using the same classification for uterine disease described above (d 7 vaginal mucus grade ≥ 3 or d 21 grade ≥ 2), cows with uterine disease at d 7 (Fig. 2-8A-E) or d 21 (Fig. 2-8F-J) had increased ($P < 0.01$) total bacterial 16S rRNA content in vaginal mucus compared to cows with no uterine disease, but there was no effect of calving season. Vaginal mucus content of *E. coli* at d 21 was increased ($P < 0.05$) in cows with uterine disease compared to cows with no uterine disease (Fig. 2-8B), but not at d 7 (Fig. 2-8G). Calving during the winter increased ($P < 0.05$) vaginal mucus content of *T. pyogenes* in cows with uterine disease compared to cows with no uterine disease at d 7 (Fig. 2-8C) and 21 (Fig. 2-8H). Vaginal mucus content of *F. necrophorum* at d 7 was increased ($P < 0.05$) in cows with uterine disease compared to cows with no uterine disease (Fig. 2-8D), but not at d 21 (Fig. 2-8I). Surprisingly, vaginal mucus content of *P. melaninogenica* at d 7 tended to decrease ($P = 0.07$) in cows with uterine disease

relative to cows with no uterine disease (Fig. 2-8E), but this tendency was not observed at d 21 (Fig. 2-8J).

Discussion

Although the negative impacts of heat stress and uterine disease on milk yield and reproduction have been described in the dairy cow, there is a lack of evidence for a link between elevated environmental temperatures and reproductive tract health. Here, we reviewed records of over 3,000 animals that calved during a 5-year period at the University of Florida and found an increased incidence of metritis during warmer months in addition to recapitulating the findings of seasonal variation in milk production. In addition, we investigated if the increased incidence of uterine disease seen during the warmer months was due to an increase bacterial content in the lower reproductive tract by evaluating vaginal mucus samples from postpartum cows during the winter and summer. While our data confirmed that total bacterial, *E. coli*, *T. pyogenes* and *F. necrophorum* content is increased in cows with uterine disease, the season of calving does not influence bacterial content of vaginal mucus. However, cows that calved in the summer did not recover from uterine disease as effectively as cows that calved in the winter. This suggests that environmental factors, such as heat stress, might affect host factors that can predispose cows to the development and poorer recovery of uterine disease such as altering the immune response to pathogens or decreasing the cow's ability to tolerate pathogens.

In agreement with our findings, Gernand et al., 2019 reported an increased incidence of uterine disorders during summer associated with increased THI by using a robust model to correct for additional environmental factors. Conflicting results have been presented regarding the influence of seasonality in the incidence of uterine

diseases where seasonality had no impact in the incidence of uterine diseases (Pinedo et al., 2020) or the higher incidence of disease occurred during the winter (Markusfeld, 1984, Benzaquen et al., 2007). Those discrepancies in association might be explained by differences in management, housing conditions, diet composition and to the arbitrary definition of season between studies. For our retrospective analysis, we split the year evenly between 6 months of warm season and 6 months of a cold season in the sub-tropical region of north central Florida. This definition meant each month within the warm season had an average THI > 68, which is an environment documented to facilitate the outward signs of heat stress in in high producing dairy cows (Zimbelman et al., 2009, De Rensis et al., 2015).

The THI is a widely utilized indicator of heat stress that combines environmental temperature and relative humidity since elevated humidity reduces heat dissipation to the environment (Dikmen & Hansen, 2009). The herd utilized here was located in Florida, a subtropical region with the second highest relative humidity in the US. The decreased milk production observed in the warm season is consistent with these cows being under heat stress conditions (Kadzere et al., 2002). In our observational study using a smaller number of cows we also observed a tendency for reduced milk production in the summer and an increase in days open, characteristic of cows under heat stress. A limitation of the current study was our inability to isolate the effects of heat stress from other seasonal factors. While data presented here were collected from cows under the same management strategy at the same location, other factors affecting the observed outcomes may include changes in photoperiod, water and air quality, feed composition and dry matter intake.

Establishment of disease requires an interaction between a susceptible host, a pathogen and the environment that influences both host and pathogen. Seasonal changes in the environment can change host or pathogen factors and predispose individuals to disease by mechanisms including increased pathogen proliferation, changes in host behavior, increased transmission within a population and change in host susceptibility (Altizer et al., 2006). Epidemiological studies show that some infectious diseases have defined seasonal outbreaks explained in part by changes in pathogen and/or host factors that are influenced by environmental temperature (Dowell, 2001, Altizer et al., 2006). Rates of bloodstream infections in humans caused by Gram-negative bacteria are associated with higher environmental temperatures and have a higher prevalence during summer compared to winter. For example, *Actinobacter spp* infections are increased by 51.8% and *E. coli* infections are increased by 12.2% in patients during the summer compared to the winter (Eber et al., 2011). However, our findings show that total bacterial load in the lower reproductive tract is not influenced by season. The lack of seasonal effect on bacterial content of vaginal mucus in conjunction with increased uterine disease incidence suggests that disease incidence and persistency may be due to host factors rather than dysbiosis.

Immune resilience mechanisms include avoidance, resistance and tolerance that act to limit the establishment of disease (Sheldon et al., 2020). Avoidance mechanisms limit exposure to pathogens, while resistance mechanisms actively reduce pathogen load, and tolerance mechanism limit tissue and cellular damage caused by pathogens. The data described here suggests that the vaginal content of total bacteria and other recognized pathogens it not affected by calving season; nonetheless, calving during the

summer increased the incidence and persistence of uterine disease. This suggests that heat stress in the summer could impair cow resistance or tolerance to pathogens, increasing tissue damage and predisposing cows to the establishment of uterine disease. Studies show that heat stress has implications on the immune system of dairy cows that might depend on the physiological state of the cow when exposed to heat stress. Dry period heat stress decreased neutrophilic function during lactation, while PBMCs from lactating dairy cows exposed to heat stress have increased production of TNFα and IL-10 in response to LPS (do Amaral et al. 2011, Marins et al., 2021). Further investigation of the effect of seasonality on immune function was not performed in this study. Sheldon et al. (2019) described a similar observation involving increased metabolic stress and decrease in tolerance due to high production. Cows producing > 35 kg milk/d were described as having decreased uterine health compared to cows that produced < 35 kg milk/d; however, the pathogen content of the uterus was comparable between cows of high and low production value cows, suggesting host tolerance as compromised in the high producing cohort. Tolerance mechanisms which may contribute to reducing uterine disease include physical barriers to infections such as the epithelium or mucus, neutralization of pathogen toxins, tissue repair mechanisms or adaptive metabolic responses (Sheldon et al., 2020). For example, during calving a large proportion of the protective endometrial epithelium is lost, effectively exposing the upper reproductive tract to the environment and potential endometrial tissue damage. Underlying endometrial stromal cells are more sensitive to the cholesterol-dependent cytolysin of *T. pyogenes* compared with endometrial epithelial cells, which would permit greater endometrial tissue damage during uterine infection if the epithelium is lost

during parturition (Amos et al., 2014). *In vitro* studies demonstrate that endometrial cells are more sensitive to cytolysin damage due to increased cholesterol content compared to endometrial epithelial cells. The ability to modulate cholesterol content of endometrial cells could mediate effective strategies to limit cytolysin damage of endometrial cells, mitigating tissue damage associated with uterine disease and promoting increased host tolerance to pathogens (Griffin et al., 2018, Preta et al., 2015).

The process of immune resistance is often intertwined with immune tolerance and mediates pathogen control. Uterine disease triggers an innate immune response characterized by increased secretion of pro-inflammatory cytokines including IL-1α, IL-1β and IL-6 in the uterus (Kim et al., 2014), effectively increasing host resistance. In parallel, cellular response to heat shock is characterized by increased synthesis of heat shock proteins (HSP), a family of chaperones activated in response to cellular damage (Sorensen et al., 2003, Schiaffonati & Tiberio, 1997). Mammalian HSP can form complexes with antigenic peptides and activate cells of the innate immune system and increase secretion of pro-inflammatory cytokines, potentially increasing resistance mechanisms under heat stress conditions (Srivastava et al., 1998, Kol et al., 1999, Moroi et al., 2000, Basu et al., 2000, Basu & Srivastava, 2000). Heat shock impacts to cytokine production seems to vary according to cellular type, animal species, duration of stress and status of the animal. Broiler chickens subjected to HS 35d after pathogen inoculation had a decreased expression on IL6 and IL1B (Quinteiro-Filho, 2017). Conversely, exercise induced heat stress elevates plasma concentrations of IL-6 and TNF-α in humans, while endurance athletes also have an increase of anti-inflammatory IL-10 whereas sedentary individuals do not (Selkirk et al., 2008). Interestingly, heat-

stressed bovine endometrial epithelial cells have reduced IL-6 production compared to cells cultured in thermoneutral conditions, while endometrial stromal cells have increased IL-6 production compared to cells cultured in thermoneutral conditions (Sakai et al., 2020). Therefore, given the immunomodulatory capacity of heat stress, combined with unaltered pathogen content of the vagina observed here, a cytokine disbalance may be an important factor in altering the response of heat-stressed cows to pathogens. Moreover, a persistent inflammation caused by prolonged heat stress may generate tissue damage and interfere with tolerance mechanisms that could be involved in maintaining uterine health or promoting disease resolution. Further investigation is necessary to disentangle the mechanisms by which heat stress impacts uterine defense mechanisms in the bovine.

A

B

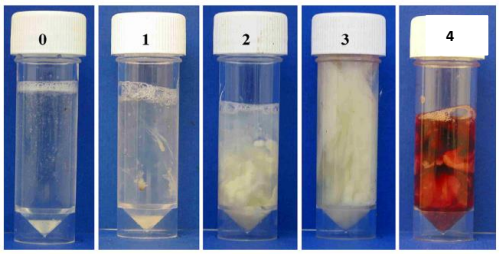

Figure 2-1. Experimental design and vaginal mucus grading. A) Cows were enrolled during summer (n = 51, September 2017; 5-day postpartum average THI = 78.6 ± 0.5) and winter (n = 51, February to March 2018; 5-day postpartum average THI = 64.4 ± 0.6). Cows were housed on pasture until 3 weeks prior to their expected calving date at which time cows were moved to a free stall barn with fans and water soakers. Vaginal mucus samples were collected on day 7 and day 21 after calving and cow performance was recorded until 60 DIM. B) Vaginal mucus was graded as 0 (clear vaginal mucus), 1 (mucus with flecks of white pus), 2 (mucus with less than 50% white pus), 3 (mucus with more than 50% white pus) or 4 (sanguinopurulent mucus) (adapted from Sheldon et al., 2009).

Table 2-1. PCR primers used for real time RT-PCR.

Bacteria (target gene)	Primer sequence (5'-3')	Annealing Temperature (ºC)	Source
E. coli (*16S*)	F–GTTAATACCTTTGCTCATTGA R–ACCAGGGTATCTAATCCTGTT	53.5	Malinen et al., 2003
T. Pyogenes (*plo*)	F–GGCCCGAATGTCACCGC R–AACTCCGCCTCTAGCGC	64.5	Belser et al., 2015
F. Necrophorum (*ikta*)	F–GATTGGGGGATAGCGGTAAT R–GAGCCTCCACATTTAGTCGC	63.0	Cunha et al., 2018
P. Melaninogenica (*phyA*)	F–ACAAAGAGGCAAACCAAGCG R–TACGAAGCATCCGTTCAGGG	55.0	In-house

Table 2-2. Descriptive table of cows enrolled during winter and summer.

	All Cows		*P*-value		
	Winter	Summer	Season	Parity	Season × Parity
Cows	51	51	-	-	-
Primiparous	15	27	-	-	-
Multiparous	36	24	-	-	-
Lactation number	2.37 ± 1.31	1.86 ± 1.15	0.75	-	-
Gestation length	273.68 ± 6.86	274.50 ± 5.74	0.11	0.11	< 0.01
Twins	8% (4/51)	4% (2/51)	0.73	0.10	1
RP	6% (3/51)	0 (0/51)	0.10	1	0.10
Avg daily milk (kg)	41.36 ± 14.45	35.35 ± 11.24	0.06	< 0.01	0.38
Avg daily ECM	40.27 ± 9.80	34.46 ± 7.41	0.11	< 0.01	0.58
Milk protein (kg)	0.99 ± 0.34	0.96 ± 0.31	0.66	< 0.01	0.81
Milk protein (%)	2.57 ± 0.36	2.79 ± 0.35	< 0.01	0.28	0.31
Milk fat (kg)	1.45 ± 0.51	1.22 ± 0.41	0.01	< 0.01	0.61
Milk fat (%)	3.82 ± 0.78	3.57 ± 0.68	0.01	0.45	0.46
Milk lactose (kg)	1.53 ± 0.65	1.46 ± 0.53	0.98	< 0.01	0.42
Milk lactose (%)	3.90 ± 0.76	4.13 ± 0.56	< 0.01	< 0.01	0.09
Metritis incidence	25% (13/51)	20% (10/51)	0.17	0.24	0.21
Ketosis incidence	67% (34/51)	51% (26/51)	0.13	0.89	0.56
Other disease incidence	35% (18/51)	25% (13/51)	0.12	0.05	0.45
Culled	12% (6/51)	2% (1/51)	0.06	0.85	0.54
Days to first insemination	77.40 ± 2.87	69.38 ± 11.96	< 0.01	0.86	0.98
Insemination: pregnancy	2.88 ± 2.02	2.68 ± 1.62	0.62	0.46	0.20
Vaginal mucus grade (d 7)	2.65 ± 1.35	3.10 ± 1.19	< 0.01	0.94	0.93
Total 16S content (d 7)	4.66 ± 11.51	6.52 ± 13.74	< 0.01	0.14	0.08
Vaginal mucus grade (d 21)	1.55 ± 1.08	1.92 ± 1.21	0.13	0.05	0.14
Total 16S content (d 21)	1.18 ± 3.01	1.39 ± 6.15	0.53	0.69	0.88
Lactation number	2.37 ± 1.31	1.86 ± 1.15	0.33	0.04	0.51
Gestation length	273.68 ± 6.86	274.50 ± 5.74	0.71	0.20	0.44

All variables are shown as mean ± std. deviation or percentage with absolute numbers. Variables were tested for the effect of season, parity and the interaction between parity and season.
For gestation length cows that had an abortion or stillbirth were excluded (7).
Metritis, ketosis and other diseases were analyzed up to 60 DIM.
Other diseases included mastitis, respiratory problems, foot problems and displaced abomasum.
IA : Preg was analyzed until cow was diagnosed pregnant or up to 200 DIM.

Table 2-3. Descriptive table from cows enrolled during winter and summer sorted by parity.

	Primiparous Cows			Multiparous Cows		
	Winter	Summer	Season	Winter	Summer	Season
Cows	15	27	-	36	24	-
Lactation	-	-	-	2.94 ± 1.14	2.83 ± 1.00	0.62
Gestation Length	269.15 ± 5.05	275.92 ± 3.82	< 0.01	275.41 ± 6.72	273.08± 6.97	0.33
Twins	0	0	-	11% (4/36)	8% (2/24)	0.54
RP	0	0	-	8% (3/36)	0	0.21
Avg daily milk (kg)	33.59 ± 10.98	31.54 ± 9.06	0.56	44.56 ± 14.49	39.73 ± 11.90	0.02
Avg daily ECM	33.43 ± 7.08	30.91 ± 3.92	0.50	43.08 ± 9.37	38.54 ± 8.31	0.09
Milk protein (kg)	0.86 ± 0.28	0.87 ± 0.25	0.66	1.07 ± 0.35	1.07 ± 0.33	0.87
Milk protein (%)	2.63 ± 0.38	2.79 ± 0.35	< 0.01	2.54 ± 0.34	2.78 ± 0.36	< 0.01
Milk fat (kg)	1.25 ± 0.41	1.10 ± 0.30	0.22	1.57 ± 0.53	1.36 ± 0.47	0.02
Milk fat (%)	3.87 ± 0.68	3.60 ± 0.66	0.04	3.79 ± 0.83	3.53 ± 0.71	0.17
Milk lactose (kg)	1.23 ± 0.50	1.29 ± 0.43	0.61	1.71 ± 0.67	1.66 ± 0.57	0.51
Milk lactose (%)	3.75 ± 0.84	4.07 ± 0.60	< 0.01	3.99 ± 0.70	4.21 ± 0.47	< 0.01
Metritis incidence	20% (3/15)	26% (7/27)	0.49	28% (10/36)	13% (3/24)	0.14
Ketosis incidence	60% (9/15)	52% (14/27)	0.43	70% (25/36)	50% (12/24)	0.11
Other disease incidence	47% (7/15)	37% (10/27)	0.39	31% (11/36)	13% (3/34)	0.09
Culled	13% (2/15)	0	0.05	11% (4/36)	4% (1/24)	0.35
Days to first insemination	77.69 ± 2.14	69.59 ± 8.82	0.01	77.28 ± 3.14	69.22 ± 15.04	< 0.01
Insemination: pregnancy	2.73 ± 2.19	3.04 ± 1.53	0.61	2.94 ± 1.97	2.26 ± 1.66	0.17
Prepartum THI	2.73 ± 1.10	3.52 ± 0.80	< 0.01	2.61 ± 1.46	2.63 ± 1.38	< 0.01
Postpartum THI	5.14 ± 15.35	7.21 ± 11.65	< 0.01	4.46 ± 9.74	5.74 ± 16.00	< 0.01
Vaginal mucus grade (d 7)	2.00 ± 1.00	2.07 ± 1.27	0.05	1.36 ± 1.07	1.75 ± 1.15	0.97
Total 16S content (d 7)	0.82 ± 2.26	0.41 ± 1.40	0.62	1.33 ± 3.29	2.49 ± 8.81	0.62
Vaginal mucus grade (d 21)	15	27	0.84	36	24	0.20
Total 16S content (d 21)	-	-	0.79	2.94 ± 1.14	2.83 ± 1.00	0.36

All variables were tested for the effect of season. Results are expressed in mean ± S.E.M. or percentage with the absolute numbers.

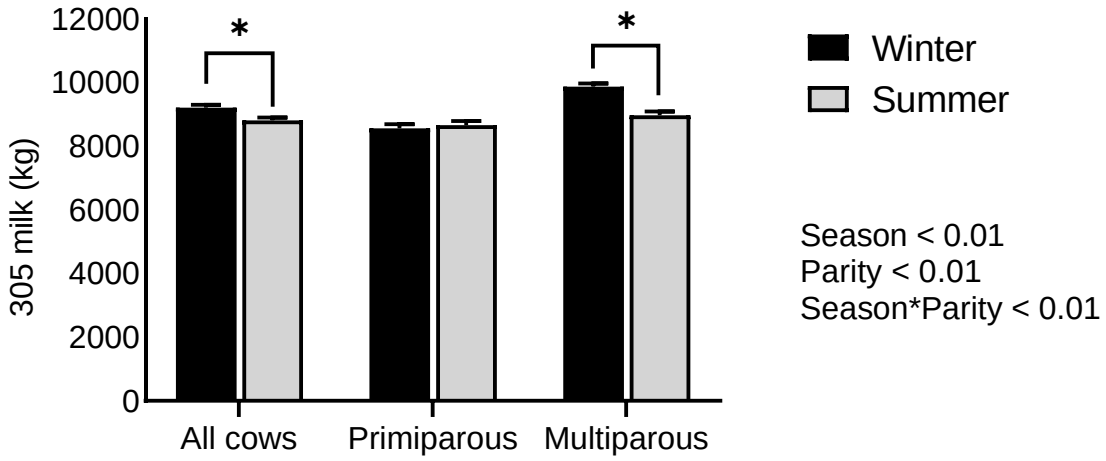

Figure 2-2. Effect of season and parity on 305-d milk yield in cows from 2012 to 2017. Estimated 305 d milk production was calculated for a total of 2,204 cows (multiparous, n = 1,354 and primiparous, n = 850) during the warm (April through September) and cool (October through March) period. Bars represent the mean ± S.E.M. * $P \leq 0.05$ for comparisons within each group.

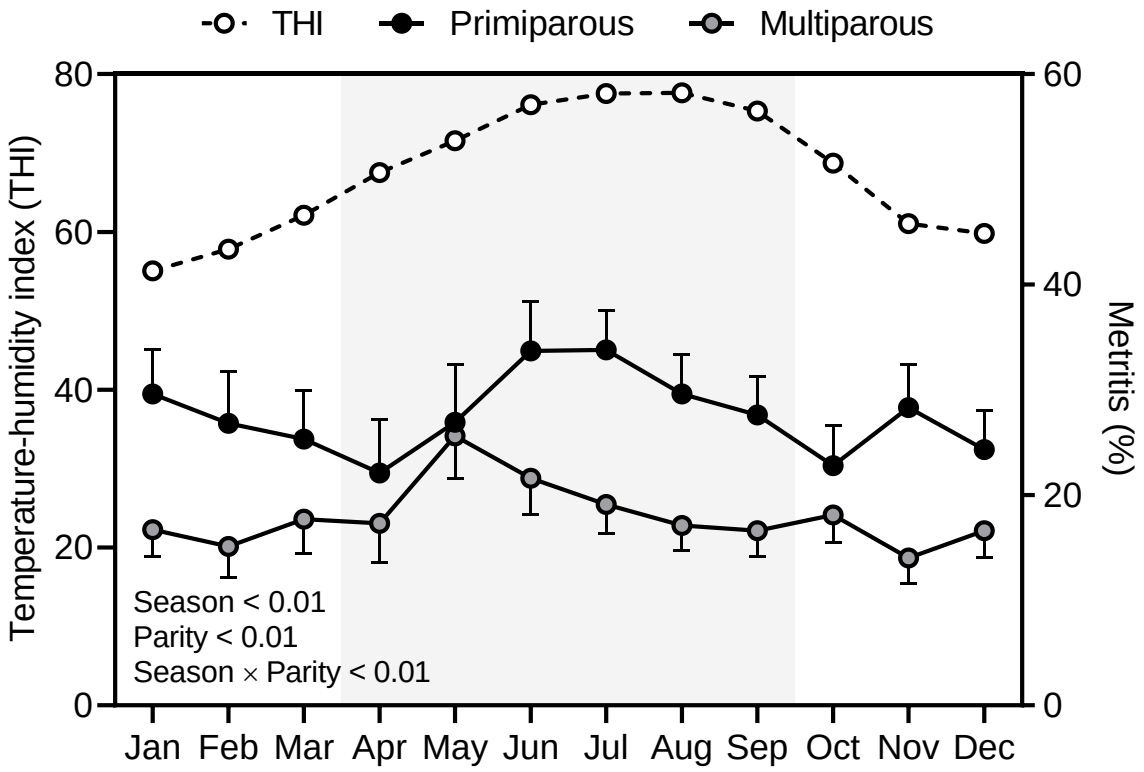

Figure 2-3. Effect of calving season on metritis incidence from 2012 to 2017 (n = 3,507). The average monthly incidence of clinical metritis was calculated by dividing the number of cows that developed metritis by total number of cows that calved in each month between January 1st 2021 and December 31st 2017 at the University of Florida Dairy Research Unit. The solid black lines represent the monthly average incidence of clinical metritis for primiparous (●) and multiparous (●) cows ± S.E.M. The dashed line (○) represents the average monthly temperature humidity index (THI) ± S.E.M.

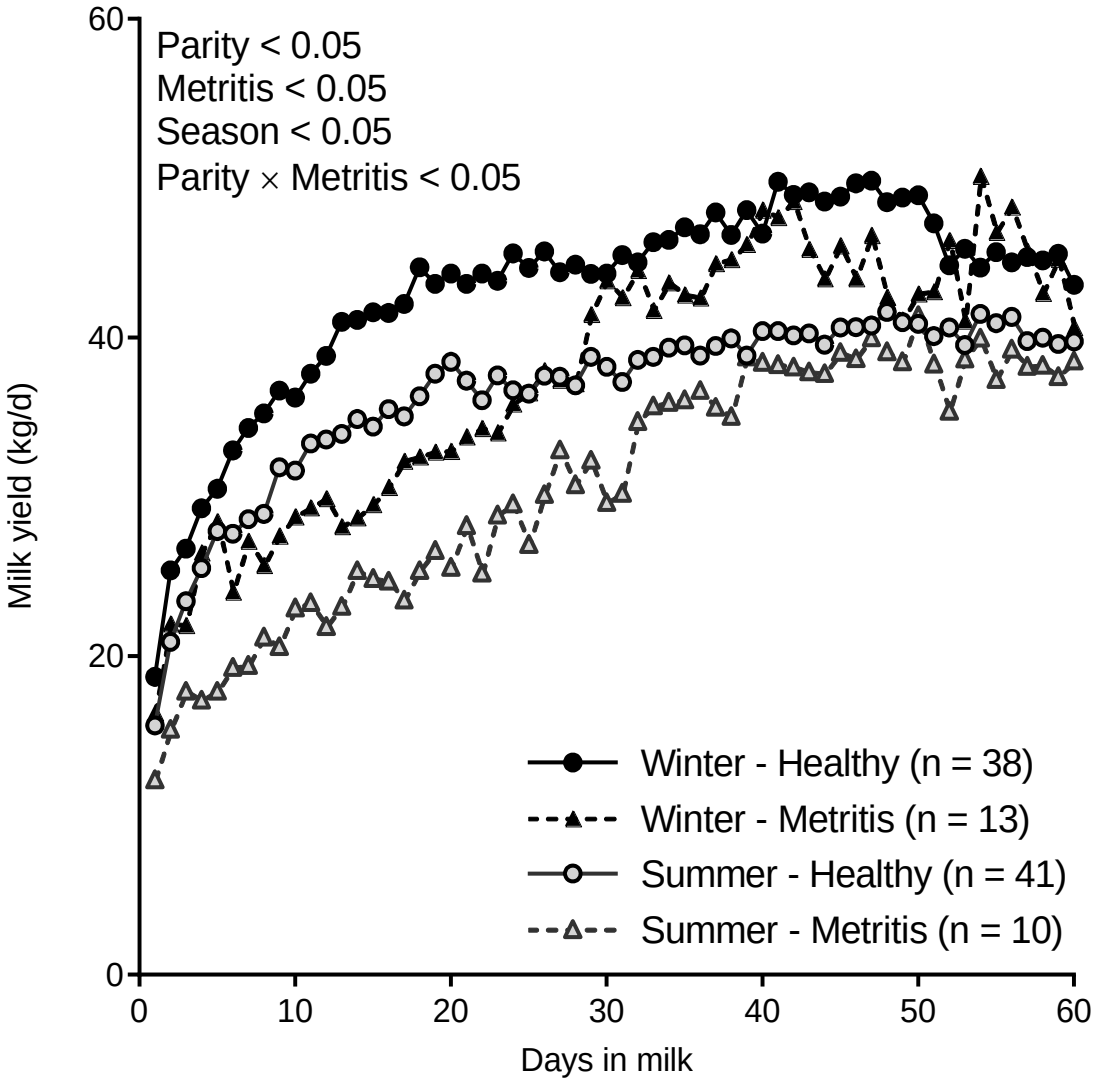

Figure 2-4. Effect of calving season and clinical metritis on daily milk yield. Average daily milk yield for the first 60 DIM from cows that calved during the winter (n = 51) and summer (n = 51). Circles represent cows that did not develop clinical metritis after calving in the winter (●) or summer (●). Triangles represent cows that did develop clinical metritis after calving in the winter (▲) or summer (▲).

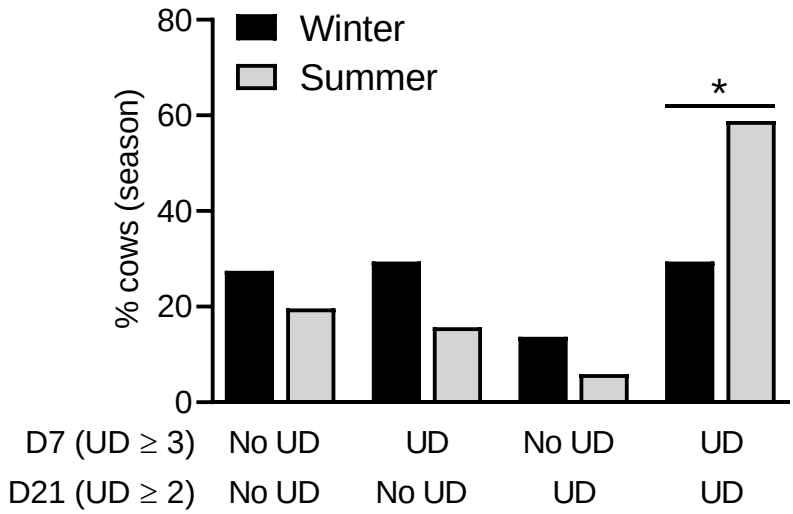

Figure 2-5. The effect of season on uterine disease incidence at day 7 and day 21 postpartum. Vaginal mucus was collected on day 7 and day 21 postpartum and graded from 0 to 4 as previously described. Cows were categorized as having uterine disease (UD) on d 7 if vaginal if mucus was ≥ grade 3, or if d 21 mucus was ≥ grade 2, and then assigned to 4 different groups: no UD d 7 and d 21; UD on d 7 and no UD on d 21; no UD on d 7 and UD on d 21, UD on d 7 and on d 21. Bars represent the percentage of cows in each category according to season. * $P \leq 0.05$ for comparisons within each group.

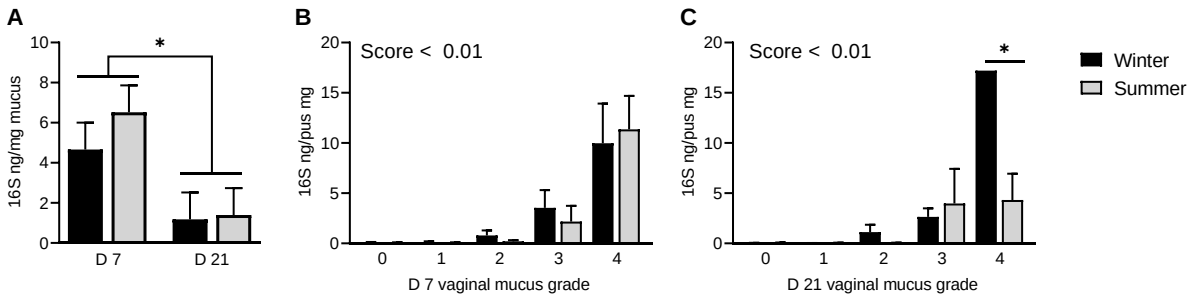

Figure 2-6. Total bacterial content of vaginal mucus according to the day of collection and vaginal mucus grade. Total bacterial content of vaginal mucus was quantified by targeting bacterial 16S rRNA using the Femto Bacterial Quantification Kit. Quantification was based on the provided standard curve and expressed as ng of targeted DNA per mg of mucus. Bars represent the mean ± S.E.M. * $P \leq 0.05$ compared to indicated group.

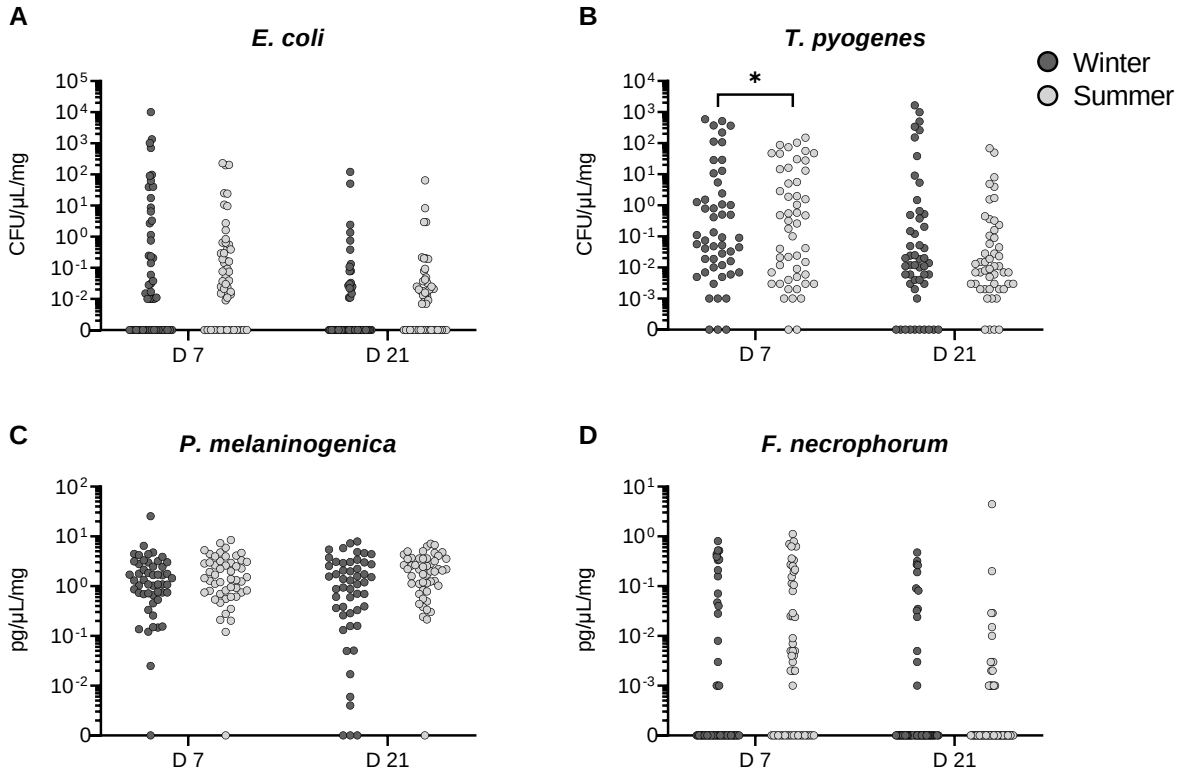

Figure 2-7. Quantification of specific bacteria in vaginal mucus according to the day of collection and season. (A) *E. coli*, (B) *T. pyogenes*, (C) *F. necrophorum* and (D) *P. melaninogenica* were quantified in vaginal mucus samples collected on d 7 and d 21 postpartum using real time RT-PCR. Quantification was based on a standard curve for each bacterium and is expressed as CFU/uL per mg of mucus for *E. coli* and *T. Pyogenes* and as pg/uL per mg of mucus for *P. Melaninogenica* and *F. Necrophorum*. Dots represent individual replicates. * $P \leq 0.05$ compared to indicated group.

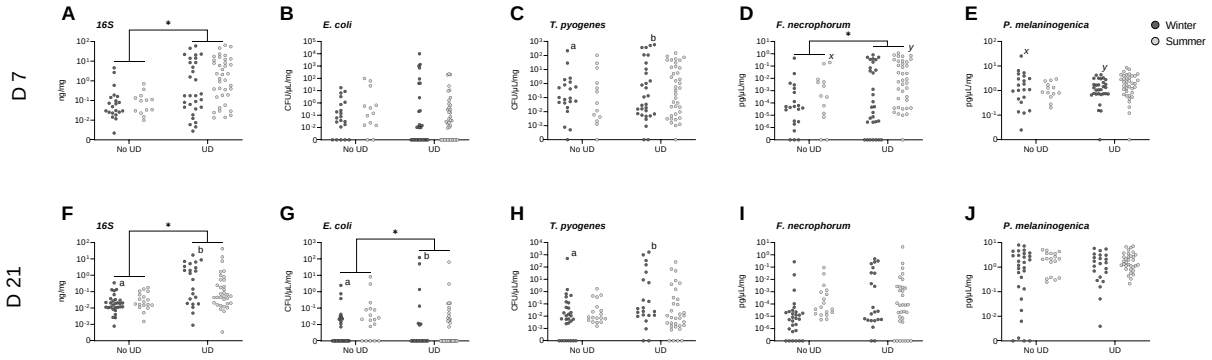

Figure 2-8. Bacterial content of vaginal mucus according to uterine health status. (A, F) Total 16S rRNA content, (B, G) *E. coli*, (C, H) *T. pyogenes*, (D, I) *F. necrophorum* and (E, J) *P. melaninogenica* were quantified in vaginal mucus samples collected on d 7 (A-E) and d 21 (F-J) postpartum using real time RT-PCR. Cows were categorized as having uterine disease (UD) based on vaginal mucus grade (uterine disease if d 7 vaginal mucus was ≥ grade 3, or if d 21 mucus was ≥ grade 2). Data are expressed as ng per mg of mucus for total 16S rRNA, CFU/uL per mg of mucus for *E. coli* and *T. pyogenes*, and pg/uL per mg of mucus for *P. melaninogenica* and *F. necrophorum*. * $P \leq 0.05$ for comparison between health status, superscript ^{a,b} indicate $P \leq 0.05$ between health status within season, superscript ^{y,x} indicates $0.05 \leq P \leq 0.08$ between health status within season.

CHAPTER 3
HEAT STRESS IN THE PREPARTUM PERIOD INCREASES IMMUNE RESPONSIVENESS OF PERIPHERAL BLOOD IN THE POSTPARUM LACTATING DAIRY COW

Heat stress occurs when elevated environmental temperature causes an imbalance between an animal's heat accumulation and heat dissipation mechanisms. Animals under heat stress display physiological and behavioral changes that attempt to reduce heat production and optimize heat loss to maintain a normal body temperature. Elevated environmental temperature combined with elevated humidity makes it harder for an animal to dissipate heat to the environment and achieve homeostasis. A well described metric for thermal stress that combines ambient temperature and relative humidity is the temperature humidity index (THI). When average THI is above 68, dairy cows exhibit signs of heat stress including increased respiratory rate and rectal temperature (Zimbelman et al., 2009). In the sub-tropical state of Florida, 257 days of the year (70% of the year) have an average THI above 68 (Ferreira et al., 2016) placing the dairy cow at risk to heat stress. Cows exposed to heat stress have lower dry matter intake, lower milk production, decreased conception rates, increased number of inseminations per pregnancy, and have greater number of days open compared to thermoneutral cows (Ouellet et al., 2020, De Rensis et al., 2002, Garcia-Ispierto et al., 2006). Moreover, heat stress can be a risk factor for the occurrence of reproductive disorders such as metritis and retained placenta (DuBois and Williams, 1980, Gernand et al., 2019).

Elevated temperatures trigger a stress response characterized by activation of the hypothalamic-pituitary-adrenal (HPA) axis and subsequent cortisol secretion. The impact of a stress response to the immune system is controversial, with reports showing

stimulation or suppression of the immune response depending on several factors including the type of stressor, length of exposure to the stressor and the status of the animal at the time of stress (Dhabhar 2009, Dhabhar & McEwen, 1997). Humans, pigs, and rats subjected to heat stress have elevated concentration of proinflammatory cytokines in peripheral blood including interleukin (IL)-1β, IL-6, TNFα and IFNγ (Bouchama et al., 1993, Ju et al., 2014, Ji et al., 2014). In parallel, peripheral lymphocytes isolated from dairy cows exposed to heat stress during the dry period have impaired proliferative potential compared to lymphocytes isolated from cooled cows (do Amaral et al., 2010). Additionally, there is also evidence suggesting a carry-over effect of heat stress on innate immune function; neutrophils from cows exposed to heat stress during the dry period and returned to cooling conditions after parturition have impaired phagocytosis and oxidative burst up to 20 days after the resolution of heat stress (do Amaral et al. 2011). Further, neutrophil numbers are greater postpartum in cows that are cooled when dry relative to those under heat stress (do Amaral et al., 2011, Thompson et al., 2014).

Uterine disease affects up to 40% of postpartum cows and is characterized by an infection of the uterus by pathogenic bacteria including *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum* or *Prevotella melaninogenica*. To prevent the development of uterine disease, cows rely on immune resilience mechanisms to avoid, tolerate, and resist pathogens. These mechanisms limit exposure to pathogens (avoidance), limit the damaged caused by pathogens (tolerance) or actively eliminate pathogens (resistance; Sheldon et al., 2020). The innate immune system is responsible for the initial recognition of pathogen-associated molecular patterns (PAMPs) present

within bacteria that cause uterine disease. Bacterial PAMPs are recognized by specific receptors, including Toll-like receptors, present on haemopoietic immune cells and endometrial cells (Cronin et al., 2012, Turner et al., 2014). Activation of the innate immune response in the endometrium by PAMPs results in the secretion proinflammatory cytokines (IL-1β and IL-6) and chemokines (IL-8) that attract and activate immune cells and induce secretion of acute phase proteins (Sheldon et al., 2019, Takeuchi & Akira, 2010, Healy et al., 2014) required to eliminate pathogens and are considered resistance mechanisms. Cellular mechanisms of innate tolerance which reduce the negative effects of pathogens and limit tissue damage are not well characterized in the endometrium; however, recent investigations suggest cellular metabolisms and cholesterol signaling may contribute to cellular tolerance to bacterial cytolysins that damage endometrial cells during uterine infections (Ormsby et al., 2021)

Given the potential of heat stress to impact the immune system, we hypothesized that heat stress alters immune resilience, predisposing cows to uterine disease. Hence, we investigated the impact of heat stress on immune resilience by evaluating bacterial content of the lower reproductive tract and immune responses of primiparous Holstein cows exposed to controlled heat stress conditions.

Materials And Methods

Animal Enrollment

This study was conducted at the University of Florida Dairy Research Unit from June to November 2019 and was approved by the Institutional Animal Care and Use Committee at the University of Florida. Nulliparous pregnant Holstein heifers were blocked by body condition score and predicted transmitting ability (PTA) for milk, housed in the same freestall barn and assigned to cool (CL) conditions (shade with

water soakers and fans; n = 14) or heat stress (HS) conditions (barn shade only; n = 16) for 60 days before their expected calving date. After calving all cows were housed in a freestall barn equipped with shade, water soakers and fans. Thermoregulatory measurements, diet information and subsequent milk yield are reported in detail by Davidson et al. (2021).

Vaginal Mucus and Blood Sampling

Vaginal mucus and blood were collected on d 7 and 21 after parturition from each cow. To collect vaginal mucus, the vulva was thoroughly cleaned with 70% ethanol and dried with paper towel prior to the insertion of a sterile Metricheck tool (Simcro, Hamilton, New Zealand) consisting of a stainless-steel rod with a rubber collection cup. Once the tool was inserted into the vagina, consistent movements were performed to sample the whole vaginal canal. After removal of the tool, the collected mucus was graded and placed in a sterile bijou tube (Thermo Fisher Scientific, Waltham, MA). The mucus was graded based on the proportion of pus in the sample and scored from 0 to 4. Score 0 = clear vaginal mucus; score 1 = mucus with flecks of white pus; 2 = mucus with less than 50% white pus; score 3 = mucus with more than 50% white pus; score 4 = sanguinopurulent mucus (adapted from Sheldon et al., 2009). Mucus samples were stored at -80°C for further analysis.

Whole blood was collected from coccygeal vessels into evacuated tubes (Vacutainer, Becton Dickson, Franklin Lakes, NJ) containing lithium-heparin anticoagulant on d 21. Samples were maintained on ice and transported to the laboratory for further processing.

Bacterial DNA Isolation and Quantification of Total Bacterial Load

Isolation of total bacterial DNA from vaginal mucus samples was performed using the Dneasy Power Soil kit (Qiagen, Hilden, Germany) as previously described (Piersanti et al., 2019). Briefly, samples were thawed on iced and then homogenized by vortexing for 5 min. Samples were weighed and 250 mg of each sample was added to a tube containing garnet particles and guanidine thiocyanate. Samples were homogenized using 3 cycles of a tissue homogenizer (30 s at 6,000 × *g*, 60 s pause, 30 s at 6,000 × *g*; Precellys 24, Bertin Technologies SAS, France) with a 5 min incubation on ice between each cycle. Supernatants were collected and added to the Dneasy Power Soil spin columns for purification of DNA following the manufacturer's instructions. Purified DNA was subjected to total bacterial 16S rRNA quantification using the Femto Bacterial DNA Quantification Kit (Zymo Research, Irvine, CA). Briefly, real time quantitative PCR was performed using the provided primer mix containing SYTO 9 fluorescent dye and primers targeting 16S rRNA in a 20 µL reaction with 2 µl of total extracted DNA in each well. A CFX Connect Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA) was used for a 3-step protocol consisting of initial denaturation at 95°C for 10 min, amplification with 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. A standard curve was provided and performed in parallel with the samples for quantification of 16S rRNA. Reactions were performed in duplicate and no-template controls were included. Results are described as nanograms of 16S rRNA per milligram of vaginal mucus.

Detection of Recognized Bacteria in Vaginal Mucus

Quantification of pathogenic bacteria associated with uterine disease in vaginal content was performed using DNA isolated from mucus samples described above. Specific primers for *E. coli, T. pyogenes, F. necrophorum* and *P. melaninogenica* were designed using the NCBI primer-design tool or were previously published and verified by BLAST (Malinen et al., 2003, Belser et al., 2015, Cunha et al., 2018). Primers were validated for amplification efficiency prior to analysis and followed MIQE guidelines (Pearson correlation coefficient $R^2 > 0.98$ and efficiency between 90 and 110%) (Bustin et al., 2009). Quantitative real time PCR was performed in 20 µL reactions containing 18 µL of SYBR Green Master Mix (Bio-Rad Laboratories), 300 nM of each forward and reverse primer and 2 µL of template DNA. PCR was performed using a CFX Connect Real-Time PCR System (Bio-Rad Laboratories) with a 3-step protocol consisting of enzyme activation at 95°C for 3 min, amplification with 40 cycles of denaturation at 95°C for 10 s, annealing for 10s (temperature varied for each target and are described in Table 3-1), and extension at 72°C for 30 s. Each reaction was performed in duplicate, and a no-template control and melt curve were included for each assay. For each pathogen, a standard curve with purified DNA was prepared and run in parallel with the samples for pathogen quantification. Total DNA from *E. coli* MS499, *T. pyogenes* MS249 (supplied by Dr. Martin Sheldon) and *F. necrophorum* (supplied by Dr. Klibs Galvão) was obtained by extraction of live cultures using the Dneasy Power Soil kit as above. Purified DNA of *P. melaninogenica* was purchased from ATCC (Manassas, VA; #25845). Results were normalized by weight of vaginal mucus and are expressed as CFU/µL/mg of mucus for *E. coli* and *T. pyogenes* and pg/µL/mg of mucus for *F. necrophorum* and *P. melaninogenica*.

Whole Blood LPS Challenge

Whole blood collected on d 21 was subjected to a whole blood challenge with LPS according to Bromfield et al. (2018) with minor modifications. Briefly, ultrapure *E. coli* O111:B4 LPS (Invivogen, San Diego, CA) was diluted in warm Dulbecco's modified Eagle medium (Thermo Fisher Scientific, Waltham, MA) to a 10 µg/mL concentration immediately prior to each challenge. Two aliquots of 980 µL of whole blood from each cow were transferred to 2 mL snap lock tubes (Eppendorf, Hamburg, Germany). Aliquots were treated with either 20 µL of warm DMEM as a negative control or with 20 µL of ultrapure LPS for a final concentration of 1 µg/mL. Samples were placed into a rotating tube holder and maintained at 38.5°C for 4 h. Following incubation, samples were centrifuged at 8500 × *g* for 10 min at 4°C to collect plasma which was stored at -80°C for further analysis.

Detection of Inflammatory Mediators

Following whole blood challenge with LPS, subsequent plasma was analyzed using the Milliplex Bovine Cytokine/Chemokine 15-plex kit (BCYT1-33K, EMD Millipore, Billerica, MA, USA) according to the manufacturer's instruction. The kit allowed for the simultaneous quantification of IFNγ, IL-1α, IL-1β, IL-4, IL-6, IL-8 (CXCL8), IL-10, IL-17A, IL-36RA (IL-1F5), IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), TNFα and VEGF-A. Briefly, plasma samples were thawed on ice, vortexed, centrifuged, and diluted 1:2 in assay buffer. Quality controls and standard stock were reconstituted with 250 µL of deionized water and allowed to sit for 10 minutes at room temperature prior to use. Standard stocks were serially diluted in assay buffer to prepare the standard curve. All reagents were used at room temperature and all reactions were run in duplicate. First, 200 µL of wash buffer was added to each well, the plate was sealed

and incubated with shaking for 10 minutes at room temperature. Wash buffer was removed and 25 µL of each standard or quality control was loaded into each designated well. Assay buffer alone was used as a background control. Subsequently, 25 µL of assay buffer was added to the sample wells followed by 25 µL of each sample. Serum matrix was reconstituted with 1 mL of deionized water and allowed to sit at room temperature for 10 minutes prior to addition of 25 µL to background, standard, and quality control wells. Premixed beads were vortexed for 1 minute and 25 µL was added to each well. The plate was sealed with aluminum foil sealing film, vortexed for 25 min at room temperature and incubated overnight at 4°C. On the following day, the plate was agitated for 1 h at room temperature prior to washing three times. Finally, 25 µL of detection antibody was added to each well. The plate was sealed with aluminum foil sealing film and incubated with agitation for 1 hour prior to the addition of 25 µL of streptavidin- phycoerythrin to each well. The plate was incubated one last time with agitation and protection from the light for 30 minutes at room temperature. After washing three times, 150 µL of MAGPIX drive fluid was added to each well, the plate was agitated for 5 minutes to resuspend the beads and samples were read using a MAGPIX instrument with xPONENT software (Luminex, Austin, TX). Samples that had a concentration above the range of detection for any analyte were diluted 1:5 in assay buffer and the assay was repeated. Quality control values for each marker were consistently within the range indicated by the manufacturer. In addition to the multiplex detection of inflammatory mediators, additional quantification of IL-1β was evaluated using a commercial bovine IL-1β ELISA kit (ESS0027; Thermo Scientific, Rockford, IL) according to the manufacturer's instructions.

Statistical Analysis

Data were analyzed using generalized mixed models on SPSS Statistics v26 (IBM Corporation, Armonk, NY). Total bacterial load and specific pathogen load were analyzed with day as a repeated measure and fixed effects of heat stress, day and the interaction between heat stress and day with pairwise comparisons. The effect of vaginal mucus grade on bacterial load was analyzed with fixed effects of mucus score, heat stress and the interaction between mucus score and heat stress. Concentration of cytokines was analyzed using a generalized mixed model with pairwise comparisons of fixed effects of treatment (LPS *vs* medium), dry period heat stress or cooling and the interaction between treatment and heat stress/cooling with heat stress/cooling as contrast field. Cow was added to the model as a random effect. Statistical significance was set at $P \leq 0.05$ and tendency at $P \leq 0.10$.

Results

Induction of Heat Stress During the Pre-Calving Period

Detailed data pertaining to thermoregulatory measurements and subsequent milk yield of cows are reported by Davidson et al. (2021). Briefly, increases in respiratory rate and rectal temperature were observed in HS cows during the pre-calving period when cows did not have access to fans or water soakers. After calving when all cows had access to fans and water soakers, milk production was decreased in HS cows compared with CL cows. These data are consistent with the induction of heat stress in the HS cows. The average vaginal mucus grade of cows was higher on d 7 compared to d 21 (3.08 ± 1.65 *vs* 0.15 ± 0.05), while the average vaginal mucus grade on day 7 (CL, 1.9 ± 3.1 *vs* HS, 1.8 ± 3.1) and d 21 (CL, 0.3 ± 0.1 *vs* HS, 0.1 ± 0.1) was not affected by

heat stress. The proportion of cows with a vaginal mucus grade ≥ 3 on d 7 (CL, 62.5% *vs* HS, 46.2%) or d 21 (CL, 12.5% *vs* HS, 15.4%) was not affected by heat stress.

The Effect of Heat Stress on Bacterial Content in the Lower Reproductive Tract

Total bacterial content of the lower reproductive tract was not affected by heat stress but tended to be increased on d 7 compared to d 21 (3.08 ± 1.65 ng/mg *vs* 0.15 ± 0.05 ng/mg, $P = 0.08$, Fig. 3-1A), and it was not affected by the interaction between day and heat stress. Total bacterial content was not affected by vaginal mucus grade, heat stress or the interaction on d 7 or d 21 (Fig. 3-1B-C).

The presence of vaginal *E. coli* was detected in 89.3% of cows at d 7 and 82.1% of cows at d 21 (Fig. 3-2A). The presence of vaginal *F. necrophorum* was detected in 69.0% of cows at d 7 and 28.6% of cows at d 21 (Fig. 3-2B). The presence of vaginal *P. melaninogenica* was detected in 100% of cows at d 7 and at d 21 (Fig. 3-2C), while the presence of vaginal *T. pyogenes* was detected in 96.6% of cows at d 7 and 78.6% of cows at d 21 (Fig. 3-2D). Vaginal content of *E. coli*, *F. necrophorum* and *P. melaninogenica* were not affected by heat stress, day of collection or the interaction of day and heat stress (Fig. 3-2). Vaginal content of *T. pyogenes* on d 7 tended to be higher relative to d 21 (266.8 ± 147.6 CFU/μL/mg *vs* 2.1 ± 9.1 CFU/μL/mg, $P = 0.08$, Fig. 3-2D), however vaginal content of *T. pyogenes* was not affected by heat stress on either day of collection.

The Effect of Heat Stress on LPS Responses in Whole Blood

Accumulation of IL-1α, IL-1β, IL-4, IL-6, IL-8 (CXCL8), IL-10, IL-17A, IL-36RA (IL-1F5), IFNγ, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), TNFα and VEGF-A were evaluated in blood treated with 1 μg/mL of LPS or medium alone for 4 h (Fig. 3-3). Treatment of whole blood with LPS increased the accumulation of IL-1α,

IL-1β, IL-6, IL-10, IL-17, IL-36RA, IFNγ, IP-10, MIP-1α, MIP-1β, TNFα and VEGF-A compared to blood treated with medium alone ($P < 0.01$, Fig. 3-3). LPS induced accumulation of IL-1β, IL-10 and MIP-1α was increased ($P < 0.01$) in blood collected from HS cows compared to CL cows, while LPS induced accumulation of IL-1α tended ($P \leq 0.08$) to be increased in blood of HS cows compared with blood of CL cows (Fig. 3-3B, C, E, G). Regardless of LPS treatment, blood from HS cows tended ($P < 0.08$) to have increased accumulation of IL-1β, IL-10 and MIP-1α after culture compared to blood of CL cows. Concentrations of IL-4 were undetectable for all samples.

A commercial bovine ELISA for IL-1β was used to confirm the findings of the multiplex assay (Fig. 3-4). As expected, treatment of whole blood with LPS increased the accumulation of IL-1β compared to blood treated with medium alone ($P = 0.03$). LPS induced accumulation of IL-1β was increased ($P < 0.01$) in blood collected from HS cows compared to CL cows in a similar fashion to what was measured using the multiplex assay. A simple linear regression of IL-1β concentration in samples measured using ELISA and the multiplex assay showed a linear correlation between the two assay types ($P < 0.01$, $r^2 = 0.69$); however, the values obtained using the ELISA are lower than those reported using the multiplex assay.

Discussion

We have previously demonstrated (Chapter 2) that the incidence of uterine disease is increased during hotter months without any apparent increase in bacteria of the lower reproductive tract. The question therefore remains; if greater bacterial content of the reproductive tract is not responsible for increased uterine disease during the summer, could heat stress impact host immunity and effectively reduce immune

resilience to pathogens? Herein, we investigated how prior exposure to heat stress impacted bacterial content of the lower reproductive tract and cytokine production of whole blood following an *in vitro* LPS challenge. Cows in the HS group had decreased milk yield and increased vaginal temperatures and respiration rates compared to CL group, indicating that our model was suitable to study the effect of heat stress in the cow's response to pathogens. Our results suggest that prior exposure to heat stress during late pregnancy has minimal impact on bacterial content of the vagina but does affect resilience to pathogens by altering host immune function, whereby blood of HS cows stimulated with LPS had increased accumulation of pro-inflammatory (IL-1β and MIP-1α) and anti-inflammatory (IL-10) cytokines compared to the blood of CL cows.

Exposure of dairy cows to temperatures above their thermoneutral zone induces a stress response which includes activation of the hypothalamic-pituitary-adrenal (HPA) axis and secretion of glucocorticoids, mainly cortisol and prolactin, into the blood (Wise et al., 1988, Sejian et al., 2018). Basal plasma levels of inflammatory cytokines IL-1β, IL-6, IFNγ and TNFα are higher in cows concurrently exposed to HS conditions compared to cooled cows (Chen et al., 2018). We did not find differences in basal level of cytokines between CL and HS cows; however, in our experiment, cytokine levels were analyzed when all cows were being cooled, 21 d after the removal of HS conditions.

Immune resistance is the ability of the animal to avoid disease by limiting pathogen proliferation, primarily using inflammation and innate immunity. Exposure of animals to stressors impacts the host immune system; however, the subsequent effect depends on the species and status of the animal and the type and length of the stressor

(Dhabhar 2009). For example, dairy cows exposed to elevated environmental temperature during the dry period upregulate molecular pathways related to immunity in the mammary gland (Dado-Senn et al., 2018). Moreover, plasma proteomic analysis revealed that long-term exposure to elevated temperatures induced an inflammatory state in lactating dairy cows (Min et al., 2016). Marins et al. (2021) reported a similar finding to ours, where mononuclear cells collected from dairy cows during heat stress produced greater concentrations of cytokines after *in vitro* stimulation with LPS compared to mononuclear cells from cooled cows. The divergent characteristic in our study was that our whole blood challenge was performed 21 days after cows were removed from HS conditions. Collectively, our data suggests that exposure to HS during the dry period has carry over effects on the cow's response to pathogens that are not overcome by subsequent cooling of cows during lactation. Moreover, our data highlights the importance of cooling dairy cows during the dry period, especially in regions like Florida, where 70% of the year is composed by days that will induce heat stress in dairy cows (Ferreira et al., 2016).

The innate immune response is the first line of defense against pathogens associated with uterine disease. Pathogenic bacteria such as *E. coli*, *T. pyogenes*, *F. necrophorum* and *P. melaninogenica* trigger an inflammatory response in the uterus characterized by secretion of pro-inflammatory cytokines. While production of inflammatory cytokines is key to innate immunity toward pathogens and prevention of disease, an even greater production of inflammatory cytokines in response to pathogens likely exacerbates or prolongs inflammatory responses and impairs tissue repair and resolution of disease (Barth et al., 2013, Qian et al., 2016).

Our findings suggest that the increased incidence and persistence of uterine disease observed during elevated environmental temperatures is not due to an increase in pathogen load, at least as quantified in the lower reproductive tract. The increased incidence and persistence of uterine disease during periods of elevated environmental temperature might be due to heat stress-induced perturbations in the systemic inflammatory response, reducing the resistance of cows to uterine disease. It is important to note that it is unclear if the effects observed here on whole blood cells could be directly translated to the uterine environment. Further investigation is necessary to evaluate the effects of heat stress on the uterine response to pathogens and to determine the mechanisms responsible for heat stress induced chronic inflammation in the dairy cow.

Table 3-1. PCR primers. Primer sequences used for real time RT-PCR to quantify specific pathogens in vaginal mucus samples.

Target Bacteria	Gene	Primer Sequence (5'-3")	Annealing temperature (ºC)	Source
E. coli	*16S*	Fwd – GTTAATACCTTTGCTCATTGA Rev –ACCAGGGTATCTAATCCTGTT	53.5	(Malinen et al., 2003)
T. pyogenes	*PLO*	Fwd – GGCCCGAATGTCACCGC Rev –AACTCCGCCTCTAGCGC	64.5	(Belser et al., 2015)
F. necrophorum	*Ikta*	Fwd – GATTGGGGGATAGCGGTAAT Rev – GAGCCTCCACATTTAGTCGC	63	(Cunha et al., 2018)
P. melaninogenica	*phyA*	Fwd – ACAAAGAGGCAAACCAAGCG Rev – TACGAAGCATCCGTTCAGGG	55	(Designed)

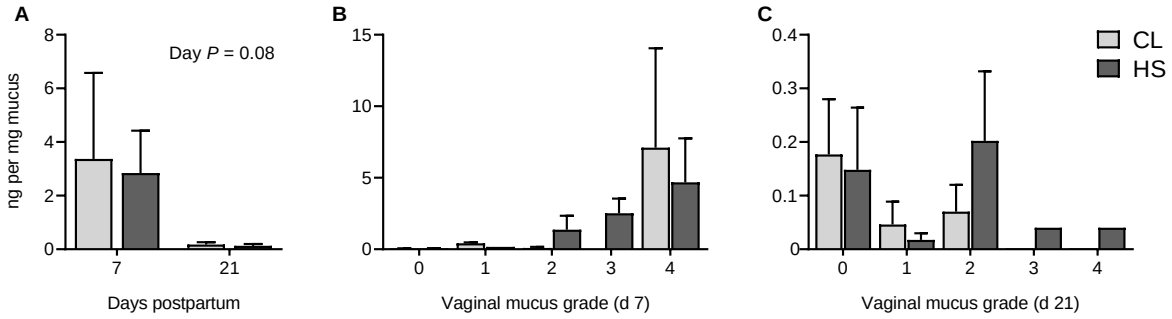

Figure 3-1. Total bacterial content of the lower reproductive tract. A) Total bacterial content of vaginal mucus from cows previously exposed to heat stress (HS) or cool (CL) housing conditions during the dry period was quantified by measuring bacterial 16S rRNA collected on d 7 and d 21 after calving. B) Total bacterial content on d 7 according to vaginal mucus grade. C) Total bacterial content on d 21 according to vaginal mucus grade. Bars represent the mean ± S.E.M. Data are expressed as ng of 16S rRNA per mg of vaginal mucus and were analyzed for the fixed effect for heat stress, day of collection, and vaginal mucus grade.

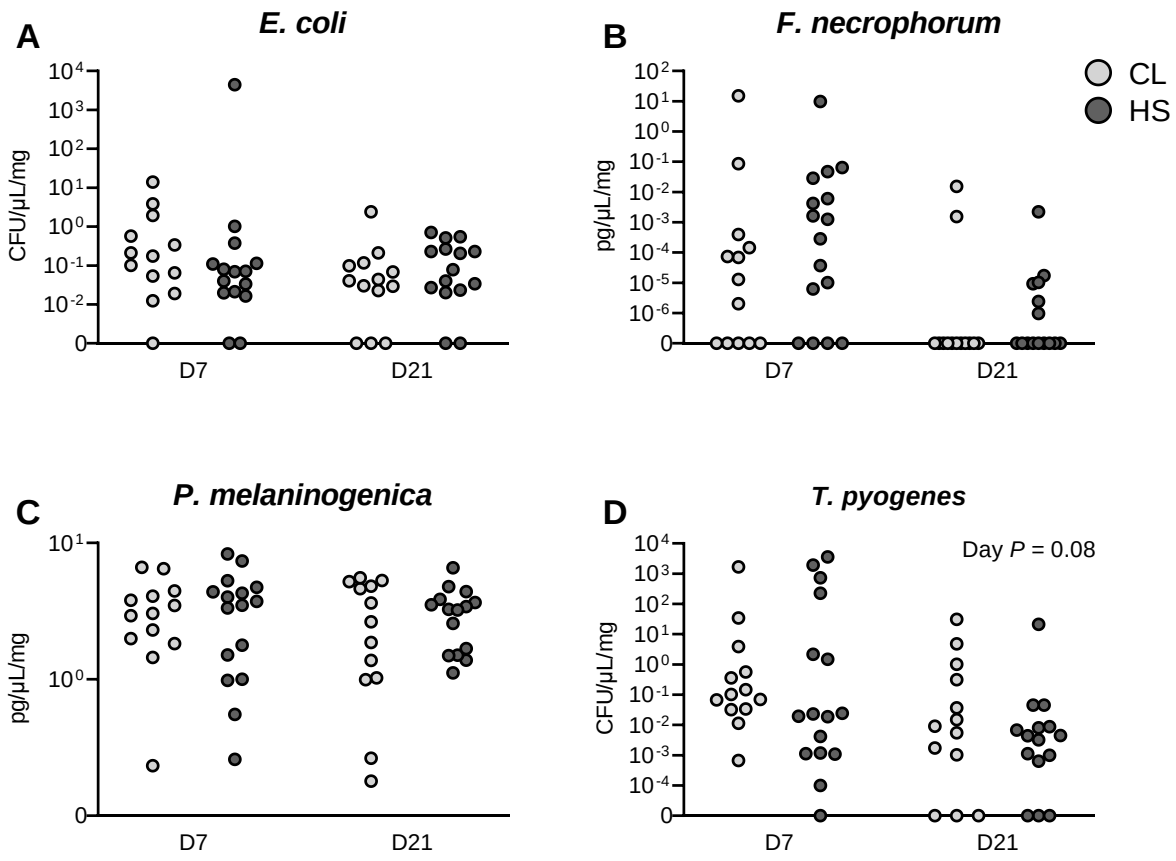

Figure 3-2. Specific pathogen content of the lower reproductive tract. Vaginal mucus from cows previously exposed to heat stress (HS) or cool (CL) housing conditions during the dry period was collected on d 7 and d 21. Content of *E. coli* (A), *T. pyogenes* (B), *F. necrophorum* (C) and *P. melaninogenica* (D) were quantified in vaginal mucus using quantitative real time RT-PCR. Data for *E. coli* and *T. pyogenes* are expressed as CFU/µL per mg of vaginal mucus, and data for *P. melaninogenica* and *F. necrophorum* are expressed as pg/µL per mg of vaginal mucus. Each dot represents an individual cow. Data were analyzed for the fixed effect for heat stress, day of collection, and the interaction of heat stress and day of collection. Vaginal mucus was not collected for two CL cows, one on d 7 and one on d 21.

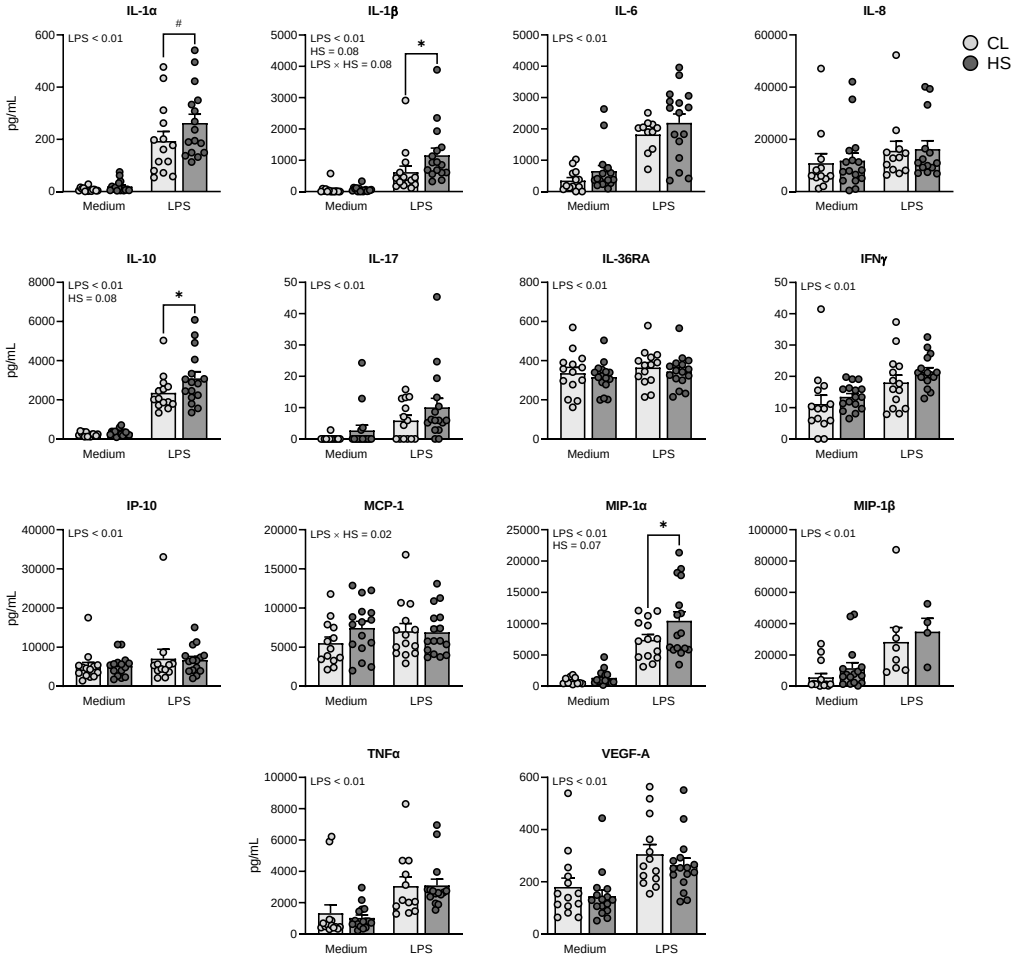

Figure 3-3. Accumulation of inflammatory cytokines following a whole blood LPS challenge. Whole blood from cows previously exposed to heat stress (HS) or cool (CL) housing conditions during the dry period was collected on d 21 after calving and exposed to 1 μg/mL of LPS or medium alone for 4 h. Accumulation of IL-1α, IL-1β, IL-6, IL-8 (CXCL8), IL-10, IL-17A, IL-36RA (IL-1F5), IFNγ, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), TNFα and VEGF-A were evaluated using a multiplex assay. Bars represent the mean ± S.E.M and dots represent individual samples. Data were analyzed for the fixed effect for heat stress, LPS treatment, and the interaction of heat stress and LPS treatment. * $P \leq 0.05$ and # $P \leq 0.08$.

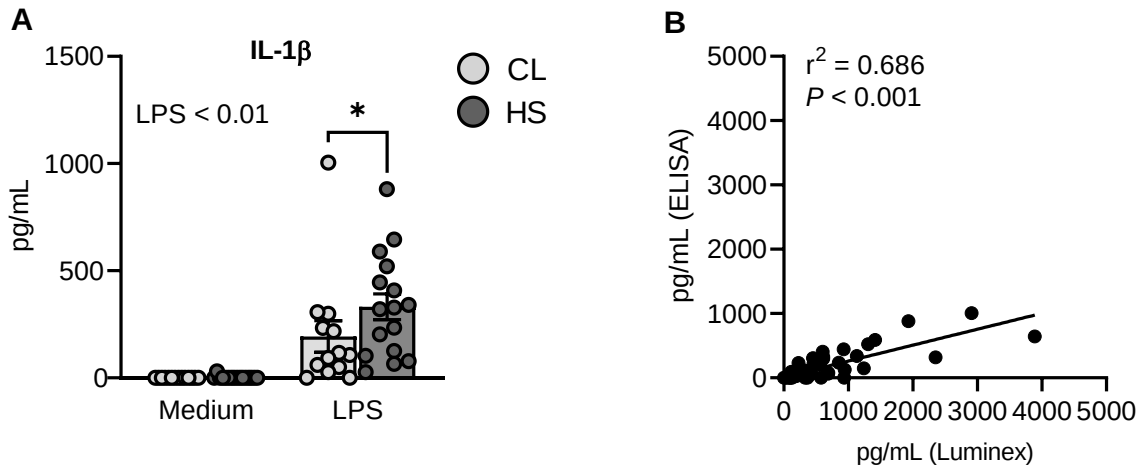

Figure 3-4. Accumulation of IL-1β following a whole blood LPS challenge. Whole blood from cows previously exposed to heat stress (HS) or cool (CL) housing conditions during the dry period was collected on d 21 after calving and exposed to 1 μg/mL of LPS or medium alone for 4 h. (A) Accumulation of IL-1β was evaluated using a commercial ELISA kit. Bars represent the mean ± S.E.M and dots represent individual samples. Data were analyzed for the fixed effect for heat stress, LPS treatment, and the interaction of heat stress and LPS treatment. * $P \leq 0.05$. (B) Data acquired from ELISA analysis was compared to data acquired from the multiplex assay using identical samples. Each dot represents an individual sample. Data were analyzed using a simple linear regression.

CHAPTER 4
INFLAMMATORY RESPONSES OF BOVINE ENDOMETRIAL EPITHELIAL CELLS ARE EXACERBATED UNDER HEAT STRESS CONDITIONS

Environmental factors including heat stress have a significant impact on dairy production across the globe. Elevated environmental temperatures trigger adaptation mechanisms in the cow to counteract the effects of heat stress and maintain optimal body temperature. However, those same adaptation mechanisms that ensure survival can also have detrimental effects on cow performance (Pragna et al., 2018). Cows exposed to heat stress have reduced DMI, subfertility and are more likely to develop mastitis, metabolic disorders, and uterine diseases (Ouellet et al., 2020, Gernand et al., 2019, Das et al., 2016).

At the cellular level, exposure to heat stress causes protein misfolding that activates heat shock transcription factors such as HSF1. Activation of HSF1 promotes transcription of heat shock proteins (HSP), including HSP1A1 (known as HSP70) that has an important role as a chaperone and aids in cellular repair following heat stress induced damage (Archana et al., 2017). In ruminants, HSPs are associated with thermotolerance and play a regulatory role in the immune system (Lacetera et al., 2006, Mishra et al., 2011). In humans and pigs, HSP1A1 regulates expression of proinflammatory cytokines in immune cells by modulating Toll-like receptor (TLR) 2 and TLR4 signaling pathways (Zhou et al., 2005, Ju et al., 2014, Chen et al., 2009). We have previously observed (Chapter 3) that whole blood from lactating dairy cows exposed to heat stress during late gestation increased synthesis of the proinflammatory cytokines interleukin (IL)-1β and IL-6 in response to lipopolysaccharide (LPS) compared to blood of cows cooled prepartum.

Uterine diseases are characterized by pathogenic bacteria entering the uterus and causing inflammation and tissue damage. Endometrial epithelial and stromal cells recognize pathogen associated molecule patterns (PAMPs) through TLRs and elicit an inflammatory response characterized by secretion of proinflammatory cytokines IL-1β, IL-6 and CXCL8 (known as IL-8). However, the role of heat stress in mediating changes to endometrial immune function, and potentiating uterine disease are not known. Therefore, we hypothesize that exposure of endometrial cells to heat stress would exacerbate expression of proinflammatory cytokines in response to PAMPs due to altered heat-shock proteins. Bovine endometrial epithelial (BEND) cells were exposed to either ultrapure LPS, a TLR4 agonist, Pam3CSK4, a synthetic triacylated lipopeptide TLR1/2 agonist, or medium alone, under thermoneutral (TN, 38.5°C) conditions or heat stress (HS, 41°C) conditions for 24 h. In an attempt to determine if heat shock proteins mediate altered expression of proinflammatory cytokines, *HSF1* and *HSPA1A* were targeted using siRNA prior to BEND cell exposure to LPS or Pam3CSK4 under heat stress conditions. Understanding the molecular mechanisms by which heat stress affects uterine immune function may be used to optimize health management of dairy cows exposed to heat stress, and aid in the development of pharmaceutical interventions to reduce heat stress associated uterine disease in the dairy cow.

Materials And Methods

Bovine Endometrial Epithelial Cell Culture

Bovine endometrial epithelial (BEND) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA; CRL-2398) and cultured according to suppliers' instructions. Briefly, cells were cultured in 75 cm² flasks (Greiner Bio-One, Monroe, NC) in complete culture medium (40% Ham F-12, 40% MEM, 10% fetal bovine

serum, 10% horse serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, 1.5 g/L of sodium bicarbonate, 0.034 mg/mL D-valine; Thermo Fisher Scientific, Waltham, MA). Cultures were maintained at 38.5°C in a humidified atmosphere containing 95% air and 5% CO2, with culture medium changed every 48 h until cells were sub-confluent. Cells were isolated using 0.25% trypsin with 0.1% EDTA for 5 minutes, centrifuged, resuspended at 10^5 cells/mL in complete medium and 500 µL was seeded into 24-well plates (TPP, Trasadingen, Switzerland). Cells were equilibrated at 38.5°C for 24 h prior to treatment.

Treatment of BEND Cells Using Pathogen Associated Molecular Patterns Under Heat Stress Conditions

Bovine endometrial epithelial cells were treated with either ultrapure *E. coli* O111:B4 LPS (Invivogen, San Diego, CA), Pam3CSK4 (Invivogen), or medium alone as a control. Doses of each treatment started at 10,000 ng/mL and decreased ten-fold to 1 ng/mL. Immediately following the application of each treatment, cells were incubated for 24 h at 38.5 °C (thermoneutral conditions, TN) or at 41°C (heat-stress conditions, HS). Each experiment was repeated six times, with BEND cells isolated between passage 4 and 12. Following treatment, total cellular RNA was stabilized in 350 µL of RLT buffer (Qiagen, Germany) and stored at −80°C for further processing.

Bovine Endometrial Cells siRNA Transfection

Cells used for siRNA transfection were cultured in 75 cm^2 flasks (Greiner Bio-One) in antibiotic free complete culture medium (40% Ham F-12, 40% MEM, 10% fetal bovine serum, 10% horse serum, 1.5 g/L of sodium bicarbonate, 0.034 mg/mL D-valine; Thermo Fisher Scientific). Cells underwent reverse transfection using the following approach. Transfection reagent solutions were established directly into the culture well

of a 24-well plate prior to the addition of cells. A total of 100 µL of warm Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific) and 1.5 µL of RNAiMAX lipofectamine (Thermo Fisher Scientific) was added to each well in the presence of either 20 nM of *HSF1* siRNA, 20 nM *HSPA1A* siRNA or 20 nM of scramble siRNA as a control (Table 4-1 Silencer Select, Thermo Fischer Scientific). Transfection reagent solutions were incubated for 20 min at room temperature prior to seeding of cells at 10^5 cells/mL. Cells were incubated with transfection reagent solution for 24 h at 38.5°C before the addition of treatments.

Following the 24 h transfection BEND cells were treated with either 1,000 ng/mL of ultrapure *E. coli* O111:B4 LPS (Invivogen), 1,000 ng/mL of Pam3CSK4 (Invivogen), or antibiotic free medium alone as a control. Immediately following the application of each treatment, cells were incubated for 24 h at 38.5 °C (TN) or at 41°C (HS). Each experiment was repeated six times, with cells isolated between passage 3 and 9. Following treatment, total cellular RNA was stabilized in 350 µL of RLT buffer (Qiagen, Hilden, Germany) and stored at −80°C for further processing.

RNA Extraction and Real-Time RT-PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Samples were quantified and checked for RNA quality by spectrophotometry (Nanodrop ND1000, Thermo Fisher Scientific) and cDNA was synthesized using the Verso cDNA kit according to manufacturer's instructions (Thermo Fisher Scientific). Primers for real-time RT-PCR were designed using the NCBI primer-design tool and verified by BLAST (Table 4-2**)**. All primers were validated for amplification efficiency prior to sample analysis and conformed to MIQE guidelines (Pearson correlation coefficient $r^2 > 0.98$ and efficiency between 90 and 110%) (Bustin

et al., 2009). Real-time PCR was performed in 20 µL reactions, containing 18 µL of SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA) with 500 nM of each forward and reverse primer and 2 µL of template cDNA. A CFX Connect Real-Time PCR System (Bio-Rad Laboratories) was utilized to perform PCR using a protocol consisting of an initial denaturation/enzyme activation step for 30 s at 95°C followed by 40 cycles of denaturation at 95°C for 5 s, annealing/extension at 60°C for 30 s. Reactions were performed in duplicate, with inclusion of a melt-curve analysis and a no-template control with no cDNA for each primer set to demonstrate the absence of non-specific amplification. Relative expression for genes of interest was calculated using the $2^{-\Delta Ct}$ method relative to either *GAPDH* or the geometric mean of *GAPDH* and *RLP19* (Livak & Schmittgen 2001). Expression of *GAPDH* and *RLP19* was stable ($P > 0.05$) across all treatments.

Assessment of Endometrial Cell Viability After Exposure to Heat Stress

Viability of BEND cells after treatment was assessed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT; Thermo Fisher Scientific) assay as previously described (Rizo et al., 2019). Briefly, BEND cells were seeded into 96-well culture plates (TPP) at a density of 105 cells/mL in 200 µL of complete culture medium and equilibrated for 24 h at 38.5°C in a humidified atmosphere containing 95% air and 5% CO^2. Cells were then exposed to 1,000 ng/mL of LPS, Pam3CSK4, or control medium in duplicate for 24 h at 38.5 °C (TN) or at 41°C (HS). At the completion of the treatment period, 10 µL of MTT (5 mg/mL) was added to each well and all plates were incubated at 38.5°C for 4 h. Supernatants were removed, and cells were washed once in warm DPBS. Supernatants were removed and 100 µL of dimethyl sulfoxide was added to each well to lyse cells and solubilize cellular formazan and incubated for 15

minutes in the dark at room temperature. Optical density of each well was measured at OD 540 nm using a microplate reader (BioTek Instruments, Winooski, VT). Each well was corrected to the average blank value and the average OD for each duplicate was calculated. Data were normalized to cells treated with control medium alone, and data were expressed as fold change from control.

Statistical Analysis

SPSS software V26.0 (IBM Analytics, Armonk, NY) was used for statistical analysis. Gene expression data was analyzed using a generalized linear mixed model with pairwise comparisons. Fixed effects included treatment, temperature, the interaction between treatment and concentration, and the interaction between temperature and treatment. For experiments using siRNA technology, fixed effects included treatment, temperature, transfection, treatment within transfection and the interaction between treatment and temperature within transfection. Statistical significance was set at $P \leq 0.05$ and tendency at $P \leq 0.10$.

Results

Induction of Heat Stress and Effect on Cell Viability

To determine the impact of increased temperature on endometrial cell immune response, BEND cells were exposed to either LPS, Pam3CSK4 or medium alone under thermoneutral (TN) conditions or heat stress (HS) conditions. Exposure of BEND cells to HS increased expression of *HSP1A1* compared to TN cells, regardless of treatment with LPS or Pam3CSK (Fig. 4-1A), indicating the establishment of heat stress. Interestingly, exposure of BEND cells to LPS reduced *HSP1A1* expression compared with cells exposed to Pam3CSK4 or medium alone, regardless of temperature.

Incubation of BEND cells under HS conditions for 24 h had no effect on cell viability compared to cells incubated under TN (Fig. 4-1B). In addition, exposure of BEND cells to LPS or Pam3CSK4 had no effect on cell viability compared to control cells. There was no interaction between incubation temperature and treatment, suggesting the conditions in which cells were cultured were not cytotoxic.

Effect of Heat Stress and Bacterial Components on the Expression of Inflammatory Mediators in BEND Cells

Overall, cells exposed to LPS had an increased expression of *IL6* and *CXCL8* and tended to have an increased expression of *IL1B* compared to medium alone controls (Fig. 4-2A-C). There was no overall effect of incubation temperature or the interaction between treatment and temperature for any of the cytokines analyzed. Specifically, exposure of BEND cells to 1,000 or 10,000 ng/µL of LPS increased expression of *IL1B*, *IL6* and *CXCL8* compared to medium alone controls, regardless of temperature (Fig. 4-2A-C). However, BEND cells exposed to 1,000 or 10,000 ng/µL of LPS and incubated under HS conditions increased expression of *IL1B* and *IL6*, and decreased expression of *CXCL8* compared to cells exposed to LPS and incubated under TN conditions (Fig. 4-2A-C).

Overall, cells exposed to Pam3CSK4 had an increased expression of *IL1B*, *IL6* and *CXCL8* compared to medium alone controls. There was no overall effect of incubation temperature or the interaction between treatment and temperature for any of the cytokines analyzed. Exposure of BEND cells to 100, 1,000 or 10,000 ng/µL of Pam3CSK increased expression of *IL1B*, *IL6* and *CXCL8* relative to medium alone controls, regardless of temperature (Fig. 4-2D-F). However, BEND cells exposed to 100, 1,000 or 10,000 ng/µL of Pam3CSK4 and incubated under HS conditions had an

increased expression of *IL1B* and *IL6* compared to cells exposed to Pam3CSK4 and incubated under TN conditions. There was no effect of incubation temperature on the expression *CXCL8* in BEND cells exposed to Pam3CSK4 at any concentration (Fig. 4-2D-F).

Effect of Heat Stress and Bacterial Components on the Expression of Toll-Like Receptors in BEND Cells

Previous data suggests that HS conditions alters inflammatory responses by increasing expression of *TLR4* in haemopoietic cells. Expression of TLR2 and TLR4 was evaluated in BEND cells exposed to either LPS, Pam3CSK4 or medium alone under HS or TN conditions (Fig. 4-3). There was no effect of incubation temperature on expression of *TLR2* or *TLR4* in BEND cells. Interestingly, exposure of BEND cells to LPS or Pam3CSK4 had no effect on the expression of *TLR2*; however, expression of *TLR4* was decreased in BEND cells exposed to LPS or Pam3CSK4 compared to control cells regardless of temperature. Specifically, expression of *TLR4* was decreased after exposure to 100, 1,000 or 10,000 ng/µL of LPS or Pam3CSK compared to control cells (Fig. 4-3B and D).

Effect of *HSP1A1* Knockdown on Inflammatory Responses of BEND Cells

To determine the mechanisms by which HS mediates an altered innate immune response to LPS or Pam3CSK, a key heat-shock protein chaperone, *HSP1A1* was targeted for knock down using siRNA technology (Fig. 4-4 and 4-5). As expected, incubation under HS conditions increased BEND cell expression of *HSPA1A* compared to incubation at TN, regardless of treatment (Fig. 4-4A). Overall, treatment of BEND cells with siRNA-HSP1A1 reduced *HSP1A1* expression compared to treatment with scramble siRNA and prevented the HS induced expression of *HSP1A1* (Fig. 4-4A and

4-5A). Knockdown of *HSP1A1* induced a 1.8-fold decrease in the expression of HSPA1A in BEND cells exposed to LPS (Fig. 4-4A). The knockdown of *HSP1A1* in HS cells did not prevent LPS mediated increased expression of *IL1B* or *IL6*, indeed *HSP1A1* knockdown further increased the expression of *IL1B* and *IL6* cells exposed to LPS under HS conditions compared to TN conditions (Fig. 4-4D-E). While LPS exposure decreased expression of *TLR2* and *TLR4*, and HS increased *TLR4* expression and tended to increase *TLR2* expression, there was no effect of *HSP1A1* knock down on expression of either *TLR2* or *TLR4* (Fig. 4-4B-C). As shown above (Fig. 4-2), expression of *CXCL8* was increased by LPS but was not affected by HS; however, *HSP1A1* knockdown increased LPS induced *CXCL8* expression compared with control cells exposed to LPS (Fig. 4-4F).

Knockdown of *HSP1A1* induced a 1.5-fold decrease in the expression of HSPA1A in BEND cells exposed to Pam3CSK4 (Fig. 4-5A). The knockdown of *HSP1A1* in HS cells did not prevent Pam3CSK4 mediated increased expression of *IL1B, IL6* or *CXCL8*, indeed the expression of *IL1B* and *IL6* were further increased in cells exposed to Pam3CSK4 under HS conditions compared to TN conditions (Fig. 4-5D-F). Expression of *CXCL8* was not altered by HS or knockdown of *HSPA1A*. Treatment with Pam3CSK4 tended to decrease *TLR2* expression whereas temperature and knockdown of *HSP1A1* had no effect on expression of *TLR2* (Fig. 4-5B). While Pam3CSK4 exposure decreased expression of *TLR4* and HS increased *TLR4* expression, there was no effect of *HSP1A1* knock down on expression of *TLR4* (Fig. 4-5C).

Effect of *HSF1* Knockdown on Inflammatory Responses of BEND Cells

To determine the mechanisms by which HS mediates an altered innate immune repose to LPS or Pam3CSK, the heat-shock response protein, *HSF1* was targeted for

knock down using siRNA technology (Fig. 4-6 and 4-7). Overall, treatment of BEND cells with siRNA-HSF1 reduced *HSF1* expression compared to treatment with scramble siRNA (Fig. 4-6A and 4-7A). Knockdown of *HSF1* induced a 1.8-fold decrease in the expression of *HSF1* in BEND cells exposed to LPS (Fig. 4-6A). Exposure of BEND cells to LPS or HS had no effect on *HSF1* expression (Fig. 4-6A). Expression of *IL1B*, *IL6* and *CXCL8* were increased after exposure to LPS, while expression of both *IL1B* and *IL6* were further increased in cells exposed to HS (Fig. 4-6D-F). Interestingly, knockdown of *HSF1* further increased HS mediated expression of *IL1B*, *IL6* and *CXCL8* in response to LPS. Expression of BEND cell *TLR2* was increased by HS but not effected by either LPS or *HSF1* knockdown (Fig. 4-6B). Similarly, expression of *TLR4* was increased by HS, however *TLR4* expression was decreased by LPS, while *HSF1* knockdown further reduced expression of *TLR4* (Fig. 4-6C).

Knockdown of *HSF1* induced a 2-fold decrease in the expression of *HSF1* in BEND cells exposed to LPS (Fig. 4-7A). Exposure of BEND cells to Pam3CSK4 or HS had no effect on *HSF1* expression (Fig. 4-7A). Expression of *IL1B*, *IL6* and *CXCL8* were increased after exposure to Pam3CSK4, while Pam3CSK4 mediated expression of both *IL1B* and *IL6* were further increased in cells exposed to HS (Fig. 4-7D-F). Interestingly, *HSF1* knockdown further increased HS mediated expression of *IL1B* and *IL6* in response to Pam3CSK4. Expression of *CXCL8* was not affected by HS or *HSF1* knockdown. Expression of BEND cell *TLR2* was not affected by Pam3CSK4, HS or *HSF1* knockdown (Fig. 4-7B). However, expression of *TLR4* was increased by HS and decreased by Pam3CSK4, and *HSF1* knockdown further reduced expression of *TLR4* (Fig. 4-7C).

Discussion

Heat stress is a major challenge for dairy production due to adaptive mechanisms that can be detrimental to a cow's performance. Cows exposed to heat stress have reduced DMI, subfertility and are more likely to develop mastitis, metabolic disorders, and uterine diseases (Ouellet et al., 2020, Gernand et al., 2019, Das et al., 2016). The innate immune system is the first line of defense against uterine disease and is negatively impacted by heat stress. However, it is unclear how heat stress mediated changes to innate immunity impacts uterine resilience to pathogens. Here, we simultaneously exposed bovine endometrial epithelial cells to bacterial components and heat stress to evaluate innate immune responses. Bovine endometrial epithelial cells exposed to heat stress conditions increased expression of the inflammatory cytokines *IL1B* and *IL6* in response to LPS or Pam3CSK4 relative to cells cultured under thermoneutral conditions. Heat stress at 41°C had no impact on cell viability but did induce increased expression of *HSPA1A* which encodes for a chaperone protein known for its role in the heat stress response. To understand if stress response genes are involved in the exacerbated inflammatory response to heat stress, *HSPA1A* and *HSF1* were targeted for knockdown in endometrial epithelial cells siRNA technology. Our transfection model successfully reduced the heat stress mediated increased expression of *HSPA1A* and *HSF1*; however, knock down of either gene did not rescue the exacerbated inflammatory response observed in BEND cells under heat stress conditions. Curiously, in some instances, knockdown of *HSPA1A* or *HSF1* further increased heat stress inflammatory responses to LPS or Pam3CSK4. These data suggest that the exacerbated inflammatory response to LPS or Pam3CSK4 induced by heat stress is not mediated by *HSPA1A* or *HSF1*.

Bovine endometrial cells respond to bacterial components, such as LPS and Pam3CSK4 using TLR4 and TLR1/2 respectively. Activation of the TLR complex following ligand binding results in increased endometrial cell expression of pro-inflammatory mediators including *IL1B*, *IL6* and *CXCL8* (Cronin et al., 2012, Turner et al., 2014). Exacerbated expression of pro-inflammatory mediators in response to pathogens is associated with persistent inflammation; for example, endometrial expression of *IL1A*, *IL1B* and *IL6* within the one week of calving are increased in cows that later develop endometritis compared to cows that remain healthy (Herath et al., 2009). We previously observed that prepartum heat stress increases the incidence and persistence of uterine disease (Chapter 2) and increased whole blood synthesis of inflammatory mediators in response to LPS (Chapter 3). In parallel, our data here shows that heat stress induces an upregulation of inflammatory cytokines by bovine endometrial epithelial cells in response to bacterial components. Interestingly, there is a potential for heat stress to modulate cytokine expression dependent on cell type. Sakai et al. (2020) reported that HS conditions induced an exacerbated production of IL-6 in bovine endometrial epithelial cells and conversely suppressed IL-6 production in endometrial stromal cells. However, recent conflicting reports suggest that in the absence of LPS, primary bovine endometrial epithelial cells decrease expression of inflammatory *PTGS2* and *IL8* in response to culture at 40.5°C for 3 to 12 h, while *IL1B*, *IL6* and *TNFA* remain unaffected by heat stress (Murata et al., 2021).

Heat shock proteins are promptly activated during heat stress and have an essential role in preventing cellular damage, restoring misfolded proteins, and maintaining cellular homeostasis. The main regulator of the heat shock response is

HSF1, a transcription factor activated during cellular stress that regulates a variety of factors including heat shock protein (HSP) 70, encoded by *HSPA1A* (Hentze et al., 2016). The chaperone protein HSP70 is involved in thermotolerance of cattle, preventing deleterious effects caused by exposure to elevated temperatures (Lacetera et al., 2006, Collier et al., 2008, Kishore et al., 2014). In human monocytes, HSP70 binds to receptors in the plasma membrane and activates the NF-κB pathway, resulting in upregulation of *IL1B*, *IL6* and *TNF* expression (Asea et al., 2020). Under our experimental conditions, targeted knockdown of *HSF1* or *HSPA1A* in endometrial epithelial cells was not able to reduce the exacerbated increase of *IL1B* or *IL6* in response to heat stress. This suggests that the exacerbated inflammatory response induced by heat stress in bovine endometrial epithelial cells is not regulated by heat shock proteins, or that the siRNA knockdown did not reduce HSF1 or HSP70 protein abundance in cells (which was not evaluated here). However, knockdown of *HSF1* or *HSPA1A* induced a further increase in expression of inflammatory cytokines in response to heat stress and PAMP, suggesting a potential inhibitory role for heat shock response factors in regulating cytokine expression. While we hypothesized that targeted knockdown of *HSF1* or *HSPA1A* would prevent or reduce heat stress mediated inflammation in endometrial cells, we found that exacerbated response was somewhat increased in response to heat stress. The knockdown of *HSF1* or *HSPA1A* may have increased inflammatory responses due to reduced endometrial cell tolerance to heat stress, or perhaps decreased cell viability under HS condition. Both of these mechanisms where not investigated in the current experimental system but will require

further investigation to determine the role of HSF1 and HSP70 in endometrial inflammation under heat stress conditions.

Caution is needed when extrapolating *in vitro* findings to the whole animal where a complex system is in place with specialized immune cells and inflammatory mediators. It is unclear if a 24 h continuous period of heat stress at 41°C is truly reflective of cows under heat stress conditions and does not take into account the various physiological adaptations that occur in cows such as altered blood flow. Nonetheless, this study demonstrates that heat stress induces an exacerbated inflammatory response in bovine endometrial epithelial cells and helps us understand how heat stress may increase the incidence of uterine disease in dairy cows.

Table 4-1. siRNA sequences used for gene knockdown in BEND cells.

Gene	Forward	Reverse	siRNA ID #
HSPA1A	GGAGAGAGCUGAUAAAACUtt	AGUUUUAUCAGCUCUCUCCag	s555404
HSF1	GAGCGAGGACAUAAAGAUUtt	AAUCUUUAUGUCCUCGCUCcg	s555402

Table 4-2. PCR primers sequences used for RT-PCR.

Gene	Forward	Reverse	Accession number
GAPDH	AGGTCGGAGTGAACGGATTC	ATGGCGACGATGTCCACTTT	NM_001034034.2
RLP19	ATGCCAACTCCCGCCAGCAGAT	TGTTTTTCCGGCATCGAGCCCG	NM_001040516.2
CXCL8	GCAGGTATTTGTGAAGAGAGCTG	CACAGAACATGAGGCACTGAA	NM_173925.2
IL1B	CTTCATTGCCCAGGTTTCTG	CAGGTGTTGGATGCAGCTCT	NM_174093.1
IL6	ATGACTTCTGCTTTCCCTACCC	GCTGCTTTCACACTCATCATTC	NM_173923.2
TLR2	CGGACAGTCAGCGCACCACA	GCTGTCCACAAAGCACGTGGCA	NM_174197.2
TLR4	AGCCACGGCCATCCTCTCCT	AGCTCAGGTCCAGCATCTTGGT	NM_174198.6
HSPA1A	GACAAGTGCCAGGAGGTGATTT	CAGTCTGCTGATGATGGGGTTA	NM_203322.3
HSF1	TCAAGCCAGAGAGGGACGA	CAGAGTGGACACACTGGTCA	NM_001076809.1

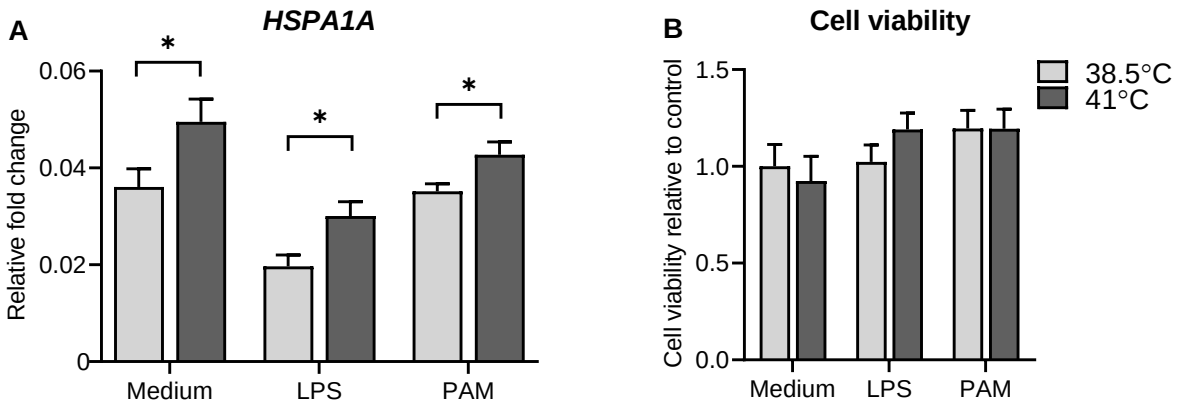

Figure 4-1. Induction of heat stress and cell viability after heat shock**.** A) Expression of *HSPA1A* in BEND cells exposed to 1,000 ng/mL of *E. coli* LPS or Pam3CSK4 (PAM) under thermoneutral (38.5°C) or heat stress (41°C) conditions. Expression is expressed relative to the geometric mean of *GAPDH* and *RLP19*. B) Cell viability was assessed in BEND cells after 24 h exposure to 1,000 ng/mL of *E. coli* LPS or Pam3CSK4 under thermoneutral (38.5°C) or heat stress (41°C) conditions. Cell viability was measured using the MTT assay and data are expressed as relative to medium alone only controls. Data were analyzed for the fixed effect for temperature, treatment, and the interaction between temperature and treatment. Bars represent the mean ± S.E.M.* $P \leq 0.05$.

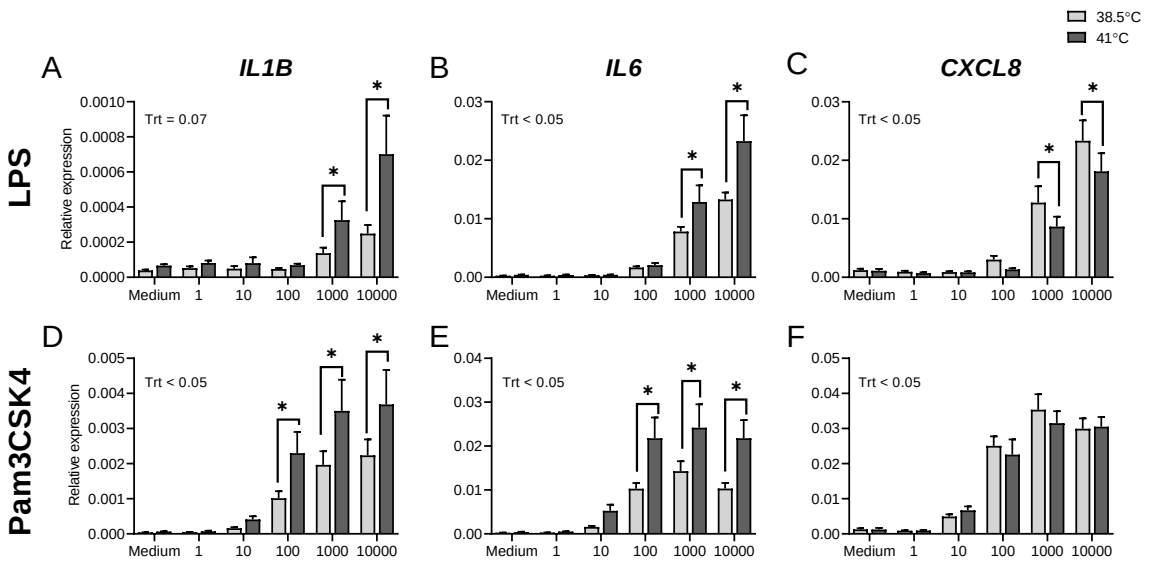

Figure 4-2. Effect of heat stress and bacterial components on the expression of inflammatory mediators in BEND cells. BEND cells were exposed to *E. coli* LPS (A - C) or Pam3CSK4 (D - E) under thermoneutral (38.5°C) or heat stress (41°C) conditions. Expression of *IL1B*, *IL6*, and *CXCL8* is expressed relative to the geometric mean of *GAPDH* and *RLP19*. Each treatment was repeated in seven independent replicates. Data were analyzed for the fixed effect for temperature, treatment, and the interaction between temperature and treatment. Bars represent the mean ± S.E.M. * $P \leq 0.05$.

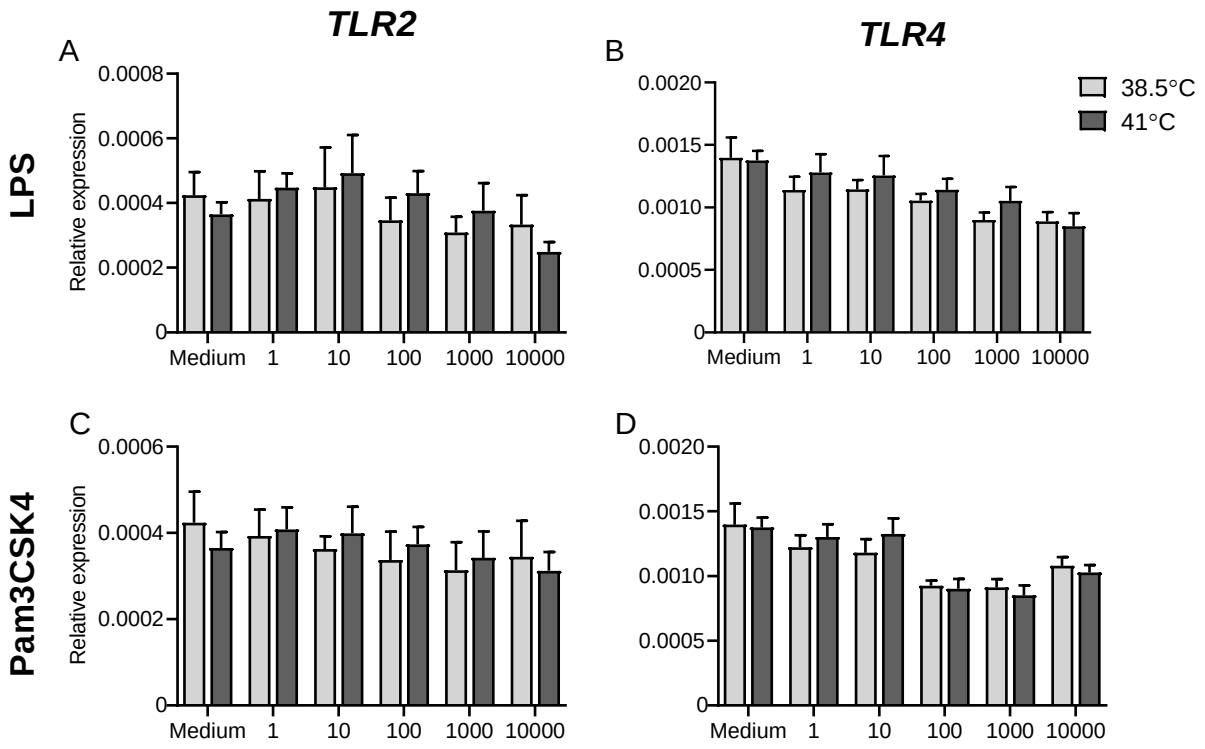

Figure 4-3. Effect of heat stress and bacterial components on the expression of Toll-like receptors in BEND cells. BEND cells were exposed to *E. coli* LPS (A - B) or Pam3CSK4 (C - D) under thermoneutral (38.5°C) or heat stress (41°C) conditions. Expression of *TLR2* and *TLR4* are expressed relative to the geometric mean of *GAPDH* and *RLP19*. Each treatment was repeated in seven independent replicates. Data were analyzed for the fixed effect for temperature, treatment, and the interaction between temperature and treatment. Bars represent the mean ± S.E.M. * $P \leq 0.05$.

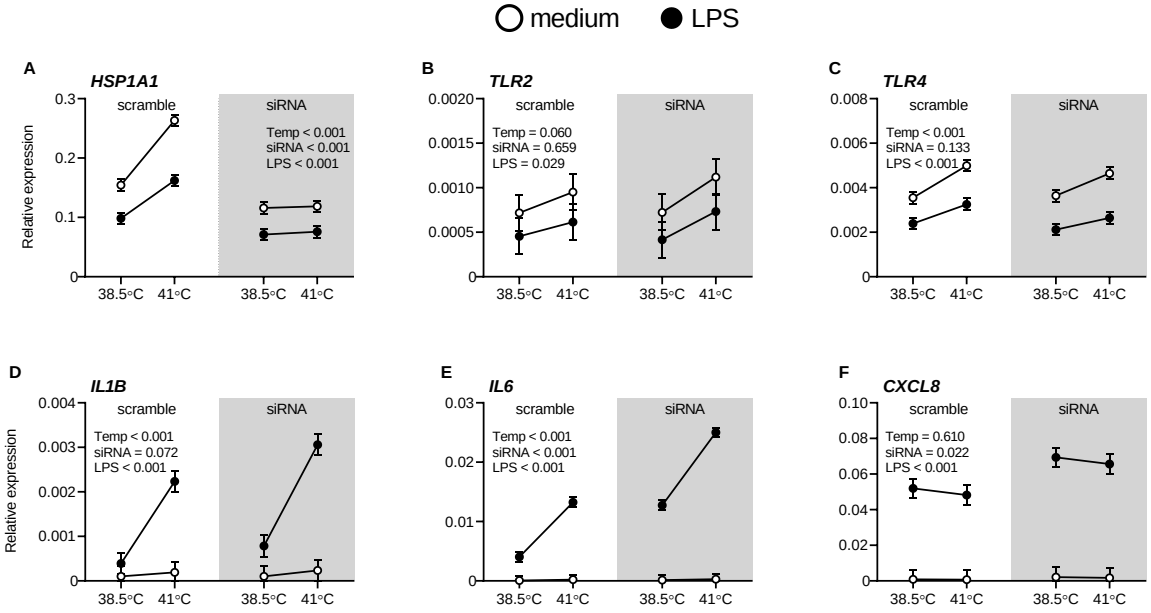

Figure 4-4. Effect of *HSP1A1* knockdown on LPS responses of BEND cells. BEND cells were transfected with siRNA targeting *HSP1A1* or a non-specific scramble control. Following transfections BEND cells were exposed to 1,000 ng/mL of *E. coli* LPS (●) or control medium (○) for 24 h under thermoneutral (38.5°C) or heat stress (41°C) conditions. Gene expression is expressed relative to *GAPDH*. Each treatment was repeated in six independent replicates. Data were analyzed for the fixed effect for temperature, LPS, and siRNA transfection. Each dot represents the mean ± S.E.M. * $P \leq 0.05$.

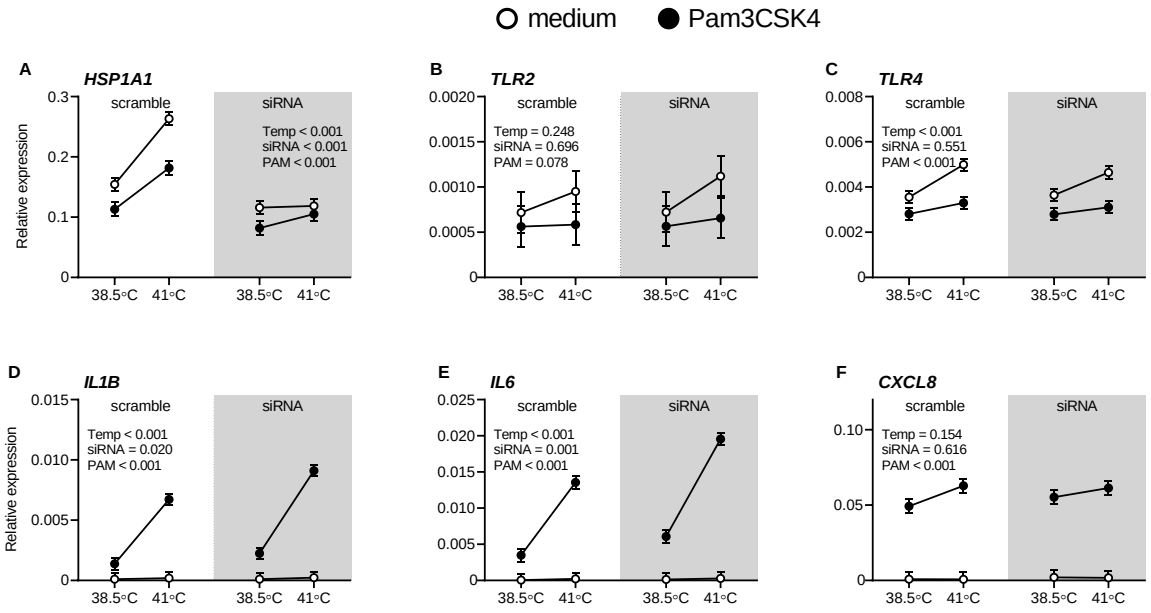

Figure 4-5. Effect of *HSP1A1* knockdown on Pam3CSK4 responses of BEND cells. BEND cells were transfected with siRNA targeting *HSP1A1* or a non-specific scramble control. Following transfections BEND cells were exposed to 1,000 ng/mL of Pam3CSK4 (●) or control medium (○) for 24 h under thermoneutral (38.5°C) or heat stress (41°C) conditions. Gene expression is expressed relative to *GAPDH*. Each treatment was repeated in six independent replicates. Data were analyzed for the fixed effect for temperature, Pam3CSK4 (PAM), and siRNA transfection. Each dot represents the mean ± S.E.M. * $P \leq 0.05$.

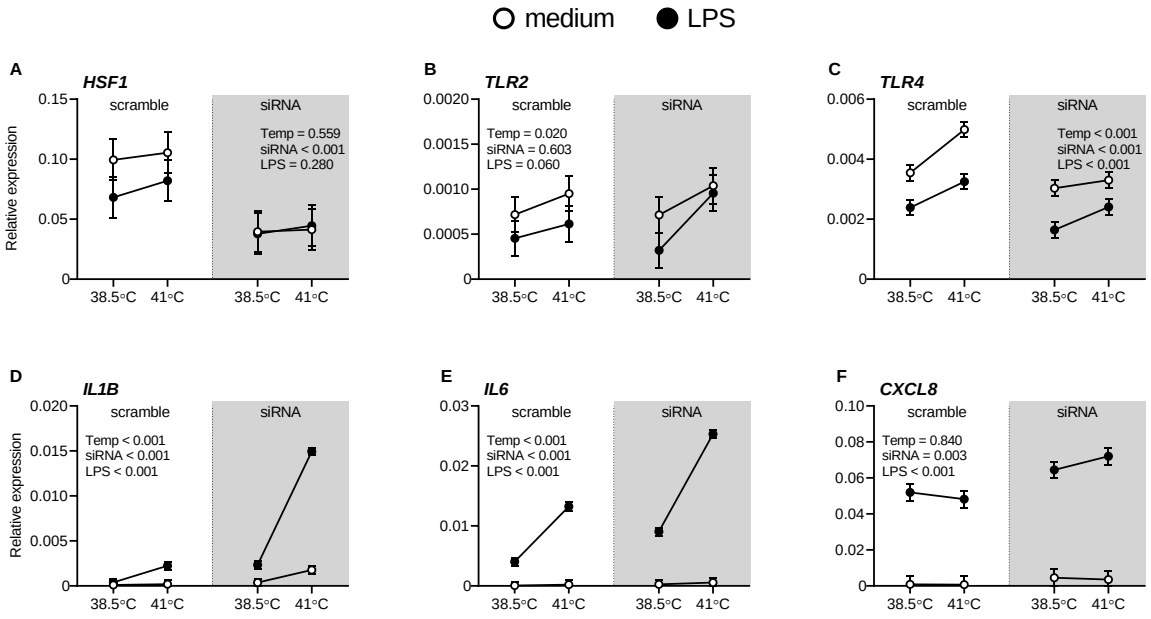

Figure 4-6. Effect of *HSF1* knockdown on LPS responses of BEND cells. BEND cells were transfected with siRNA targeting *HSF1* or a non-specific scramble control. Following transfections BEND cells were exposed to 1,000 ng/mL of *E. coli* LPS (●) or control medium (○) for 24 h under thermoneutral (38.5°C) or heat stress (41°C) conditions. Gene expression is expressed relative to *GAPDH*. Each treatment was repeated in six independent replicates. Data were analyzed for the fixed effect for temperature, LPS, and siRNA transfection. Each dot represents the mean ± S.E.M. * $P \leq 0.05$.

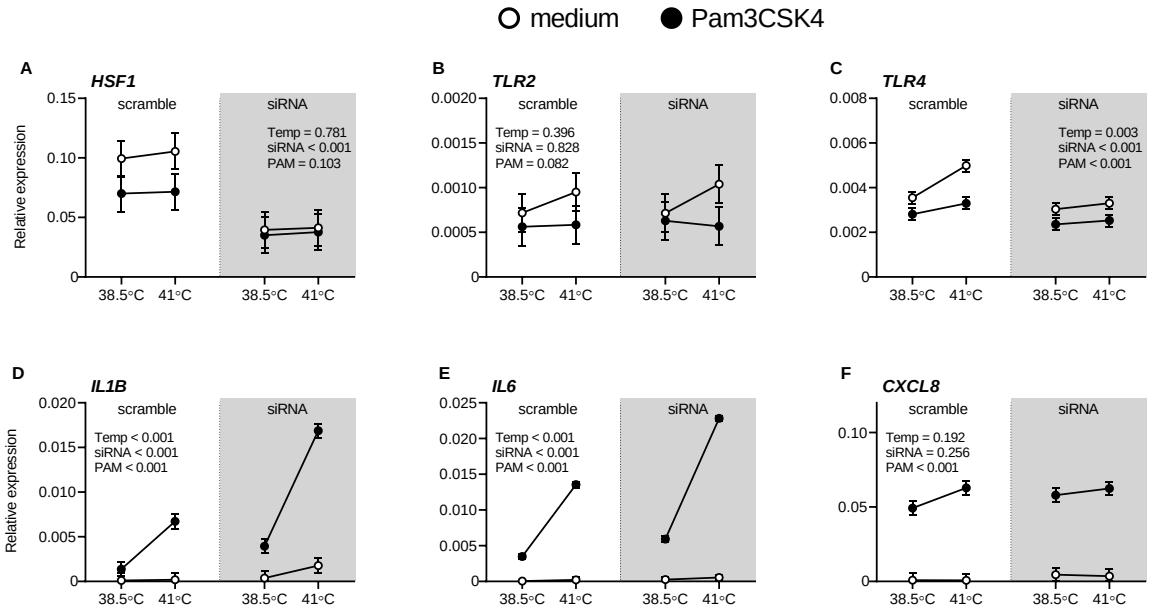

Figure 4-7. Effect of *HSF1* knockdown on Pam3CSK4 responses of BEND cells. BEND cells were transfected with siRNA targeting *HSF1* or a non-specific scramble control. Following transfections BEND cells were exposed to 1,000 ng/mL of Pam3CSK4 (●) or control medium (○) for 24 h under thermoneutral (38.5°C) or heat stress (41°C) conditions. Gene expression is expressed relative to *GAPDH*. Each treatment was repeated in six independent replicates. Data were analyzed for the fixed effect for temperature, Pam3CSK4 (PAM), and siRNA transfection. Each dot represents the mean ± S.E.M. * $P \leq 0.05$.

CHAPTER 5
GENERAL DISCUSSION AND CONCLUSIONS

Uterine disease is a pervasive issue to dairy cattle, affecting up to 40% of cows in the postpartum period (Sheldon et al., 2002). Another substantial problem for dairy cows is heat stress, especially in warmer areas of the globe. In the U.S., cows in Alabama, Florida, Louisiana, Mississippi, and Texas are severely affected by the length and intensity of exposure to high environmental temperatures; in Florida, environmental conditions meet the criteria for heat stress for at least 70% of the year, which can have a significant impact on cows if heat abatement management strategies are not implemented (St. Pierre et al., 2003, Ferreira et al., 2016). Heat stress not only decreases milk production but also impacts reproductive parameters and can predisposes cows to metabolic diseases (Dahl et al., 2020, Kadzere et al., 2002). The incidence of metritis and endometritis are increased during warmer months, suggesting that exposure of dairy cows to elevated environmental temperatures predisposes the individual to uterine disease (Gernard et al., 2019, DuBois & Williams, 1980, Gautam et al., 2010, Pascottini et al., 2017). In data presented here (Chapter 2), retrospective analysis of 3,507 calving events at the University of Florida Dairy Unit from 2012 to 2017 demonstrates that the incidence of metritis is increased during summer months.

The overall objective of the studies reported in this dissertation is to determine the impact of heat stress on uterine disease incidence and resilience mechanisms in dairy cattle. In Chapter 2 I hypothesized that seasonal variations in environmental temperature would influence uterine disease incidence and severity in the dairy cow by favoring bacterial proliferation in the lower reproductive tract. To further understand how heat stress impacts resilience mechanisms toward uterine pathogens, I hypothesized

that induced heat stress during the prepartum period alters immune resilience in the postpartum period (Chapter 3), and that acute heat stress would alter endometrial epithelial cell immune function due to altered heat shock protein signaling (Chapter 4).

To address the first hypothesis (Chapter 2), I analyzed two groups of cows that calved in two distinct seasons of the year (winter *vs*. summer). Vaginal mucus samples were collected on day 7 and day 21 postpartum and total bacteria content and specific pathogen content (*E. coli*, *T. pyogenes*, *F. necrophorum* and *P. melaninogenica*) were quantified in the lower reproductive tract. Total bacteria content and specific pathogen content of vaginal mucus did not differ between cows that calved during the winter and those that calved during the summer. Total bacteria content was associated with vaginal mucus grade, with higher grades containing higher bacteria content. Based on vaginal mucus grade, the number of cows that had uterine disease on both d 7 and d 21 was higher during summer compared to the winter, suggesting an impaired capacity to overcome an equivalent pathogen burden in the reproductive tract. Our findings suggest that the increased incidence and persistence of uterine disease during summer months is not due to increased pathogen content of the lower reproductive tract (Chapter 2) and may therefore be due to other host related factors including immune resilience mechanisms.

To isolate the effects of heat stress and reduce confounding effects related to seasonality, I performed a second study with two groups of cows in the summer that were simultaneous either exposed to heat stress (shade only) or cooled (shade, fans, and sprinklers) during the prepartum period (Chapter 3). After the prepartum treatment, all cows were cooled in the postpartum period. In agreeance with my previous

experiment (Chapter 2), total bacteria content and specific pathogen content of vaginal mucus on d 7 or d 21 postpartum did not differ between heat stress cows and cooled cows. Whole blood collected on d 21 after parturition from heat stressed cows had an exacerbated response to LPS compared to whole blood from cooled cows. Synthesis of IL-1α, IL-1β, IL-10 and MIP-1α were increased in the blood of previously heat stressed cows. Interestingly, basal levels of inflammatory mediators were not elevated in whole blood of cows previously heat stressed. This data suggests that heat stress during the prepartum period has carry over effects on innate immune resilience mechanisms of cows, inducing an exacerbated response that might predispose animals to the development of disease, increases the duration or even severity of uterine infection.

I utilized an endometrial epithelial cell culture model to assess the impacts of acute heat stress on the endometrial responses to pathogens, and to further investigate molecular pathways involved in heat stress mediated inflammation (Chapter 4). Endometrial epithelial cells that were heat stressed (41°C) during exposure to PAMPs increased expression of *IL1B* and *IL6* compared to cells exposed to PAMPs in thermoneutral (38.5°C) conditions. Heat shock protein 70, encoded by the gene *HSPA1A*, is suggested to have a dual role as a chaperone protein and to stimulate the immune system. In my model, heat stress effectively increased the expression of *HSPA1A* with no change in cell viability. Using siRNA technology, I successfully knocked down expression of *HSPA1A* and *HSF1*, an upstream regulator of heat shock proteins. Here, the increased expression of *IL1B* and *IL6* under heat stress conditions was not prevented by knocking down *HSF1* or *HSPA1A* expression. Of note, in some cases knockdown of *HSF1* or *HSPA1A* further increased expression of inflammatory

mediators in response to PAMPs, reiterating their important role in thermotolerance in the cow. In humans and pigs, HSP70 induces the expression of proinflammatory cytokines via TLR2 and TLR4 pathways (Ju et al., 2014, Chen et al., 2018). In our experimental conditions, in general, exposure to PAMPs decreased TLR2 and TLR4 expression. We observed an upregulation of *TLR2* and *TLR4* under heat stress for cells treated with LPS that was prevented by knock down of *HSF1*. Our findings did not suggest that the exacerbated response to pathogens was related to altered expression of *TLR2* and *TLR4*. In accordance with what I observed in whole blood, expression of inflammatory cytokines was not increased by heat stress alone in the absence of PAMPs. Therefore, the data suggests that acute heat stress induces an exacerbated response to pathogens in the endometrium that is not mediated by *HSF1* or *HSPA1A*.

While data from the large retrospective cohort (Chapter 2) suggests a season effect on metritis incidence, additional studies evaluating season effects or heat stress in smaller cohorts of cows (Chapter 2 and 3) did not demonstrate the same increase in uterine disease incidence. This is likely due to a reduced sample size in the two additional studies using smaller numbers of cows, which did not provide enough statistical power to detect a variation in disease incidence based on a binomial variable. Future studies should consider assessing pathogen load from a larger cohort of cows which may provide stronger evidence for any impacts of season or heat stress on uterine disease incidence and persistence. Additionally, assessment of the uterine microflora may provide a clearer insight into the effects of heat stress or season on the microbiology associated with uterine disease, something that has not been consider in other microbiome studies related to uterine disease. A larger sample size or additional

sampling sites might be necessary to detect nuances in pathogen content that were not detected in our model, due to large variation between cows. Nonetheless, the data presented here suggests a seasonal increase in uterine disease incidence in the absence of increased bacterial content even when cows receive heat abatement management during lactation.

The data presented in Chapter 2, when cows were sampled in two distinct seasons, suggests that uterine disease persistence is increased in warmer months and compounds seasonal changes in milk production. However, it is not possible to simply suggest this effect is due to heat stress or increased environmental temperatures. Factors other than heat stress which are seasonal may also impact the findings observed, such as photoperiod, feed quality, water quality and dry matter intake. I approached the studies presented in Chapter 3 to partially resolve this caveat, where two simultaneous groups of cows, housed in the same barn were either cooled using heat abatement management, or not which resulted in heat stress. My results here indicate that cows exposed to heat stress have an exacerbated peripheral immune response to bacterial components. However, we did not collect endometrial tissue from cows that had previously been under heat stress to evaluate immune function in the uterus itself. Future studies may wish to evaluate the local immune function of the endometrium which may provide an insight into the susceptibility of the uterus to infection by specific pathogens.

The *in vivo* model of heat stress presented in Chapter 3 provides evidence that heat stress during the prepartum period results in an exacerbated immune response much later, even after the cow is receiving heat abatement management during the

postpartum period. However, it is intriguing that the *in vitro* heat stress experiments presented in Chapter 4 describe a similar exacerbated immune response of endometrial epithelial cells exposed to acute heat stress for only 24 h. Therefore, these two studies are not directly comparable, but do result in the same immune phenotype whether heat stress is acute, or whether prior heat stress was chronic. Future experiments using endometrial tissues and cells collected from lactating dairy cows exposed to heat stress in the dry period would be an exciting model to determine if/how heat stress impacts long term immune function of the endometrium.

The acute exposure of endometrial epithelial cells to elevated temperatures in Chapter 4 caused an exacerbated immune response to bacterial components. However, further investigation is needed to evaluate how this altered inflammatory response may influence establishment or resolution of uterine disease. Caution warranted when attempting to extrapolate *in vitro* findings to *in vivo* situations; however, our results provide evidence that heat stress could be affecting the uterine response to pathogens in the cow, which may ultimately result in an increased susceptibility to uterine disease.

In summary, the data presented in this dissertation provides evidence that heat stress can increase the occurrence and reduce recovery of uterine diseases in dairy cattle (Fig. 5-1). Elevated environmental temperatures provide optimal conditions for bacterial proliferation; however, I found that the increased incidence and persistence of uterine disease in dairy cattle is independent of increased bacterial content of the lower reproductive tract. Moreover, chronic heat stress in the prepartum period has carry over effects on immune resilience to pathogens in the postpartum period after heat abatement strategies are employed. Finally, exposure of endometrial epithelial cells to

PAMPs under acute heat stress have exacerbated inflammatory responses independent of *HSF1* and *HSPA1A*. Together, these results highlight the importance of providing heat abatement to dairy cows throughout the productive life cycle and pave the way for further investigation into the mechanisms by which heat stress modulates the uterine immune response in dairy cows.

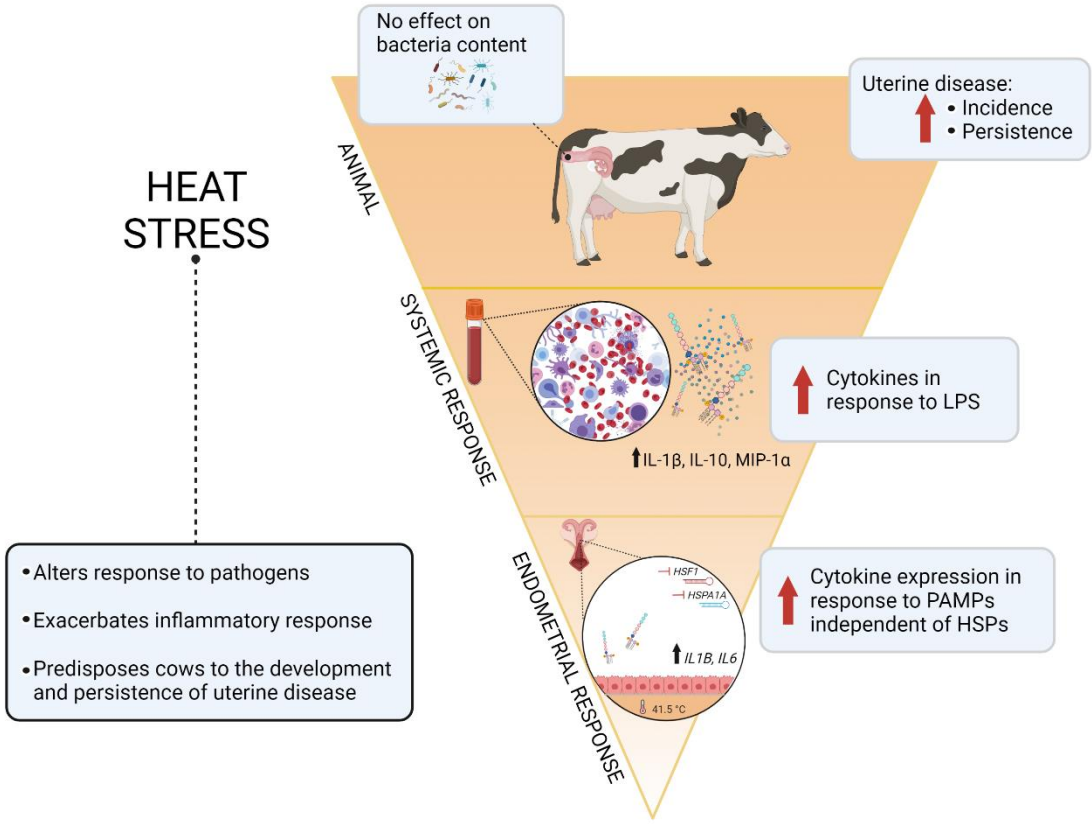

Figure 5-1. Influence of heat stress on bovine uterine health and immune cell function. Cows under heat stress have an increased incidence and persistence of uterine disease independent of vaginal bacteria content compared to cooled cows. Moreover, cows exposed to heat stress in the dry period presented an exacerbated systemic immune response to pathogens. In a similar fashion, exposure of endometrial epithelial cells to PAMPs while under acute heat stress increased expression of inflammatory mediators. Compiled, these findings suggest that heat stress predisposes cows to the development and persistence of uterine disease by altering host immune response to pathogens.

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BIOGRAPHICAL SKETCH

Paula Molinari received a Veterinary Medicine degree in 2017 from the University of Sao Paulo, Brazil. While in veterinary school she found her passion for dairy cows and small ruminants and since her second year in her program, she became heavily involved with dairy production, participating in internships at the Dairy Cattle Research Lab (LPBL- Pirassununga), the Small Ruminant Unit (Pirassununga) and at the Bovine Immunology Lab at the Clinical Department (VCM - Sao Paulo). She was responsible for two junior projects on the immune response of dairy cows to pathogens associated with mastitis. During that time Paula also received an Academic Merit Scholarship that allowed her to come to the University of Florida as a short time scholar, working closely with graduate students on projects related to nutrition. The time Paula invested in internships and on junior research reassured her passion for science and she knew then she wanted to pursue a career in research. She returned to the University of Florida for her final internship before receiving her Veterinary degree and was offered a position to start her doctoral studies in January 2018 with Dr. John Bromfield.

During her time in her PhD, she switched gears to dairy cattle reproduction, focusing mainly on the impacts of heat stress in the development of uterine diseases. Her goal as a veterinarian and as a researcher is to be able to share and apply the knowledge she learned while in academia to make dairy cattle production more efficient and sustainable.