The Ovine Inflammatory Response to Lipopolysaccharide and Association with the Stress Response Phenotype

by

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ABSTRACT

THE OVINE INFLAMMATORY RESPONSE TO LIPOPOLYSACCHARIDE AND

ASSOCIATION WITH THE STRESS RESPONSE PHENOTYPE

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Improving the stress resiliency of livestock is a strategy that can be used to mitigate the negative impacts of stressors that are anticipated to worsen due to climate change. The hypothalamic-pituitary-adrenal axis is the major neuroendocrine stress response system, and its activation demonstrates individual variation and heritability, giving rise to a novel health phenotype—the stress response phenotype—that could be considered in selection criteria for livestock breeding programs. The immune system is also activated during stress events and the interaction with the neuroendocrine system could dictate stress resiliency. Therefore, the purpose of this thesis was to assess a comprehensive panel of circulatory mediators as potential immune stress biomarkers during a systemic lipopolysaccharide challenge, and whether these biomarkers are differentially regulated in variable stress responding sheep.

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ACE2	angiotensin-converting enzyme 2
ACTH	adrenocorticotropic hormone
Arf6	ADP ribosylation factor 6
AVP	arginine vasopressin
CCL	chemokine ligand
CD	cluster of differentiation
COX-2	cyclooxygenase-2
CRH	corticotropin-releasing hormone
CXCL	C-X-C motif chemokine
CXCR	C-X-C chemokine receptor
DC	dendritic cell
DTH	delayed-type hypersensitivity
G-CSF	granulocyte-colony stimulating factor
GI	gastrointestinal
GR	glucocorticoid
GRE	glucocorticoid responsive elements
H	high
HCR	high cortisol responder
HPA	hypothalamic-pituitary-adrenal
HSR	high stress responder
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IRF3	Interferon Regulatory Factor 3
L	low
LCR	low cortisol responder
LOD	limit of detection
LPS	lipopolysaccharide
LSR	low stress responder

MD-2	myeloid differentiation protein 2
MFI	median fluorescence intensity
MHC II	major histocompatibility class II
miRNA/miR	microRNA
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NLPR3	NACHT, LRR and PYD domains-containing protein 3
NLR	neutrophil-lymphocyte ratio
PAMP	pathogen associated molecular pattern
PGE2	prostaglandin E2
PMVEC	pulmonary microvascular endothelial cell
PVN	paraventricular nucleus
ROS	reactive oxygen species
SAM	sympatho-adrenomedullary
SARA	subacute ruminal acidosis
Th	T-helper
THI	temperature-humidity index
TIR	toll/interleukin-1 receptor
TIRAP	toll-interleukin 1 receptor domain containing adaptor protein
TLR4	toll-like receptor 4
TNFα	tumour necrosis factor α
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
WBC	white blood cell

Chapter I: Introduction

1.1 Climate change as a source of stress in ruminant livestock production

The Intergovernmental Panel on Climate Change (IPCC) (IPCC, 2014) has predicted increases in global average temperatures by 0.5-2.5 °C by 2050 and this change in climate is predicted to be accompanied by an increase in extreme weather events (e.g. heat waves) and precipitation variability. Aside from the impacts these factors are predicted to have on water and feed availability for livestock, they also will have direct and indirect effects on livestock physiology and health by increasing exposure to environmental stressors like heat stress (Rojas-Downing et al., 2017), parasites (Short et al., 2017), and vector borne diseases such as bluetongue disease (Wittmann et al., 2001). There is a rising interest in improving the robustness of livestock to deal with these anticipated changes in the agricultural landscape (Mormède et al., 2011; Phocas et al., 2016). Robustness in livestock is the ability to express a high level of production, along with a high level of welfare, by being resilient to stressors (König and May, 2019). Berghof et al. (2019) defined resilience as "the capacity of the animal to be minimally affected by disturbances or to rapidly return to the state pertained before exposure to a disturbance", and this underpins the robustness trait (Knap, 2005). The host immune and stress responses are interconnected through the neuroendocrine system and can dictate outcomes to stress challenges that may manifest as resilience. Due to evidence of genetic regulation of the stress response (Hough et al., 2013; Pant et al., 2016; You, et al., 2008a), there is the potential to incorporate this trait into breeding programs. However, understanding the dynamics of the immune response during stress is needed to assess the feasibility of this strategy. Therefore, this chapter will review the stressors livestock are expected to face attributed to climate change and the resulting consequences to production and health. Further, evidence of genetics underlying resilience and the stress response, and finally, the connection between the stress response to bacterial lipopolysaccharide and the immune response.

1.1.1 Direct effects of climate change: heat stress

Increases in temperature associated with climate change are regarded as the most critical climate factor impacting livestock production due to direct impacts on performance, reproduction, and health (Rojas-Downing et al., 2017). Every species has a thermoneutral or 'comfort' zone, a range of environmental temperatures at which an animal does not have to expend extra energy to maintain a normal body temperature (FAO, 1986). When environmental temperatures are out of this range, the animal begins to experience thermal stress (FAO, 1986). The thermoneutral range for fleeced sheep has been described as -12°C to 32°C (Wojtas et al., 2014), but this range is very fluid based on numerous factors including breed, wool covering, age, bodyweight, health, and environmental conditions (National Research Council, 1981; Wojtas et al., 2014). For instance, heat stress is exacerbated by high humidity, so a widely used measure to evaluate the severity of heat stress conditions is the temperature-humidity index (THI), that factors in ambient temperature and relative humidity (Marai et al., 2007; LPHSI, 1990). Most of the research detailing the negative effects of heat stress in ruminants has involved beef and dairy cattle as opposed to small ruminants because cattle experience heat stress at a lower THI. However, reduced performance, reproduction, and health of sheep have also been attributed to heat stress (Marai et al., 2007; Rojas-Downing et al., 2017).

1.1.1.1 Impacts of heat stress on small ruminant performance

There is evidence that heat stress may impair milk yield and quality in small ruminants. Dairy goats exposed to moderate or severe heat stress have reduced milk yields, with the greatest losses during early lactation (Das et al., 2016; Sano et al., 1985). However, a study by Sevi et al. (2001) assessing milk production in Comisana ewes did not find differences in milk yield between ewes either exposed to, or protected from solar radiation. Despite this, the quality of milk, based

on decreased casein and fat levels, was diminished; it should also be noted that the ewes in this study were sampled towards the end of their lactation, which can diminish the impact that heat stress may have on yield (Hamzaoui et al., 2013). Furthermore, breeding programs focusing on developing animals with high milk production can inadvertently decrease heat tolerance, as these animals typically generate more metabolic heat (Finocchiaro et al., 2005).

Impacts of heat stress are also evident in other production traits like feed efficiency and possibly carcass quality. When sheep are under heat stress, feed intake and feed efficiency typically decrease, which can have negative impacts on growth as measured by body weight and condition (Maurya et al., 2018). Additionally, goat and sheep carcass traits can be negatively affected by heat stress, likely due to an increase in muscle pH, and biological processes shifting away from protein synthesis (Archana et al., 2018; Gregory, 2010; Kadim et al., 2008). Lamb birth weights have also been shown to decline over generations when raised in hot and semi-arid regions (Singh and Karim, 1995), possibly due to heat stress-induced intrauterine growth restriction (Regnault et al., 2002). Even after birth, lamb growth may be impaired based on observations that lamb average daily gain is lower in the summer than winter (Marai et al., 1997), but whether this is due solely to heat stress cannot be determined. Heat stress however, has been associated with increased lamb morbidity (Dwyer, 2008).

Overall, heat stress is responsible for increasing the maintenance requirements for animals to maintain normal internal temperatures; according to NRC (2007) there is a 30% increase in maintenance requirements under heat stress conditions. Compounding this problem is that heat stress also reduces feed intake, which is likely to lower metabolic heat. The combined increased maintenance requirements and decreased feed intake does not support normal production ability under heat stress conditions (Das et al., 2016).

1.1.1.2 Impacts of heat stress on small ruminant reproduction

Hyperthermia, resulting from heat stress, is known to decrease fertility, implantation, and embryo survival (Naqvi et al., 2004; Sawyer, 1979; Thwaites, 1971). For example, rams experience impaired reproductive ability under heat stress conditions, through reduced sperm volume, concentration and motility (Maurya et al., 2018). Regarding embryo implantation, elevated merino ewe body temperature was negatively correlated with reduced numbers of transferrable embryos during an artificial insemination program in Australian merino ewes (Narayan et al., 2018). In another study, exposure of ewes to consistently high ambient temperatures in experimental conditions 7 days prior to conception increased embryo deformities (Hansen, 2009).

1.1.1.3 Impacts of heat stress on ruminant health

Heat stress poses a direct threat to livestock. Heat wave events in California in 2006 were purportedly responsible for the deaths of more than 30,000 dairy cows, and a more recent heat wave in 2011 in Iowa was responsible for 4000 deaths (Rhoads et al., 2013). Heat stress is likely a major factor in these deaths considering that an assessment of cattle in France found increased mortality risk was associated with increased duration and intensity of extreme temperatures (Morignat et al., 2018). This is perhaps due to less opportunity for animals to recover from extreme heat exposure. Although no such evaluation has been applied to sheep, it is expected that effects would be similar even though their thresholds for heat tolerance are higher than those in cattle. For instance, heat stress was responsible for the death of nearly 1500 sheep in July 2016, and 2400 sheep in August 2017 during ship transport between Australia and the Middle East (Australian Department of Agriculture and Water Resources, 2018; Worthington, 2018).

1.1.1.4 Impact of heat stress on gut barrier function

In a healthy animal at normal physiological temperatures, the gastrointestinal (GI) epithelium forms a protective barrier that separates microbes in the GI tract from underlying sterile host tissues; this barrier is maintained by tight junctions that hold intestinal epithelial cells closely together, mucous secretions and immune system components (e.g. leukocytes) (Lambert, 2004). The GI barrier is selectively permeable, allowing nutritional components to travel from the GI tract to the circulation (Arrieta et al., 2006). However, heat stress can increase GI permeability, which can result in the leakage of luminal contents, including microbiota, into the circulation (Lambert, 2004; Vargas & Marino, 2016).

One adaption strategy of dealing with rising core temperatures during heat stress is the redirection of blood flow away from the GI epithelium to peripheral tissues to help dissipate excess heat (Hales, 1997; Lambert, 2004). Reduced blood flow in the splanchnic vessels that supply the GI tract for example, has been noted during hyperthermia (Kregel et al., 1988). An outcome of this redirected blood flow is hypoxia that can subsequently damage the GI epithelium tight junction proteins due to production of reactive oxygen species (ROS) (Hall et al., 1994; Hall et al., 1999). The mechanisms that open the tight junctions and result in increased intestinal permeability however, are multifactorial. Moseley et al. (1994) and Lambert et al. (2002) found that hyperthermia can induce intestinal permeability without cell death and independently of ROS.

The opening of tight junctions during heat stress allows gut bacterial endotoxins such as lipopolysaccharide (LPS) to travel into the systemic circulation (Vargas and Marino, 2016); the presence of these endotoxins in the circulation has been used as a measure of GI permeability (Lambert, 2004). These endotoxins activate immune cells such as tissue macrophages, T cells and natural killer (NK) cells, which produce pro-inflammatory cytokines and ROS (Lambert, 2004),

and these can further reduce the integrity of GI tight junctions—potentiating endotoxin translocation across the gut barrier (Capaldo and Nusrat, 2009; Capaldo et al., 2014). Endotoxins in the circulation can then lead to systemic inflammation, and this is thought to play a role in heat stress-related pathologies (Hales, 1997). In fact, a detailed assessment of the sheep mortality that occurred during transport from Australia to the Middle East in August 2017 found that many animals died of either enteritis or septicemia (Australian Department of Agriculture and Water Resources, 2018). Endotoxin, or LPS, is regarded as the most significant microbial agent in the pathogenesis of septicemia (Opal, 2010) and a prominent factor in cases of gastroenteritis (Liang et al., 2005). Further evidence of LPS involvement in the pathologic process of heat stress has been demonstrated by DuBose et al. (1983) where tolerance to gut-derived bacterial endotoxins also increased tolerance to heat stress (DuBose et al., 1983).

1.1.1.5 Impact of heat stress on the gut microbiome

Heat stress can disrupt the microbial balance in the GI tract of ruminants (Li et al., 2019), which is considered to be linked to animal health, nutrient digestion and absorption, and feed efficiency (McCormack et al., 2017; Park, 2018)*;* microbiota and their metabolites can also regulate gut barrier function (Kelly et al., 2015). Li et al. (2019) observed a decrease in fecal microbiota diversity in summer heat stress conditions compared to spring thermoneutral conditions, and also noted increases in the abundance of *Firmicutes* and decrease in *Bacteroidetes* during summer heat stress. This dynamic between *Firmicutes* and *Bacteroidetes* is well-documented in inflammatory diseases like obesity and type 2 diabetes, for example (Park, 2018). Conversely, Zhao et al. (2019) did not find major shifts in gut microbial communities between dairy cows in spring (non-heat stressed) compared to summer (heat stressed). However, the length of time that the cows were exposed to the treatment conditions in the study by Zhao et al. (2019)

were shorter, so it is possible that the cows needed to be exposed to the heat stress conditions longer for alterations in microbial communities to be detectable. In pigs, He et al. (2019) and Xiong et al. (Xiong et al., 2019) found that exposure to heat stress had decreased *Bacteroidetes* microbial communities, but increased the abundance of other bacteria phyla. Xiong et al. (2019) also found decreased microbial metabolites in the feces of the heat stressed pigs, including short chain fatty acids that are beneficial to intestinal function and health (Kles and Chang, 2006). In contrast, increased microbial metabolites were observed in the serum of heat stressed pigs; these metabolites were correlated with serum inflammatory acute phase proteins and were proposed as biomarkers of heat stress (Cui et al., 2019). Overall, it appears that heat stress alters microbiota populations and metabolites, which may play a role in inflammatory processes by reducing beneficial microbes and metabolites that support intestinal barrier health. Of note, Ducray et al., (2019) found that oral administration of *Saccharomyces cerevisiae* fermentate prebiotic protected rats from heat stress-induced changes in tight junction proteins and decreases in Paneth and goblet cells, important in antimicrobial defence and tolerance.

1.1.2 Indirect effects of climate change: disease challenges

The anticipated changes in the global climate would not only directly affect the physiological status of an animal but could also indirectly affect animals through increased parasite and bacterial loads, fungal mycotoxin exposure, and the spread of vector borne diseases. Some of these factors contribute to the emergence of new diseases in regions where animals have not been exposed to them before (Thornton et al., 2009). Increases in annual rainfall in the UK along with warmer winters for example, have been implicated in earlier and unpredicted outbreaks of parasites including *Haemonchus contortus, Nematodirus battus, Teladorsagia circumcincta*, and *Fasciola hepatica* (Short et al., 2017). These same conditions increase the spread of disease-carrying vectors

like *Culicoides imicola*—the vector of Bluetongue virus that affects sheep, which has been modelled to have increased prevalence at an increased global average temperature of 2 °C (Wittmann et al., 2001). Warmer temperatures and increased humidity also promote the development of harmful mycotoxins in livestock grains and forages (Mannaa and Kim, 2017, 2018). Similarly, increased temperatures favour the survival and replication of bacteria, which have been hypothesized as the cause of increased incidences of bovine mastitis during the summer months (Chirico et al., 1997; Hogan et al., 1989). Furthermore, ewes exposed to higher ambient temperatures had more mastitis-related pathogens in their milk (Sevi et al., 2001).

Climate change is also implicated in reduced host resistance to disease. Chronic heat stress can elicit an immune-suppressed phenotype demonstrated by reduced leukocyte counts and ability to respond to pathogens that could impair resistance to disease challenges, a phenomenon documented in several species (Jin et al., 2011; Sevi et al., 2001; Xiang-hong et al., 2011). In mice under chronic heat stress conditions (4 hrs at 38°C for 21 days) (Jin et al., 2011), reduced numbers of alveolar macrophages, increased lesions in the respiratory tract, and impaired dendritic cell (DC) maturation were observed. Additionally, mice were challenged with avian influenza at the end of the heat stress treatment and experienced higher mortality and viral loads in the lung. Miniature pigs monitored over 21 days in heat stress conditions developed a low CD4:CD8 ratio by day 14, which is an indicator of immunosuppression (Xiang-hong et al., 2011). However, the heat stress treatment did not alter neutrophil or NK cell function and the CD4:CD8 ratio in heat stressed pigs was not different from the control by day 21, indicating that heat stress differentially influences components of the immune system. Furthermore, the impacts of heat stress on disease susceptibility are likely determined, in part, by the severity of the heat stress and timing of the disease challenge.

1.1.3 Current mitigation strategies for heat stress

The indirect and direct consequences of heat stress on sheep are broad and detrimental and several mitigation strategies have been investigated. The major ones include use of shelters for shade, altered feeding schedules, and nutritional supplementation, or a combination of these. Lees et al. (2018) for example, used devices that measure rumen temperature to investigate the use of shelters against heat stress in two feedlot cattle breeds. Shade was found to help regulate temperature in Angus steers, but Brahman steers, native to tropical climates, did not need provision of shade for normal thermoregulation. A similar study by Sevi et al. (2001) found that protecting ewes from solar radiation and changing feeding times to the afternoon improved immune status and udder health; body temperature however, was not monitored, so the assistance that shade provides in thermoregulation could not be determined. However, this was later explored by Caroprese et al. (2012), who found that shaded ewes had lower recorded rectal temperatures than un-shaded ewes, and adding flaxseed into the diet additionally lowered respiration rate and enhanced humoral immune responses in both shaded and un-shaded animals. Flaxseed is thought to be beneficial to livestock during heat stress due to its high energy density that can increase energy intake while minimizing metabolic heat, in addition to increased omega-3 fatty acid content that can enhance immune function (e.g. cytokine, humoral, and cellular immune responses) (Caroprese et al., 2012). Although provision of shade offers some protection from solar radiation and helps livestock regulate internal temperature, it does not completely protect them from heat stress, as sheep reared indoors without climate control can still experience heat stress through high air temperatures and humidity. Furthermore, a study comparing sheep reared outdoors and indoors with poor ventilation found that indicators of heat stress, namely cortisol levels and immune response, were not different (Casamassima et al., 2001); unfortunately, body temperature was not

recorded in this study. The lack of differences between the sheep in the different housing treatments could be explained by the similarly recorded THIs in the indoor and outdoor environments.

Antioxidant supplementation with vitamin E, selenium, or a combination, in sheep has also been investigated for protection against heat stress. Gene expression analysis of skeletal muscle in antioxidant-supplemented sheep under heat stress showed downregulation of inflammatory genes and upregulation of the ROS scavenger, heat shock protein 90; heart rate was also significantly lowered by antioxidant supplementation (Chauhan et al., 2014). In this same study, however, respiration rate and body temperature were not significantly different between supplemented and un-supplemented sheep, although there was a statistical trend. This was followed up in another study by Chauhan et al. (2015) that found supplementing sheep with vitamin E and selenium was able to lower the respiration rate and rectal temperature of sheep. In addition, antioxidant gene expression and activity were increased in the supplemented sheep. Supplementation with vitamin E and selenium has also been shown to ameliorate bodyweight loss during periods of heat stress (Alhidary et al., 2015).

Ultimately, adjusting the management strategies of sheep can aid in ameliorating some of the negative effects of heat stress, but currently does not form an encompassing solution. Moreover, the cost of implementing some of these strategies is high and more detailed cost-benefit analyses and long-term studies that assess the relevance of dietary supplement regimes are needed.

1.2 Potential to breed ruminants for increased stress resilience

1.2.1 Variation in stress resilience

As heat stress challenges are anticipated to worsen for livestock in light of climate change, additional strategies need to be explored. A sustainable solution that can supplement improved management is genetically selecting animals that have enhanced heat stress resistance or resilience. The potential for this is apparent by examining differences in stress resilience among breeds and individuals. As such, livestock native to arid and harsh climates can tolerate those conditions better than livestock developed in cooler, more temperate climates. For example, cattle from India and South America, like the Brahman breed, have better ability to maintain normal body temperatures during heat stress conditions than Angus, which are native to Scotland (Lees et al., 2018). Similarly, McManus et al. (2016) used respiration rate and eye temperature as measures of heat tolerance to outline the geographic regions in Brazil that 11 different sheep breeds could be raised. The study found that British breeds, which were developed for cooler climates, had very restricted rearing limits compared to those developed in continental Europe, South Africa, and Brazil. However, there is still considerable variation in the ability to tolerate heat stress even amongst indigenous breeds found within arid regions. For example, heat stress resilience in three goat breeds indigenous to India was quite variable based on measured physiological parameters such as respiration rate, rectal temperature, as well as the cellular expression of heat shock protein 70 (Aleena et al., 2018).

Despite the more robust qualities of the breeds native to hotter climates, breeds from more temperate climates have higher production ability, when raised in their native regions, than do those from harsher climates raised in theirs. Because of this, there have been attempts to increase the production capacity of livestock in hotter countries by cross breeding with higher producing

breeds, like Holstein for dairy cattle or Suffolk for sheep. These crossbreds are more susceptible to heat stress, however, and ultimately have poorer performance in the harsher climates than their native counterparts. This can be explained partly because as production traits increase, like milk yield and growth rates, metabolic heat production increases and reduces the capacity to tolerate heat (Bhanuprakash et al., 2016; Hoffmann, 2010).

Interestingly, breeds local to arid regions are not only more heat tolerant, but more adapted to parasitic and disease stressors as well (Hoffmann, 2010). The Food and Agriculture Organization of the United Nations (2007) compiled a list of livestock breeds reported to be more resistant or tolerant to a range of tick-transmitted diseases, parasites, and some bacterial infections, and most of these breeds were from developing countries known for their harsher climates. These reports, however, were based on anecdotal evidence, and more scientific studies are needed to validate findings and elucidate potential mechanisms involved.

Although breeds known for their high production ability are less adaptable to adverse environmental conditions, and more susceptible to heat stress and disease challenges, there is opportunity to improve these breeds' stress resilience. Because differences between individual breeds can be observed, some genetic basis underlying stress resilience in livestock is likely. Within-breed variability for heat tolerance and disease resistant traits has been explored for several species. For example, heritability estimates have been determined for higher producing *Bos taurus* cattle for resistance to mastitis, ketosis, parasitic nematodes, mycotoxins, and heat stress (Garner et al., 2016; Morris, 2007). Similarly, genetic regulation has been observed in sheep for resistance to parasitic infections and bacterial infections; namely mastitis and foot rot (Sayers et al., 2005; Bishop and Morris, 2007; Oget et al., 2019) . While progress has been made, selection for increased resistance to these stressors is based on indicator traits, like fecal egg counts for parasite resistance,

somatic cell score for mastitis, and lesion scoring for foot rot, but these phenotypes have low to moderate heritability, suggesting further genetic improvements can be made. Within-breed variation in heat tolerance has also been described in a population of Mediterranean dairy sheep, highlighting the potential to breed high producing animals for this trait as well (Finocchiaro et al., 2005). Although these pathologies are all significant for the livestock sector, research has not addressed potential relationships among them, and genetic correlations have not been established.

1.2.2 The neuroendocrine stress response pathway

When faced with disease or heat stress, animals need to adapt to overcome the challenge. This adaptation involves behavioural and physiological changes that constitute the stress response, and the major product from activation of the stress response is the hormone cortisol. The two primary systems involved in the stress response are the sympatho-adrenomedullary (SAM) and hypothalamic-pituitary-adrenal (HPA) axes (Ulrich-Lai and Herman, 2009). Activation of the SAM axis results in a rapid release of stored catecholamines (dopamine, epinephrine, and norepinephrine) from postganglionic sympathetic axons and adrenal medullary chromaffin cells (Takiyyuddin et al., 1994). Although the half-life of catecholamines is measured in seconds, they have powerful effects on heart rate, peripheral vasoconstriction, and energy mobilization. Parallel activation of the HPA axis potentiates the sympathetic-induced physiological changes in response to stress. The HPA axis activation results in the release of glucocorticoids (cortisol in sheep, humans, and fish, and corticosterone in rats and birds) from the adrenal cortex (Figure 1). The release of glucocorticoids begins by stimulation of neurons in the paraventricular nucleus (PVN) of the hypothalamus, which then release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) that travel to the anterior pituitary to signal the release of adrenocorticotropic

hormone (ACTH) into the systemic circulation (Herman et al., 2016). When ACTH reaches the adrenal cortex, glucocorticoids are synthesized and released into the systemic circulation where they impact the function of the cardiovascular, metabolic, musculoskeletal, neuroendocrine, and immune systems via glucocorticoid (GR) and mineralocorticoid nuclear receptors. The physiological and immunological changes induced from activation of the SAM and HPA axes ultimately help prepare the animal to overcome the stress challenge that it is faced with.

Control of HPA axis activation is important for managing cortisol production and is partly regulated through a negative feedback loop. When cortisol is released into the systemic circulation, the concentration also increases at the level of the central nervous system where it binds to glucocorticoid receptors, in the PVN for example, to inhibit further release of CRH (Herman et al., 2016); by preventing CRH release, cortisol production is down-regulated and circulating concentrations can return to basal levels (Herman et al., 2012).

Although the above describes conventional understanding of the stress response, it is more nuanced than this. The production of cortisol for example, can bypass activation of the HPA axis via direct stimulation of the adrenal glands by cytokines and bacterial ligands (Bornstein et al., 2008). Additionally, at the level of the adrenal gland, there is communication between the cortisol-producing adrenocortical cells and catecholamine-producing chromaffin cells; chromaffin cells for example can produce neuropeptides like CRH and AVP, and catecholamines that can directly stimulate cortisol production (Schinner and Bornstein, 2005). This local modulation of cortisol production may also be influenced by immune cells, specifically macrophages and neutrophils, that are also capable of producing catecholamines (Flierl et al., 2007).

1.2.3 Heritability of the neuroendocrine stress response pathway

Glucocorticoid production is highly conserved across species, which is unsurprising considering this hormone's influence on behaviour and function of biological systems during stressful situations, in addition to normal physiological functioning, including metabolic processes and energy balance (Romero, 2004). Because of this, HPA axis function in wild populations has been investigated and found to predict survival (Rivers et al., 2012; Romero, 2004; Virgin & Rosvall, 2018). However, in one study that examined overwinter survival of sparrows, HPA axis reactivity was associated with survival in one year, but not another (MacDougall-Shackleton et al., 2013). It is thought that the discrepancies in the associations of HPA axis reactivity and survival could be influenced by life stage (Bonier et al., 2009) and environment (Crespi et al., 2013). Despite the complexities regarding the HPA axis and its association with survival, there is enough evidence to support that it has been a possible target for natural selection. The genetic contribution to cortisol production has been explored extensively in numerous wildlife and domestic species and heritability estimates have been calculated (Taff et al., 2018), but these estimates are dependent on how and when cortisol is measured. For example, basal cortisol concentrations have very low heritability, whereas stress-induced cortisol production has moderate heritability in part because measurements are more repeatable (Taff et al., 2018). This difference could be due to the challenges of controlling pre-stress levels, as measuring cortisol often requires restraint, which is itself stressful. Stress-induced cortisol production also has a greater range than at basal levels, making it easier to identify phenotypic extremes.

Repeatability and heritability of stress-induced cortisol production has been measured several ways; most commonly, restraint and handling stress are used (Nemeth et al., 2016; Overli et al., 2005; Romero, 2004; Taff et al., 2018), although stimulation with pharmacological

compounds like ACTH has also been explored. Repeated intravenous injections of synthetic ACTH in healthy horses for example, demonstrated short- and long-term repeatability in salivary cortisol measurements (Scheidegger et al., 2016), and produced a heritability estimate of 0.68 in pigs (Larzul et al., 2015).

Breeding programs have also been established based on stress-induced cortisol levels, creating high (H) and low (L) stress responsive lines (Overli et al., 2005). In this study, third generation rainbow trout, divergently selected based on cortisol production after confinement stress, were produced and heritability estimates of 0.44-0.73 were calculated (Overli et al., 2005). Also, variable turkey lines were developed based on plasma corticosterone levels after cold stress, and corticosterone responses were confirmed using an ACTH stimulation test (Brown and Nestor, 1973). Furthermore, indirect selection of high cortisol responsiveness to a hypoglycemic stress test was achieved using South African Merino sheep through a selective breeding program targeting multiple rearing ability; in this study, sheep selected for increased multiple rearing ability were found to have stronger cortisol responses (Hough et al., 2013).

The importance of the stress response in animals' life history, combined with the genetic control of this pathway, have created an opportunity to use this trait to develop stress resilience in livestock populations.

1.3 Modelling the stress response using an immunological stressor

1.3.1 Activation of the stress response by bacterial LPS

In addition to restraint stress and stimulation with pharmacological compounds, the HPA axis can also be activated by immunological stressors (Figure 1). One such stressor is bacterial LPS endotoxin, a pathogen-associated molecular pattern (PAMP) of the outer membrane of Gram-

negative bacteria. Activation of the HPA axis begins by recognition of LPS through toll-like receptor 4 (TLR4), which is found on the surface of numerous cell types including many leukocytes and epithelial cells and requires association with MD-2 and CD14 proteins to promote effective recognition and intracellular signalling by LPS. After binding to LPS, TLR4 receptors homodimerize and interactions between their intracellular TIR-domains lead to conformational changes in the molecule that facilitate recruitment of adapter molecules in the cytoplasm that are required for the initiation of two signalling pathways. The first involves association of the MyD88 and TIRAP proteins, which leads to early activation of the transcription factor NFκB that upregulates pro-inflammatory cytokine production, TNFα and IL-6 for example (Takeuchi and Akira, 2010). The second pathway involves internalization of the TLR4 dimer into an endosome where adapter molecules TRAM and TRIF interact, which facilitates the activation of transcription factor IRF-3 that upregulates type I interferon (Kawai and Akira, 2011) and anti-inflammatory cytokine IL-10 production (Iyer et al., 2010). The TRAM-TRIF pathway also leads to late activation of NFκB, potentiating inflammatory cytokine transcription (Kawai and Akira, 2011). In addition to cytokines, NFκB is also involved in inducing other inflammatory mediators, including chemokines and enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) for example (Rivest, 2003). These mediators are involved in coordinating the fever response and elimination of pathogens (Takeuchi and Akira, 2010), and simultaneously activate the HPA axis (Beishuizen and Thijs, 2003).

Activation of the HPA axis by pro-inflammatory cytokines and these other inflammatory mediators can occur at numerous levels including the PVN, pituitary, and adrenals, as demonstrated by increased cytokine receptor expression in these tissues following LPS challenge (Nazarloo et al., 2003; Vallières & Rivest, 1999). Additionally, vascular endothelial cells and

brain-residing macrophages can increase the expression of COX-2 and subsequently PGE2 production when activated by pro-inflammatory cytokines or LPS, which then stimulate neurons in the PVN to secrete CRH (Serrats et al., 2010). Activation of the HPA axis in response to LPS at various levels, combined with the different effector molecules and cell types that regulate this activation, highlight the complexity of the immune stress response.

1.3.2 Cortisol as a mediator of LPS-induced inflammation

Although a strong (i.e. protective) immune response is necessary to control an infection, it also must be tightly regulated to prevent excessive inflammation from occurring that can lead to tissue damage evident in numerous inflammatory pathologies (Karrow, 2006). Cortisol is critical for suppressing immune-mediated inflammation and facilitates bidirectional communication between the neuroendocrine and immune systems. The importance of HPA axis activation during bacterial infection is evident in studies that find increased lethality after adrenalectomy or after blocking cortisol signalling with GR antagonists (Webster and Sternberg, 2004). Moreover, adrenal insufficiency leads to a higher risk of mortality in patients with septic shock (Annane et al., 2006). In fact, corticosteroids are one of the most widely used drugs in the world for their effectiveness in treating inflammatory and immune-mediated diseases (Barnes, 2006).

The usefulness of corticosteroids for treating inflammatory and immune-mediated diseases is due to the widespread expression of the GR in immune cells, which allows cortisol to have extensive immunomodulatory effects (Bellavance and Rivest, 2014). Cortisol can increase the phagocytic activity and survival of monocytes and macrophages (Bellavance and Rivest, 2014), which helps to clear pathogens and resolve infections. In other innate immune cells like dendritic cells, cortisol can enhance apoptosis and inhibit maturation, while also down-regulating their expression of major histocompatibility class II (MHC II) molecules, which are responsible for

presenting antigen to T cells during T cell activation. In addition to suppressing the activation of T cells by DCs, cortisol reduces the number of T cells in the circulation by directing their migration back to the bone marrow and secondary lymphoid organs. This is possibly mediated by cortisol up-regulating the expression of chemokine receptor CXCR4 on the surface of T cells leading to their extravasation from the blood (Besedovsky et al., 2013). Depending on the subtype of immune cell, however, cortisol can have opposite effects. For example, glucocorticoids have been shown to stabilize the T regulatory cell phenotype and function during inflammation (Rocamora-Reverte et al., 2019). Cortisol can also down-regulate pro-inflammatory cytokine and upregulate anti-inflammatory cytokine production through genomic and nongenomic mechanisms of action (Oakley and Cidlowski, 2013). When cortisol-GR binding occurs in the cytoplasm for example, the GR can translocate to the nucleus either as a dimer, or monomer; as a dimer, the GR binds glucocorticoid responsive elements (GRE) within the prompter region of certain genes leading to transcription (of IRF3 for example), whereas, as a monomer, the GR can tether with the transcription factor NFκB to prevent the transcription of pro-inflammatory cytokines (Tian et al., 2014). The large array of actions of cortisol on immune cells make it an important immunomodulator for fine-tuning the inflammatory response.

1.3.3 Relevance of LPS in climate-associated stressors

Inflammation is a characteristic of numerous livestock pathologies and disease processes, including mastitis (Herry et al., 2017; Morimoto et al., 2011), endometritis (Bilal et al., 2016), sepsis (Opal et al., 1999; Zweigner et al., 2001), acidosis (Abaker et al., 2017), and heat stress (Vargas and Marino, 2016), and LPS is also associated with all these conditions. The major causative pathogen of mastitis is *Escherichia coli*, a Gram-negative bacteria, and vaccination using LPS or an *E. coli* strain, improves short-term resistance to, and resolution from, this disease in

dairy cows (Herry et al., 2017; Morimoto et al., 2011). LPS is also relevant in subacute ruminal acidosis (SARA), a condition caused by feeding high grain diets. The decrease in rumen pH during SARA damages the gut epithelia, allowing LPS to translocate into the bloodstream (Abaker et al., 2017). LPS derived from the digestive tract has been detected in the systemic circulation of dairy cows and goats with SARA, leading to inflammation in the uterus (Bilal et al., 2016) and liver (Abaker et al., 2017; Dong et al., 2013). Heat stress is another driver of ruminal acidosis, due to a combination of increased respiration rate, which alters the availability of HCO_3^- as a ruminal buffer, and reduced saliva entering the rumen due to reduced feed intake (Kadzere et al., 2002). As previously described, heat stress can also damage the GI epithelia by redirecting blood flow away from the gut, causing hypoxia and oxidative stress; this allows LPS to enter into the bloodstream (Vargas and Marino, 2016), which leads to immune system activation and systemic inflammation that causes sickness behaviour due to pro-inflammatory cytokine production (Nordgreen et al., 2018). Interestingly, heat tolerance improves in dogs and rabbits that have experimentally reduced bacterial contents in their gut (Hales, 1997), supporting the role endotoxins have in heat stress. Due to the significant contribution LPS has on inflammatory pathologies and processes, investigating the immune and stress responses of livestock to LPS will help to understand and develop better strategies to manage them.

1.3.4 Genetic regulation of the stress response to LPS and relationship with the immune response

Variation in the stress response to systemic administration of LPS in sheep demonstrates regulation by genetic processes. Using an intravenous injection of low dose LPS, a population of sheep were identified as high (HCR) and low (LCR) cortisol responders, based on peak serum cortisol concentrations measured 4 hours after administration (You et al., 2008a). Importantly, this response was repeatable over time (You et al., 2008a), and a study by Pant et al. (2016) found it to

be moderately heritable (h^2=0.3). Additionally, Pant et al. (2016) identified 16 single nucleotide polymorphisms associated with the cortisol response to LPS. You et al., (2008a) also investigated the relationship between the cortisol response phenotype in sheep and immune responses and found IL-1 and IL-6 cytokine production were not associated with the phenotype. However, another study comparing immune and stress responses to LPS between Dorset and Suffolk sheep found Suffolk ewes to have greater temperature and cortisol responses, as well as higher gene expression of pro-inflammatory cytokines (Hadfield et al., 2018). Notably, You et al. (2008b) also performed a follow-up study in the HCR and LCR cortisol responding sheep and found a relationship between the stress phenotype and cell- and antibody-mediated immune responses, linking another facet of immunomodulation to variation in the cortisol response. Furthermore, piglets divergently selected for cortisol response to ACTH had different immune cell subset compositions and TNFα production after *ex vivo* stimulation of their blood (Hervé et al., 2019); however, these differences were lost by the time the pigs reached 8 weeks of age. Similar connections in divergent cortisol responses are evident in European sea bass; these fish had altered hepatic immune transcript profiles (Samaras et al., 2016), further supporting the interconnectedness between stress and immune responses. The stress response clearly demonstrates genetic regulation and potential for selection in sheep and other livestock. This complex health phenotype also appears to be linked to altered immune parameters and responses. Unfortunately, the few studies investigating this relationship so far have mainly assessed gene expression or the production of a small number of pro-inflammatory cytokines, when regulation of the immune response involves a large array of cells and effector molecules.

Summary

Bacterial LPS has a major role in numerous livestock inflammatory pathologies and is a potent activator of stress and immune responses. Furthermore, this immunological stressor can be used to identify variable stress responding sheep; a novel health phenotype that could potentially improve the stress resiliency of livestock. However, it is likely that stress resiliency is dictated by the functional dynamics of both the stress and immune responses, and it is unclear to what extent the stress response phenotype in sheep is associated to immune function. Therefore, research is needed to characterize the relevant effector molecules of LPS-induced inflammation and investigate immune function in variable stress responders for improved stress resilience. Consequently, the studies in this thesis will focus on making a more comprehensive assessment of the circulatory molecules released in response to systemic LPS challenge and how these molecules are differentially produced in variable stress responding sheep.

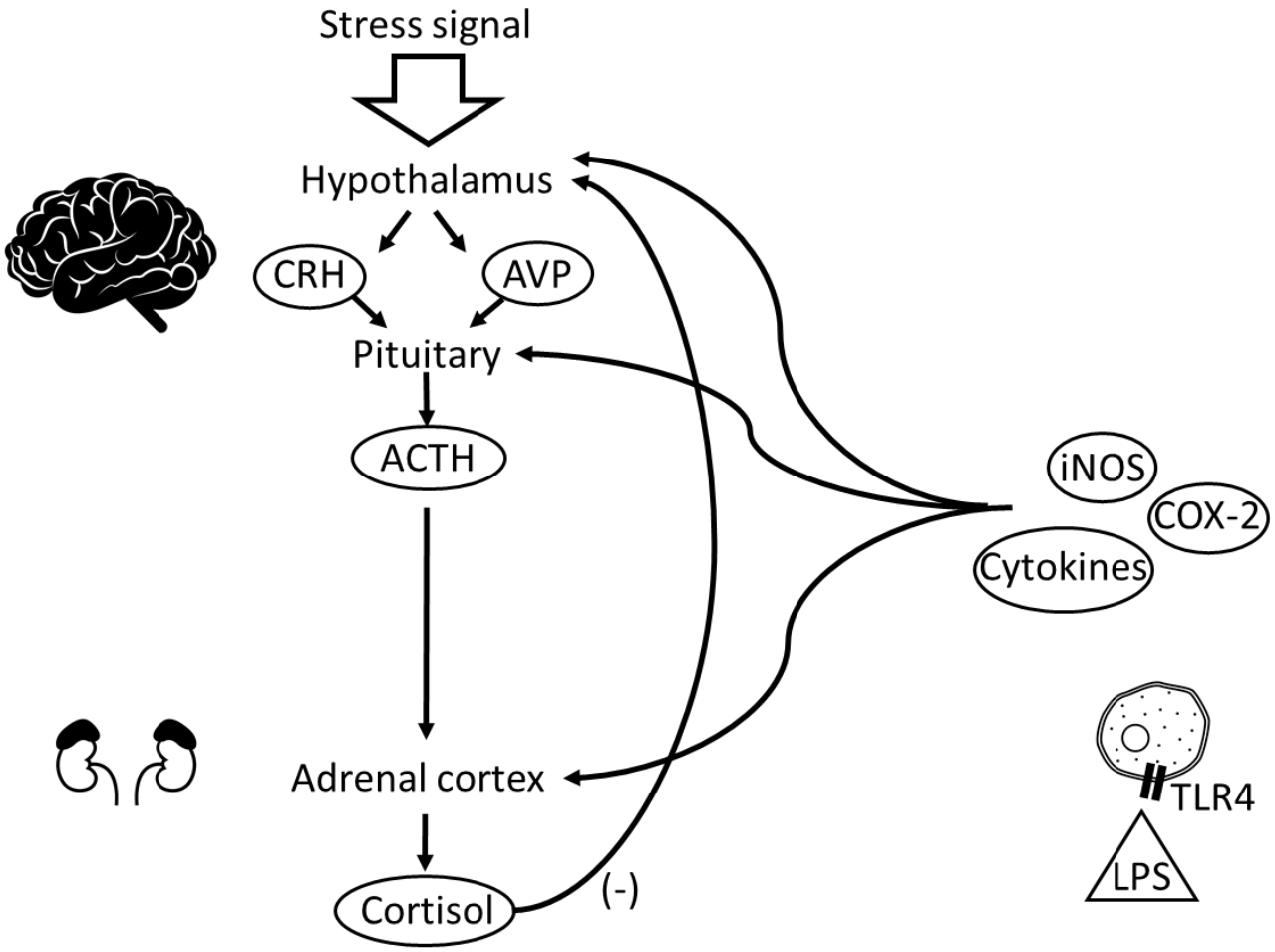

Figure 1: Activation of the HPA axis and release of cortisol to stress signals (i.e. physiological, perceived, or immune stress) and the negative feedback of cortisol on the HPA axis;

CRH: corticotropin-releasing hormone

AVP: arginine vasopressin

ACTH: adrenocorticotropic hormone

iNOS: inducible nitric oxide synthase

COX-2: cyclooxygenase-2

Chapter II: Objectives

Climate change is predicted to exacerbate microbial and environmental stressors on livestock that will have negative impacts on animal health and production. While some management strategies could aide in reducing the negative effects of these stressors, a more comprehensive solution is needed. This may be possible by leveraging genetically regulated traits that could confer resilience. Since the stress response is variable and moderately heritable in sheep, there is an opportunity to investigate this phenotype for improved stress resilience.

Observations of genetic regulation of resilience and resistance in livestock have already been made by comparing different breeds and within-breeds. For example, Brahman cattle can maintain normal body temperatures in heat stress conditions compared to Angus (Lees et al., 2018). Similarly, for sheep, breeds native to cooler climates have more restrictions in where they can be raised in Brazil, compared to sheep from hotter, more arid, countries, based on measures of heat tolerance (McManus et al., 2016). Unfortunately, the breeds that demonstrate enhanced resilience have lower production ability and are not extensively used in North American farming operations. Even though the higher producing breeds are more susceptible to environmental stress and disease challenges, progress has been made in increasing their resistance to these stressors as well. The majority of this evidence is in *Bos taurus* cattle breeds for traits like mastitis, ketosis, parasitic nematodes, mycotoxins, and heat stress (Garner et al., 2016; Morris, 2007), but breeding programs for sheep have only targeted resistance to either parasitic (Aguerre et al., 2018), or bacterial infections like mastitis and foot rot (Bishop and Morris, 2007). The indicator traits used to measure resistance to these stressors, like fecal egg count and lesion scoring, can be time-consuming, costly, imprecise, and have provided low to moderate heritability estimates. Despite this, improvements can be made in health traits through genetic

selection, but to improve stress resiliency of livestock, a better understanding of how animals respond to stress is needed.

Due to the strong interplay seen between the stress and immune response, functioning of the immune system could also play a role in outcomes of stress resiliency. And this connection is particularly evident with LPS, which is a relevant bacterial stressor to livestock, since it is involved in several inflammatory pathologies (Abaker et al., 2017; Herry et al., 2017; Bilal et al., 2016; Vargas & Marino, 2016; Morimoto et al., 2011; Zweigner et al., 2001; Opal et al., 1999). LPS is recognized by innate immune cells and this will initiate an inflammatory response, characterized by an increase in pro-inflammatory cytokine production. These pro-inflammatory cytokines can then activate the HPA axis at various levels to produce cortisol (Nazarloo et al., 2003). This LPS-induced inflammatory pathway has been utilized to identify H, M, and L stress responding sheep, based on their cortisol response, and permits the study of the neuroendocrine-immune axis (You et al., 2008a). The stress response phenotype has demonstrated connection to certain facets of the immune system including cell- and antibody-mediated immune responses (You et al., 2008b). Specifically, H and L cortisol responders had enhanced cell- and diminished primary antibody-mediated immune responses compared to M cortisol responders. There were no observed differences, however, among the variable cortisol responding sheep in serum IL-6, IL-1, and INF-γ production after intravenous LPS challenge (You et al., 2008a; You et al., 2008b). But, in another study utilizing a systemic LPS challenge in sheep, there were significant increases in serum cortisol, TNFα, and other inflammatory mediators (Hadfield et al., 2018). Additionally, changes were noted in white blood cell counts (i.e. initial decrease, then increase) and gene expression of other pro- and anti-inflammatory cytokines, chemokines, and regulatory enzymes. It should be noted though, that the goal of this study was not to test the association of

cortisol with these immune parameters, but this should be explored further considering the concurrent changes in cortisol with the numerous immune-related parameters.

Clearly, the immune response to LPS involves an extensive array of circulatory pro- and anti-inflammatory cytokines, chemotactic molecules, and immune cells, among other mediators, that dictate the dynamics of the immune response. Due to the bi-directional communication between the stress and immune response, it is possible that selecting sheep for the stress response phenotype alters their immune functioning, and possibly stress resilience. Therefore, the objectives of these studies were to:

1) Comprehensively assess the immune and stress-related circulatory mediators of an LPS-induced inflammatory response in sheep.

2) Investigate how this response varies when sheep were selected based on the stress response phenotype.

Chapter III: Characterizing ovine serum stress biomarkers during endotoxemia

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3.1 Abstract

Breeding stress resilient livestock is a potential strategy to help mitigate the negative impact of environmental and pathogenic stressors. The hypothalamic-pituitary-adrenal (HPA) axis and immune system are activated during stress events and release mediators into the circulation that help to restore physiological homeostasis. The purpose of this study was to assess a comprehensive set of circulatory mediators released in response to an acute immune stress challenge to identify candidate biomarkers that can be used for the selection of stress resilient animals. Fifteen female lambs were stress challenged with an i.v. bolus of lipopolysaccharide (LPS) (400 ng/kg), and blood was collected from the jugular vein at 0, 2, 4, and 6 hr post LPS challenge to identify and monitor candidate stress biomarkers; temperature was also recorded over time. Biomarker responses were evaluated with a repeated measures model to compare time points back to baseline values. As expected, all sheep had a monophasic febrile response to LPS challenge and cortisol increased and returned to baseline by 6 hr. The cytokines TNF-α, IL-6, IFN-γ (pro-inflammatory), and IL-10 (anti-inflammatory) increased, but only TNF-α returned to baseline during the monitoring period. The cytokines IL-1α, IL-1β, IL-17α (pro-inflammatory), and IL-4 (anti-inflammatory) did not respond to LPS challenge. All chemokines (CCL2, CCL3, CCL4, CXCL10, and IL-8) responded to LPS challenge; however, only CCL2, CCL3, CCL4, and CXCL10 increased over time, and only CCL3, CCL4, and CXCL10 returned to baseline during the monitoring period. MicroRNA (miR-145, -233, and -1246) also increased and remained elevated during the study. In summary, the LPS challenge induced a strong stress response in Rideau-Dorset sheep that could be monitored with a distinct profile of circulatory biomarkers.

3.2 Introduction

Breeding stress resilient livestock is a potential strategy to help mitigate the negative impact of environmental and pathogenic stressors. Contributing to these concerns, climate change is expected to exacerbate the impact of environmental and microbial stressors on animals that have known negative consequences on health, production, and welfare (Rojas-Downing et al., 2017). Current concerns over the sustainability of livestock production and increasing antimicrobial resistance can potentially be addressed through genetic selection programs which focus on animal resiliency, defined here as the ability to be minimally affected by a stressor through rapid adaptation. Incorporating stress resiliency into selection criteria could help mitigate negative impacts of increased stress exposure and reduce reliance on antimicrobials.

The neuroendocrine and immune systems are activated by stressors, including physical (Sevi and Caroprese, 2012), microbial and infectious (Kabaroff et al., 2006), and psychological stressors (Caroprese et al., 2010). These systems work in concert to assist in overcoming stress challenges by restoring homeostasis. Recognition of pathogen-associated-molecular-patterns (PAMPs) and host alarmins by innate immune cells, triggers the release of pro-inflammatory cytokines and chemokines into the circulation to initiate an innate immune response, which manifests as inflammation. Pro-inflammatory cytokines such as TNF-α and IL-6 activate the hypothalamic-pituitary-adrenal (HPA) axis, the major neuroendocrine stress system—whose end-product is the stress hormone cortisol. Cortisol functions to control the inflammatory response in addition to having modulatory functions on energy metabolism and physiology (Karrow, 2006).

Lipopolysaccharide (LPS) is a PAMP derived from the cell wall of Gram-negative bacteria and contributes to several livestock illnesses including mastitis (Herry et al., 2017), endometritis (Bilal et al., 2016), acidosis (Abaker et al., 2017), and heat stress due to gut leakage (Vargas and

Marino, 2016). The relevancy of LPS to livestock health has contributed to its widespread use to model the host response and pathologies related to bacterial infection. Moreover, LPS-induced fever, pro-inflammatory cytokine (IL-6 and TNF-α) and cortisol responses have been well-characterized in ruminants (Kabaroff et al., 2006; Hadfield et al., 2018). The endogenous mediators that are activated during the response to LPS, however, are far more extensive, and may also serve as stress biomarkers.

Circulating microRNAs (miRNA) have more recently been discovered and their roles of regulating gene expression are being explored, including influencing immune cell differentiation and inflammation (Dai and Ahmed, 2011). Furthermore, LPS challenge results in the rapid upregulation of miRNA by human monocytes (Taganov et al., 2006). A similar response would be expected in sheep; however, although the miRNA transcriptome of sheep has been characterised (Zhang et al., 2013), little has been done to monitor ovine miRNA responses to systemic LPS challenge.

Since the collective mediators released into the circulation during stress fine-tune the host response to overcome a stressor and help to restore homeostasis, stress resiliency may be determined by the profile of circulatory protein and miRNA biomarkers during stress challenge. Therefore, the purpose of this study was to perform a comprehensive assessment of the ovine stress response to a microbial stressor by measuring circulating cortisol, pro- and anti-inflammatory cytokines and chemokines, miRNA and fever responses to an acute systemic LPS immune stress challenge.

3.3 Materials and Methods

Outbred Rideau-Dorset ewe-lambs at 70 days old were used in this study and housed at the University of Guelph Ponsonby sheep research station, Ontario. The day before the experiment, 15 randomly selected sheep were weighed and housed in individual pens with *ad libitum* access to hay and water. Sheep were challenged i.v. with *Escherichia coli* O111:B4 LPS endotoxin (400 ng/kg body weight, Sigma Chemical Co., St. Louis, Mo, USA) (Kabaroff et al., 2006); no animals showed signs of clinical disease at the beginning of the experiment and the average body weight was 31.4 ± 5.6 kg (mean ± SD mean). Blood samples were collected from the jugular vein in 10 mL BD Vacutainer® serum tubes just prior to LPS challenge (0 hr/ baseline), and at 2, 4, and 6 hrs following LPS challenge. Serum was isolated and aliquoted into microcentrifuge tubes and stored at -80°C until further analysis. Rectal temperatures were monitored hourly during the experiment. All experimental procedures were approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol # 3436).

Total serum cortisol concentrations were measured by a competitive chemiluminescent enzyme immunoassay using the IMMULITE/IMMULITE 1000 Cortisol kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) and an IMMULITE 1000 analyzer (INTER MEDICO, Markham, ON, CANADA) according to manufacturer's instructions; the antibody is highly specific for cortisol and the analytical sensitivity is 5.5 nmol/L.

Serum cytokine concentrations were measured in duplicate using a commercially available multiplex immunoassay kit (MILLIPLEX® MAP Custom 13-Plex Bovine Magnetic Bead Panel, EMD Millipore Corp., Burlington, MA, USA) according to manufacturer's instructions. Serum samples were diluted 1:2 with provided assay buffer prior to adding to wells. The median fluorescence intensity (MFI) of each well was obtained using the Luminex 200™ (Bio-Rad,

Mississauga, ON, CANADA) and data were processed using the Milliplex Analyst software. Concentrations were determined using the 4PL fitting model. The average intra-assay coefficient of variation for the analytes was 4.96%, and all were below 10%.

MiRNA was isolated from serum using the miRNeasy® Serum/Plasma Advanced Kit Qiagen (Hilden, Germany) according to manufacturer's instructions. A spike-in control comprised of known concentrations of CE-MIR-39-1, included in the kit, was added as reference miRNA.

The candidate miRNAs (miR-145, -223, and -1246) were selected based on previous human and bovine literature involving LPS, and forward primer sequences used were 5' GTCCAGTTTTCCCAGGAATCCCT 3', 5' CCTGTCAGTTTGTCAAATACCCCA 3', and 5' GAA TGGA TTTTTGGAGCAGGAA 3' (Li et al., 2015), respectively; the Universal reverse primer (Qiagen) was used for these miRNA assays. The spike-in control was used as a reference miRNA to normalize the expression data. The $2^{-\triangle\triangle Ct}$ method was used to determine change in miRNA expression (Pfaffl, 2001).

For cytokine/chemokine analysis, samples below the minimum limit of detection (LOD) were replaced with LOD / square root(2) as suggested by Croghan and Egeghy (2003). For IL-1β, IL-17α, and CCL4, greater than 30% of values were below the minimum LOD, and IL-8 and CXCL10 had greater than 30% of values above the maximum LOD, so MFI values of these analytes were used for analysis (Breen et al., 2016).

For all analyses, SAS software, version 9.4, was used (SAS Institute Inc 2013. SAS/STAT® 9.4. Cary, NC, USA). Cytokines/chemokines, cortisol, temperature, and miRNA expression were analysed over time using a general linear mixed model (PROC MIXED) with repeated measures. Time was modelled as a fixed-effect with plate (plate only relevant for cytokine and chemokine analysis) and sheep ID as random effects. Repeated measures were

accounted for by fitting autocorrelation structures. The Akaike information criterion was used as a guide for the best fitting model. The assumptions of the model were checked by examining the residuals for normality after passing through the model with a Shapiro-Wilk test, and data were log transformed when necessary to meet the assumptions of normality. Specifically, the data that required a log transformation were: IL-1α, IL-6, IFN-γ, TNF-α, IL-10, CCL2, CCL3, cortisol, miR-145, -223, and -1246. Post-hoc tests were used on a Dunnett adjustment and significance was set at $P \leq 0.05$. Logged data were back-transformed showing geometric means, and untransformed data were represented as the arithmetic mean, both with upper and lower 95% confidence limits.

3.4 Results and Discussion

The present study demonstrated that a 400 ng/kg systemic dose of LPS elicited a strong stress response as indicated by the induction of numerous circulatory pro- and anti-inflammatory cytokines and chemokines, cortisol and miRNAs. The LPS challenge also elicited a monophasic fever response as reported previously (Kabaroff et al., 2006) that peaked at 3 hr after LPS challenge, with temperature approaching baseline levels by 6 hr (Figure 3.1A).

Fever is considered a cardinal response to infection (Evans et al., 2015) and is thought to be driven by some pro-inflammatory cytokines, particularly IL-1, IL-6, and TNF-α. Given this, it is not surprising that TNF-α displayed a significant response over time ($P < 0.01$), peaking at 2 hr then returning to baseline by 6 hr (Figure 3.1C), and an early increase in IL-6 was observed at 2 hr and plateaued around 4 hr before beginning to drop (Figure 3.1D). Early induction of TNF-α is associated with increased blood flow to sites of infection, signalling endothelial cells to produce chemokines, and the recruitment of leukocytes (mainly neutrophils) to potentiate the innate immune response (Waters et al., 2013). Though generally considered pro-inflammatory, IL-6 can

have anti-inflammatory functions, depending on which IL-6 receptor is involved (Kaplanski et al., 2003).

Although the IL-1 family of cytokines is thought to have a primary role in innate immunity and inflammation, it was interesting to observe that IL-1α (Figure 3.1E) and IL-1β (data not shown) concentrations did not change over time ($P > 0.05$). As a soluble mediator, IL-1α can be classified as an alarmin that is released from necrotic cells (Di Paolo and Shayakhmetov, 2016); it is possible that the low LPS dose used in this study did not induce necroptosis. Although other studies have detected IL-1β responses in sheep to either high dose LPS infusion, or lethal dose of live *E. coli*, large variation was observed in these studies and it was determined that TNF-α and IL-6 had more consistent responses (Byrne et al., 2017; Peake, 2002). In addition to the IL-1s, IL-17α (data not shown) and IL-4 (Figure 3.1H) did not have an observable response over time ($P > 0.05$).

IFN-γ, known for driving T-helper 1 cell (Th1) and inhibiting T-helper 2 cell (Th2) differentiation (Deng et al., 2012), also responded to LPS challenge ($P < 0.01$) and approached baseline by 6 hr (Figure 3.1F). It is well-known that LPS induces IFN-γ production (Varma et al., 2002). IFN-γ also promotes the differentiation of monocytes to macrophages rather than dendritic cells (Delneste et al., 2003), and enhances macrophage production of TNF-α and IL-6 (Chen and Ivashkiv, 2010).

IL-10 is a major anti-inflammatory cytokine that controls immune cell differentiation and the production of pro-inflammatory cytokines by activated macrophages and antigen-presenting cells (Murray, 2005). We observed an IL-10 response ($P < 0.01$) that peaked later than most pro-inflammatory cytokines, around 4 hr, and approached baseline by 6 hr (Figure 3.1G).

Chemokines mediate immune cell trafficking and interactions during the immune response (Sokol and Luster, 2015), and we found all chemokines measured (CCL2, CCL3, CCL4, IL-8, and CXCL10) displayed a response over time ($P < 0.01$) to LPS challenge. Chemokines CCL3, CCL4, and CCL2 peaked around 2 hr (Figure 3.2A, 3.2B, 3.2C) and CXCL10 had a relatively more gradual rise over time, peaking around 4 hr (Figure 3.2D). Lastly, IL-8 (CXCL8) was different from baseline only at 6 hr, where levels decreased ($P < 0.01$; Figure 3.2E). CCL2, also known as monocyte chemoattractant protein 1, is known for promoting infiltration of macrophages and monocytes by acting through the CCR2 receptor with high affinity (Lu et al., 1998), although, CCL3 can also bind this receptor. CCL3 has been shown to mediate neutrophil migration during inflammation (Ramos et al., 2005), and its contribution to alveolar macrophage activation infection has been previously demonstrated (Lindell et al., 2001). Although CCL4 acts on the same CC receptors as CCL3 (Jing et al., 2003), one study found that activated T cells and Natural killer cells were preferentially recruited by this chemokine (Megjugorac et al., 2004). In a canine endotoxemia study, CXCL10 was found to be upregulated in the heart, lung, kidney, liver, and spleen, and was associated with T lymphocyte infiltration (Frangogiannis et al., 2000). The chemokine IL-8 has been shown to selectively recruit neutrophils during *E. coli*-induced mastitis in sheep (Gangur et al., 2002). The decreased IL-8 levels at 6 hr in our study could indicate a transition from an initial neutrophil influx to a mononuclear leukocyte population.

Expression of miR-145, -1246, and -223 was upregulated ($P < 0.05$) during the 6-hr period after LPS challenge and displayed high variation during peak expression. MiR-145 had a delayed increase in expression beginning at 6 hr ($P < 0.05$, Figure 3.3A). MiR-1246 expression increased from 4 to 6 hr (Figure 3.3B), and miR-223 increased at 4 hr before plateauing (Figure 3.3C). Given that miRNA target mRNAs, it is not surprising that they have important regulatory roles in immune

cell development and immune responses. MiR-223 for example, has been found to be upregulated during granulopoiesis leading to enhanced granulocyte differentiation (Fazi et al., 2005); however, another study involving miR-223 knockout mice found miR-223 to be a negative regulator of granulocyte differentiation and activation, with knockout mice having increased numbers of hyperactive granulocytes causing excessive tissue damage (Johnnidis et al., 2008). MiR-1246 has been described as having a role during LPS-induced inflammation (Wu et al., 2017), in addition to being upregulated in the serum of heat-stressed Holstein cows, and further enrichment analysis identified target genes related to the stress response and immune system development and response (Zheng et al., 2014). Another study assessing miRNA expression in Holstein mammary tissue during heat stress found miR-145 to be among the highest differentially expressed miRNAs (Li et al., 2018a). Over-expression of miR-145 has also been found to promote the expression of TNF-α and CCL4 (Li et al., 2018b); however, we observed opposite profiles between these factors. The miRNAs we measured were upregulated towards the end of the monitoring period, suggesting that they could be involved in resolution of acute inflammation.

As anticipated, cortisol levels increased in response to LPS ($P < 0.01$) and peaked around 2 hr before returning to baseline by the end of the sampling period; we observed the highest variation at 2 and 4 hr post LPS challenge (Figure 3.1B). The induction of cortisol is consistent with the strong pro-inflammatory cytokine response, particularly IL-6, as this cytokine can stimulate cortisol release at the level of the adrenal gland (Bethin et al., 2000) and the brain (Girotti et al., 2013). Cortisol helps control the inflammatory response by preventing transcription of pro-inflammatory cytokines and promoting the production of anti-inflammatory cytokines (Tian et al., 2014).

3.5 Conclusion

To date, this is the most comprehensive assessment of the ovine immune, stress, and miRNA responses to systemic LPS challenge. Collectively, a low dose of LPS induced a strong inflammatory response that nearly resolved within the 6-hr monitoring period based on the recovery of fever. The inflammatory response can be detected through the measurements of immune and stress biomarkers IL-6, TNF-α, IFN-γ, IL-10, CCL2, CCL3, CCL4, CXCL10, miR-145, -1246, -223, and cortisol. However, IL1β, IL-1α, IL-17α, and IL-4 did not have an observable response over time. Cytokine profiles and, more recently, miRNAs are being associated with disease susceptibility, and the high variation we observed in the responses of some of these measured variables could be investigated further to determine what contributes to this variation and whether it is associated with stress resiliency. Considering the miRNA transcriptome of sheep contains thousands of miRNAs, future work should evaluate a larger panel of miRNAs. Additionally, immune cell populations that are involved in LPS recognition and the initiation of the inflammatory response should also be evaluated, which could help to verify some functional capabilities of the measured chemokines. Lastly, cortisol responsiveness could be evaluated further as it is critical in the resolution of the inflammatory response—necessary for stress resiliency.

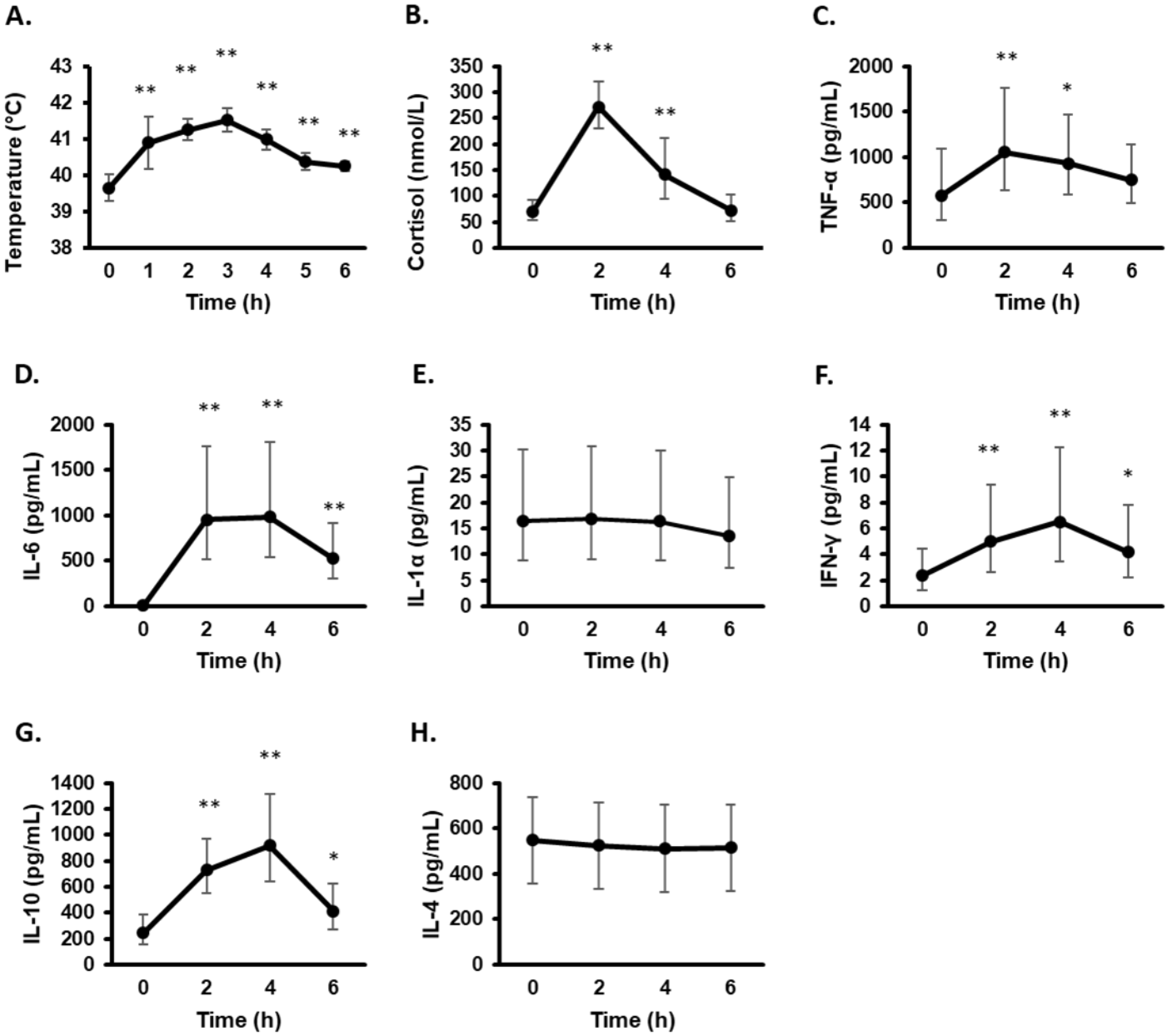

Figure 3.1: Rectal temperature (A), serum cortisol (B), TNF-α (C), IL-6 (D), IL-1α (E), IFN-γ (F), IL-10 (G), and IL-4 (H) responses in female sheep (n=15) following i.v. LPS (400 ng/kg) challenge. Results are reported as geometric means if back transformed, or the mean, with an upper and lower 95% confidence limit. An asterisk (*) represents $P < 0.05$ and double asterisk (**) represents $P \leq 0.01$ in comparison to the time zero baseline. *MFI: median fluorescence intensity*.

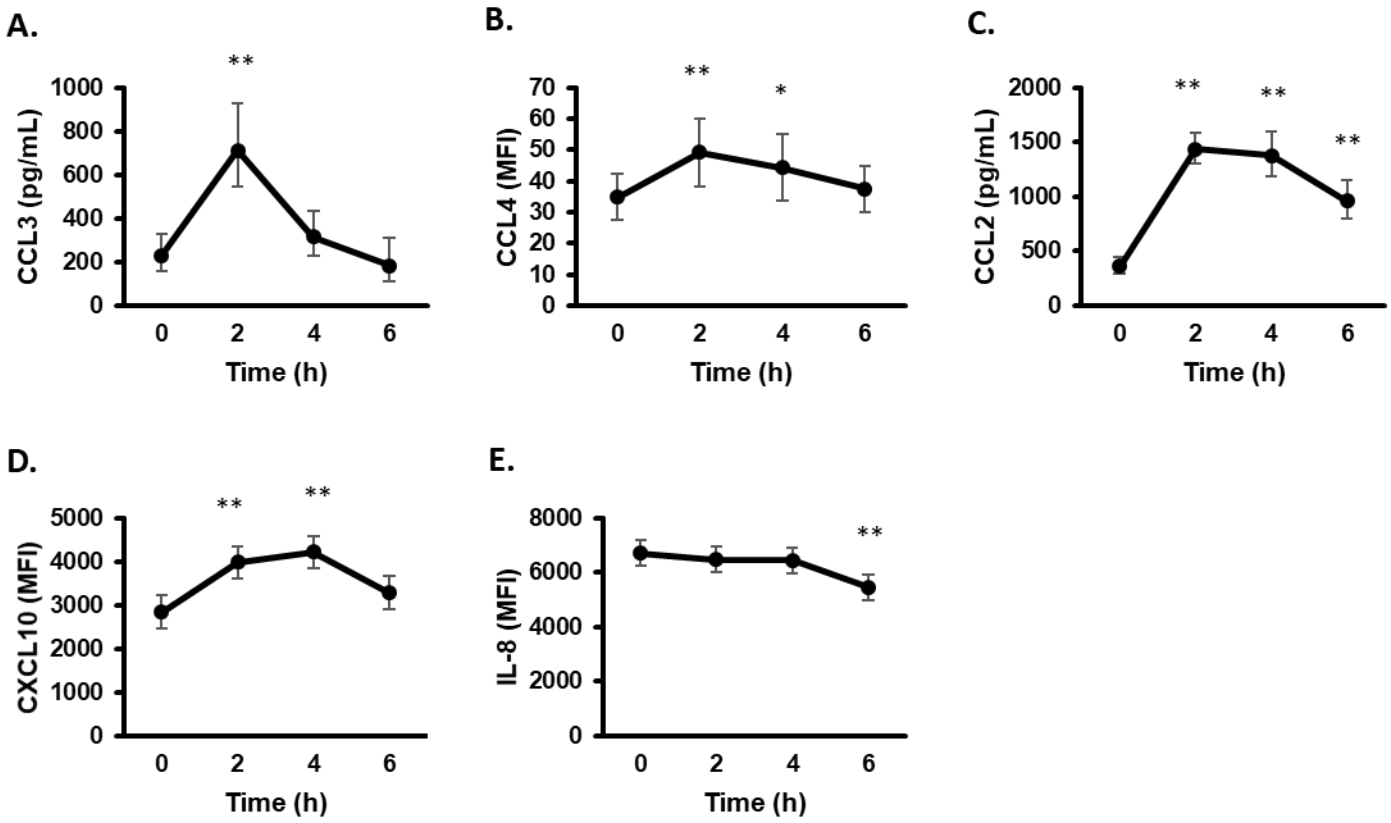

Figure 3.2: Serum CCL3 (A), CCL4 (B), CCL2 (C), CXCL10 (D) and IL-8 (E) chemokine responses in female sheep (n=15) following i.v. LPS (400 ng/kg) challenge. Results are reported as geometric means if back transformed, or the mean, with an upper and lower 95% confidence limit. An asterisk (*) represents $P < 0.05$ and double asterisk (**) represents $P \leq 0.01$ in comparison to the time zero baseline. *MFI: median fluorescence intensity*.

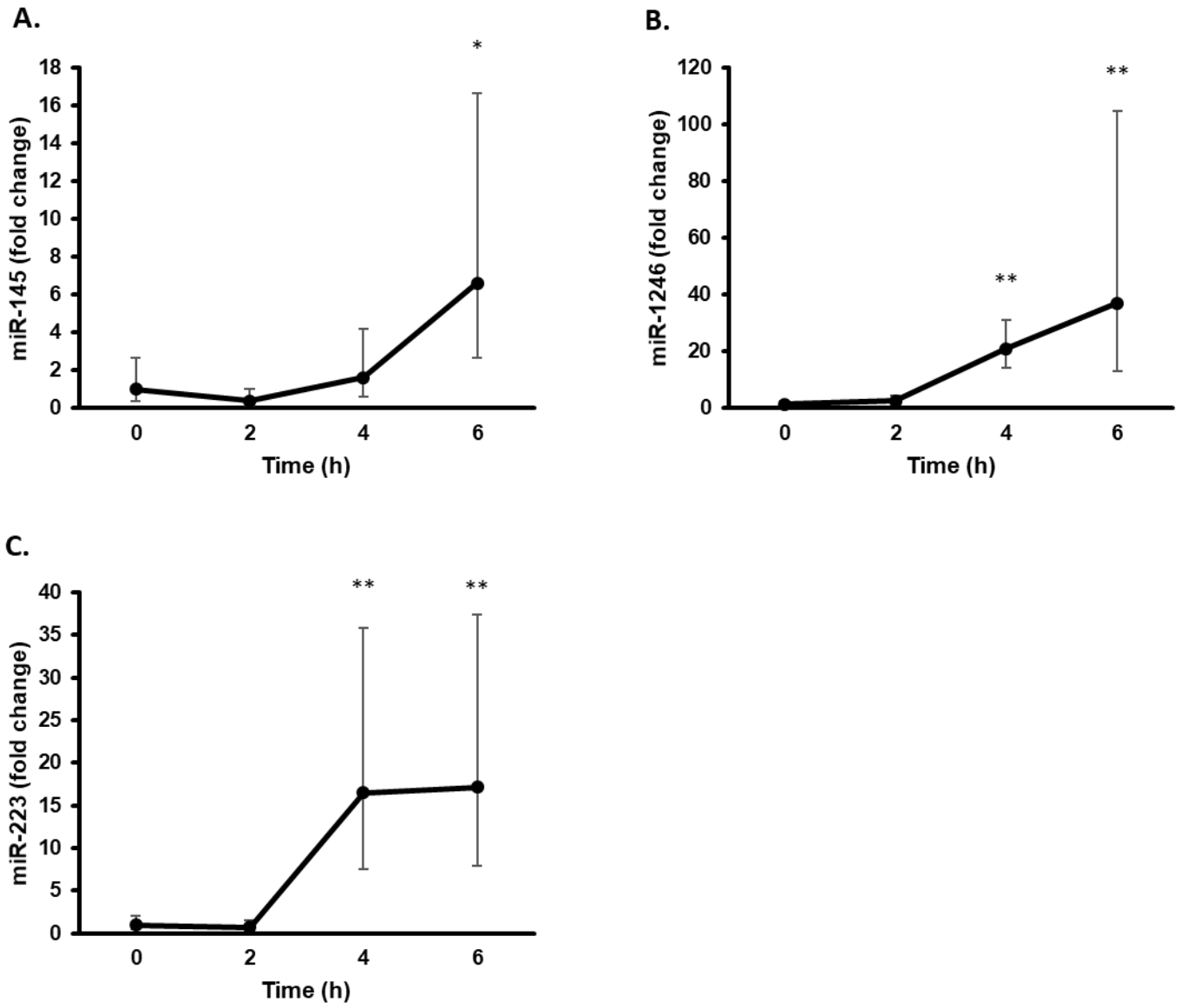

Figure 3.3: Fold-change expression of miR-145 (A), miR-1246 (B), and miR-233 (C) in female sheep (n=15) following i.v. LPS (400 ng/kg) challenge reported as geometric means with an upper and lower 95% confidence limit. Fold-change expression is reported relative to baseline levels and normalized to the spike-in control CE-MIR-39-1. An asterisk (*) represents $P < 0.05$ and double asterisk (**) represents $P \leq 0.01$ in comparison to the time zero baseline.

Chapter IV: LPS-induced cytokine, chemokine and immune cell profiles of variable stress-responding sheep

4.1 Abstract

Individual variation of the neuroendocrine system's hypothalamic-pituitary-adrenal (HPA) axis activation in response to stress could contribute to variable stress resiliency of livestock. During stress events, the immune system may also be activated and work in concert with the neuroendocrine system to restore homeostasis while minimizing tissue damage. The purpose of this study was to assess immune function and regulation in variable stress responding sheep in response to a lipopolysaccharide (LPS) immune-challenge to identify a possible mechanism of variable stress resiliency. High (HSR), middle (MSR), and low stress responders (LSR) were selected from a population of 112 female lambs that were challenged intravenously with a bolus of LPS (400 ng/kg). Blood was collected from the jugular vein at 0 and 4 hrs post LPS challenge to monitor changes in serum pro- and anti-inflammatory cytokines and chemokines, and white blood cell populations. Rectal temperature was also recorded hourly to monitor fever response. As expected, all sheep had a monophasic febrile response to LPS challenge, but HSR had a stronger fever than MSR and LSR (HSR > MSR > LSR). HSR also had a stronger pro-inflammatory cytokine response, measured by increased serum IL-6 (HSR > MSR > LSR) and IFN-γ (HSR > MSR, LSR). There was no change in serum concentrations of IL-1α, IL-1β, TNF-α, and IL-17α between 0 and 4 hrs. HSR and MSR had a stronger anti-inflammatory cytokine response than LSR, measured by serum IL-10 (HSR, MSR > LSR), but there was no change in serum IL-4 concentrations between 0 and 4 hrs. All chemokines (CCL2, CCL3, CCL4, and CXCL10), except for IL-8, responded to LPS challenge with higher concentrations at 4 hrs. Differences among the stress response groups, however, were only evident for CCL2, with HSR and MSR having higher concentrations at 4 hrs than LSR (HSR, MSR > LSR). Lymphocyte and monocyte counts decreased, and neutrophils and the neutrophil-lymphocyte ratio (NLR) increased at 4 hrs post LPS

challenge, but eosinophil counts were unchanged. Furthermore, no differences were detected among the stress response groups in the white blood cell parameters measured. In summary, the stress response phenotype contributed to distinct immune function and regulation in sheep, measured by fever, pro- and anti-inflammatory cytokines, and the chemokine CCL2. Future studies should utilize more refined methodologies for monitoring immune cell populations. Additionally, the performance and health of variable stress responders should be monitored in heat stress conditions and over the long term to assess the phenotype in a practical setting and potentially identify an optimal phenotype.

4.2 Introduction

Climate change is anticipated to increase the exposure of environmental and microbial stressors on livestock that have negative impacts on health, welfare, and production (Rojas-Downing et al., 2017). This may compound the challenges of having sustainable livestock production and controlling antimicrobial resistance. Breeding strategies are consequently being adapted and include a more balanced selection of production, conformation, and functional traits to increase resiliency of livestock than in the past (Mormede et al., 2011).

The hypothalamic-pituitary-adrenal (HPA) axis is the major stress response pathway of the neuroendocrine system. There is considerable variation within individuals with regard to its activation, and heritability estimates have been made in several species, including sheep, suggesting that genetics contribute significantly to this variation (Pant et al., 2016; Brown & Nestor, 1973; Larzul et al., 2015; Overli et al., 2005). Cortisol is the end-product of HPA axis activation and modulates inflammation, immune responses, energy metabolism, and physiology (Karrow, 2006). The vast effects cortisol has, combined with its genetic regulation, justify

exploring HPA axis activation, or more specifically, the cortisol response to stress, as a candidate phenotype for improving stress resiliency of livestock.

Lipopolysaccharide (LPS) is a component of Gram-negative bacteria that is widely used in livestock health research, as it has been implicated in numerous livestock pathologies (Bilal et al., 2016; Guo et al., 2017; Herry et al., 2017; Vargas and Marino, 2016). LPS is a pathogen-associated-molecular-pattern (PAMP) that can be recognized by innate immune cells; recognition initiates the production and release of pro-inflammatory cytokines and chemokines into the circulation—initiating an innate immune response that manifests as inflammation. Certain pro-inflammatory cytokines, such as IL-6 and TNFα, can activate the HPA axis to induce the release of cortisol (Beishuizen and Thijs, 2003). Cortisol subsequently controls inflammation to prevent excessive damage (Karrow, 2006). This pathway has been previously utilized to stress phenotype sheep as high (HCR) and low (LCR) cortisol responders in response to LPS challenge (You et al., 2008b). Serum pro-inflammatory cytokine production (IL-6 and IL-1) were evaluated in these sheep, but no differences in production were detected between the groups.

In Naylor et al. (2020), systemic biomarkers of LPS-induced inflammation, including a variety of pro- and anti-inflammatory cytokines, chemokines, microRNA (miRNA) and white blood cell populations, were found to change over time. These circulatory mediators have functional consequences in immune regulation and their production during a stress challenge may contribute to variation in the stress response, and ultimately to stress resiliency and animal health and welfare. The purpose of this study, therefore, was to investigate immune function in variable stress-responding sheep phenotyped based on an LPS immune-challenge. Immune function was assessed by examining a panel of pro- and anti-inflammatory cytokines, chemokines, and changes in immune cell populations.

4.3 Materials and Methods

One hundred and twelve outbred Rideau-Dorset ewe-lambs (75 ±6 days old and 26.3 ±5 kg (mean ±SD mean)) were housed at the University of Guelph Ponsonby sheep research station, Ontario, and were stress phenotyped between November 2017 and December 2018. During the phenotyping, the ambient temperature ranged from -7 °C (January 2018) to 26 °C (June 2018). The day before the LPS challenge, sheep were weighed, necks shaved, and then housed in adjoining 4-ft x 4-ft individual pens with *ad libitum* access to hay and water. The sheep had visual contact with each other to minimize isolation stress. Sheep were challenged i.v. with *Escherichia coli* O111:B4 LPS endotoxin (400 ng/kg body weight, Sigma Chemical Co., St. Louis, Mo, USA) according to Kabaroff et al. (2006); no animals showed signs of clinical disease at the beginning of the experiment. Rectal temperatures were recorded just prior to LPS challenge (0 hr/ baseline) and hourly for 6 hrs. For blood collection, animals were restrained with assistance; sheep were straddled and had their jaw lifted at an approximate 30-degree angle to expose the jugular vein. Blood samples were collected within 1.5 min of restraint from the jugular vein in 10 mL BD Vacutainer® serum and EDTA tubes just prior to LPS challenge and 4 hrs following LPS challenge. Previously, the 4-hr sampling time was determined to correspond to the peak cortisol response during systemic endotoxin challenge using LPS (Kabaroff et al., 2006). A subset of 12 high stress responders (HSR), 12 middle stress responders (MSR), and 12 low stress responders (LSR) were identified based on their serum cortisol concentrations measured 4 hrs post-LPS challenge. The HSR and LSR represent the extremes of the 4 hr cortisol response range and are greater than, or less than, one standard deviation unit from the mean response. The MSR were selected around the median cortisol response. Serum was isolated and aliquoted into microcentrifuge tubes and stored at -80°C until further analysis. Rectal temperatures were

monitored hourly during the experiment. All experimental procedures were approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol #3436) and followed the Canadian Council on Animal Care guidelines (CCAC, 1993).

Total serum cortisol concentrations were measured by a competitive chemiluminescent enzyme immunoassay using the IMMULITE/IMMULITE 1000 Cortisol kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) and an IMMULITE 1000 analyzer (INTER MEDICO, Markham, ON, CANADA) according to manufacturer's instructions; the antibody is highly specific for cortisol and the analytical sensitivity is 5.5 nmol/L.

Serum cytokine concentrations were measured in duplicate using a commercially available multiplex immunoassay kit (MILLIPLEX® MAP Custom 13-Plex Bovine Magnetic Bead Panel, EMD Millipore Corp., Burlington, MA, USA) according to manufacturer's instructions. Serum samples were diluted 1:2 with the provided assay buffer. The median fluorescent intensity (MFI) of each well was obtained using the Luminex 200™ (Bio-Rad, Mississauga, ON, CANADA) and data were processed using the Milliplex Analyst software. Concentrations were determined using the 5PL fitting model. The intra-assay coefficient of variation was less than 10% for all analytes. Samples below the minimum limit of detection (LOD) were replaced with LOD/square root(2) as suggested by Croghan and Egeghy (2003). For IL-1β, greater than 30% of values were below the minimum LOD, and IL-8 and CXCL10 had greater than 30% of values above the maximum LOD, so MFI values of these analytes were used for analysis (Breen et al., 2016).

For the white blood cell differential, fresh blood was used to prepare blood smears on glass microscope slides. Specifically, whole blood-containing EDTA tubes were inverted 10 times, then, using a transfer pipette, a drop of blood was applied to a glass microscope slide and the edge of another glass slide was used to push the blood into a tongue shape with a smooth feathered edge.

Slides with blood smears were then dried under a small fan and stained using a Siemens Hematek® Automated Slide Stainer (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) using the Siemens Modified Wright's Stain Hematek® Stain Pak (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). White blood cells were examined under the light microscope at 40× magnification and neutrophils, lymphocytes, monocytes, and eosinophils were counted. A total of 100 unique white blood cells in each sample were classified. The neutrophil-lymphocyte ratio (NLR) was calculated by dividing the neutrophil and lymphocyte counts for each sample.

For all analyses, SAS software, version 9.4, was used (SAS Institute Inc 2013. SAS/STAT® 9.4. Cary, NC, USA). A general linear mixed model (PROC MIXED) was used to model the relationship of cortisol concentrations between baseline and 4 hrs post-LPS challenge, while accounting for the fixed effect of month of phenotyping. Linear and quadratic terms were initially included, as well as interactions. Non-significant effects were removed to simplify the model. Data were tested for normality with Shapiro-Wilk, Cramér-von Mises, and Kolmogorov-Smirnov tests, and examination of the residuals was carried out. Significance was set at $P \leq 0.05$.

A general linear repeated measures model (PROC MIXED) was used to test for differences in rectal temperature among the stress response groups and if there was a change over time, controlling for the month phenotyped. The fixed effects of month, time post-LPS challenge, and phenotype, including the phenotype by time interaction, were included in the model. To determine the best correlation structure for repeated measures on each animal, the Akaike Information Criterion (AIC) 'smallest is best', was used. Data were tested for normality with Shapiro-Wilk, Cramér-von Mises, and Kolmogorov-Smirnov tests, and examination of the residuals was carried

out. Significance was set at $P \leq 0.05$. A post-hoc Tukey adjustment for multiple comparisons was applied to compare phenotypes at each time.

A general linear mixed model (PROC MIXED) was used to test for differences in cytokine and chemokine production, and cell counts, while controlling for the month phenotyped. The fixed effects of month, time post-LPS challenge, and phenotype, including the phenotype by time interaction, were modelled. Random effects of plate and id(plate*group) were also included in the model for the cytokine and chemokine variables. Non-significant effects were removed to simplify the model. Data were tested for normality with Shapiro-Wilk, Cramér-von Mises, and Kolmogorov-Smirnov tests, and examination of the residuals was carried out; a log transformation was applied to IL-1β, IL-6, IFN-γ, IL-10, CCL4, and TNFα. Significance was set at $P \leq 0.05$. A post-hoc Tukey adjustment for multiple comparisons was applied to compare phenotypes at baseline and 4 hrs post-LPS challenge.

4.4 Results

4.4.1 Comparison of basal and LPS-induced cortisol concentrations among the variable stress responders

For the 112 sheep, the basal serum cortisol concentration ranged from 19.8 to 171 nmol/L, and concentrations 4 hrs post-LPS challenge ranged from 19.8 to 395 nmol/L. The large variation in cortisol concentration at 4 hrs allowed for selection of 36 animals to be classified as HSR (336.2 ±27.9 nmol/L cortisol concentration (mean ± SD mean), n = 12), MSR (147.3 ±9.5 nmol/L cortisol concentration, n = 12), and LSR (32.1 ±10.4 nmol/L cortisol concentration, n = 12).

As expected, the 4 hr serum cortisol concentrations were significantly different among the stress response groups ($P < 0.01$), but there were no differences in basal cortisol concentrations among the groups ($P > 0.05$) (Figure 4.1A). The month that animals were phenotyped was not

associated with 4 hr cortisol concentrations ($P > 0.05$) but did influence basal levels ($P < 0.05$), with sheep phenotyped in the winter having higher basal concentrations than sheep phenotyped in the summer (Figure 4.1B).

4.4.2 Comparison of LPS-induced rectal temperature responses among the variable stress responders

All sheep responded to the LPS with a monophasic fever response that peaked between 2 and 3 hrs post-LPS challenge. There were no differences in basal temperature among the stress response groups ($P > 0.05$), but the groups had significantly different responses to LPS challenge over time ($P < 0.01$); the greatest differences among the stress response groups were observed at 3 and 4 hrs post-LPS challenge. The HSR had the strongest fever response, peaking at 3 hrs. This was followed by MSR, also peaking at 3 hrs, and then LSR with the weakest fever response, peaking earlier at 2 hrs (Figure 4.2).

4.4.3 Comparison of basal and LPS-induced serum cytokine and chemokine concentrations among the variable stress responders

The month that sheep were phenotyped was not significant for any of the cytokines and chemokines measured ($P > 0.05$).

4.4.3.1 Pro-inflammatory cytokines

Serum IL-1α (Figure 4.3A), TNFα (Figure 4.3C), and IL-17α (Figure 4.3D) concentrations, and IL-1β (Figure 4.3B) MFI, did not change between 0 and 4 hrs post-LPS challenge ($P > 0.05$), and no differences were detected among the stress response groups ($P > 0.05$). IL-6, on the other hand, had a significant phenotype by time interaction ($P < 0.01$)—no differences were detected between the stress response groups baseline levels, but there were differences at 4 hrs post-LPS challenge (Figure 4.3E). Specifically, all groups had a significant increase in IL-6 production ($P < 0.01$), with HSR having significantly higher serum concentrations than MSR ($P < 0.05$) and LSR

(P <0.01), and MSR higher than LSR (P < 0.01). Similarly, IFNγ also had a significant phenotype by time interaction (P < 0.01). Again, no differences were detected at baseline among the groups, but there were differences in production of IFNγ 4 hrs post-LPS challenge (Figure 4.3F). Only HSR and MSR had a significant increase in IFNγ production (P < 0.01), and HSR had significantly higher serum concentrations than MSR (P < 0.01) and LCR (P < 0.01). Although MSR had a significant IFNγ response, whereas LSR did not, MSR did not have significantly different concentrations from the LSR at 4 hrs post-LPS challenge (P > 0.05).

4.4.3.2 Anti-inflammatory cytokines

Serum IL-4 (Figure 4.3G) did not significantly increase 4 hrs post-LPS challenge and no differences were detected among the stress response groups (P > 0.05). However, a significant phenotype by time interaction was detected for IL-10 (P < 0.05), where differences in serum concentration among the stress response groups occurred 4 hrs post-LPS challenge (Figure 4.3H). All groups had a significant increase in production of IL-10 (P < 0.01), and HSR and MSR had a stronger response than LSR (P < 0.01 and P < 0.05, respectively), but HSR did not have significantly different IL-10 production than MSR (P > 0.05).

4.4.3.3 Chemokines

IL-8 (Figure 4.4A) MFI did not significantly change between 0 and 4 hrs post-LPS challenge, and no differences were detected among the groups (P > 0.05). Despite serum CCL3 (Figure 4.4B) and CCL4 concentrations (Figure 4.4C), and CXCL10 MFI (Figure 4.4D), having a significant increase at 4 hrs post-LPS challenge (P < 0.01), no differences were detected among the groups (P > 0.05). CCL2 (Figure 4.4E) was the only chemokine in which differences were observed among the groups. All groups had a significant increase in CCL2 in response to LPS challenge (P < 0.01), and the phenotype by time interaction was significant (P < 0.01). HSR and

MSR had a stronger response than LSR ($P < 0.01$) but HSR did not have significantly different production than MSR.

4.4.4 Comparison of basal and LPS-induced white blood cell populations

Despite neutrophil (Figure 4.5A), lymphocyte (Figure 4.5B), the NLR (Figure 4.5C), and monocyte (Figure 4.5D) counts being significantly different between baseline and 4 hrs post-LPS challenge ($P < 0.05$, $P < 0.01$, $P < 0.01$, $P < 0.05$, respectively), no differences were detected among the groups. Eosinophil counts did not change over time ($P > 0.05$) and again, no differences were detected among the groups ($P > 0.05$) (data not shown).

4.5 Discussion

This study utilized a systemic LPS challenge to assess immune function in variable stress-responding sheep. The high variation in cortisol production 4 hrs post-LPS challenge, with concentrations ranging from 19.8 nmol/L to 395 nmol/L, allowed for selection of phenotypically extreme and moderate animals, termed HSR, MSR, and LSR. Despite phenotyping sheep during different months of the year to reach the desired sample size of 112 animals, month did not significantly influence how sheep responded to the LPS challenge. It did, however, influence basal cortisol concentrations, with the levels appearing to follow a seasonal pattern—higher basal concentrations during the winter months and lower concentrations during the summer. This pattern of basal cortisol levels has been well documented in humans (Persson et al., 2008; Pierre et al., 2016) and in dairy cows, Braun et al. (2017) found that hair cortisol concentrations were higher in the winter than the fall, but not the spring or summer. In addition to basal cortisol levels, there is also evidence of seasonal effects on immune activity (Martin et al., 2008); however, this was not

observed in the present study; this validates the use of LPS endotoxin to stress phenotype sheep throughout the year.

Rectal temperatures were monitored to confirm that sheep had an inflammatory response to LPS challenge, as fever is a hallmark of inflammation (Evans et al., 2015). All sheep had a monophasic fever response to LPS challenge, which has been previously reported (Naylor et al., 2020; Kabaroff et al., 2006). Fever is a highly conserved response to infection that not only creates an unfavourable environment for bacteria and viruses, but can stimulate the immune response (Evans et al., 2015). For example, using an in vitro human macrophage model and an in vivo mouse model, Zhao et al. (2007) demonstrated that pre-treatment with 39.5 °C temperature for 30 minutes enhanced pro-inflammatory cytokine production in response to LPS, which was attributed to increased expression of TLR4, the receptor responsible for LPS recognition. Similarly, peripheral blood mononuclear cells from cattle exposed to heat stress conditions, had upregulated TLR4 and pro-inflammatory cytokine IL-6 expression (Bharati et al., 2017). In this study, the variable cortisol responders had distinct fever responses to LPS challenge, where the HSR had the strongest and most prolonged response, and LSR had the weakest (HSR > MSR > LSR). Based on these observations, cortisol responsiveness has a direct relationship with inflammation as measured by rectal temperature and could contribute to varied immune function.

The pro-inflammatory cytokines IL-6, IL-1, and TNFα are referred to as pyrogens, in that they induce fever (Netea et al., 2000). Despite the fever response observed in the sheep, only IL-6 increased 4 hrs post-LPS challenge in this study. In Naylor et al. (2020), however, both IL-6 and TNFα responded to LPS challenge. In the Naylor et al. study however, TNFα peaked earlier at 2 hrs, so it is likely the 4 hr timepoint measured in the current study did not sufficiently capture the TNFα response. For IL-6, HSR had the greatest response and LSR had the weakest response (HSR

> MSR > LSR). A study by Hadfield et al. (2018) investigated breed-specific immune responses to high dose (2.5 μg/kg) iv LPS challenge in ewes, and found that Suffolk sheep had stronger cortisol and fever responses, in addition to greater IL-6 expression compared to Dorsets. TNFα was also found to be significantly induced in both breeds from the Hadfield study; however, the response again peaked within 2 hrs and then dropped, consistent with the finding observed by Naylor et al., (2020). IL-6 is a pleiotropic cytokine that regulates several aspects of immune function including leukocyte trafficking, apoptosis, T cell polarization, B cell activation, and proliferation (McLoughlin et al., 2005). These varied effects are made possible by signalling through either a soluble or membrane bound receptor. During acute inflammation, this dual mode of IL-6 signalling allows for the transition from an initial influx of neutrophils to a mononuclear leukocyte population (Kaplanski et al., 2003).

Contributing to this response is the presence of IFNγ, another pro-inflammatory cytokine, since it has been shown that a deficiency in IFNγ also leads to a deficiency in IL-6 and impaired clearance of neutrophils (McLoughlin et al., 2003). The IFNγ production observed in this study followed a similar pattern as IL-6; HSR had stronger production than MSR and LSR, but no difference was detected between MSR and LSR at 4 hrs (HSR > MSR, LSR). The vast effects IL-6 has on immune cell functions could potentially influence immune competence of the different stress responders.

IL-17α was another pro-inflammatory cytokine measured in this study, and consistent with the observations by Naylor et al. (2020), there were neither changes over time, nor differences among the groups. To our knowledge, no studies have examined systemic IL-17α production in sheep in response to LPS, let alone other immune stimulants. The methods of detecting this cytokine in ruminants have been relatively limited until the past few years, which likely contributes

to the lack of research on this cytokine in sheep. Although IL-17α is mainly associated with autoimmune disorders and allergy responses (Aujla et al., 2008; Valeri and Raffatellu, 2016), it has also been described having a role in host defense against extracellular bacteria (Gu et al., 2013), which is surprising since no response was detected in this study. In pigs, an i.v. infusion of 50 ug/kg LPS over 2 hrs resulted in elevated levels of plasma IL-17α (Xu et al., 2017), so it is possible that either the low dose of 0.4 ug/kg of LPS used in this study, or a species difference, is contributing to the lack of IL-17α production. Species differences in IL-17α biology are known to exist even across ruminants; the types of immune cells responsible for IL-17α production are more limited in sheep than cattle, with $CD4^+$ and WC-1^+ T cells identified in sheep, but $CD8^+$ cells also identified in cattle (Wattegedera et al., 2017). Regarding dose, humans challenged i.v. with a very low dose of 0.8 ng/kg of LPS failed to produce Th1- or Th17-associated cytokines, including IL-17α (Brinkhoff et al., 2018). However, lack of production of other pro-inflammatory cytokines, like IFNγ and TNFα, was also observed—and in the current study, IFNγ had a strong response to LPS. Given the conflicting observations on LPS-induced IL-17α and the lack of research on this cytokine in sheep, more investigations are warranted to clarify its role during systemic inflammation in sheep.

Anti-inflammatory cytokines control pro-inflammatory cytokine production, which helps regulate inflammation and prevent excessive damage. The anti-inflammatory cytokine IL-10 was induced for all groups, but differences in production were only detected between LSR and the two other groups. Since the HSR and MSR had stronger inflammatory responses, it is important to have strong regulation of this response to protect the host. Consistent with this observation, monocytes, macrophages, and fibroblasts isolated from transgenic sheep over-expressing TLR4 had increased production of pro- and anti-inflammatory cytokines, including IL-10, following LPS

challenge (Deng et al., 2012b). Furthermore, in an experimental mouse model, Campylobacter jejuni infection in the lungs induced a strong pro-inflammatory cytokine response that was followed by anti-inflammatory cytokine production (Al-Banna et al., 2008). In addition to controlling the accumulation of immune cells, IL-10 can target monocytes and macrophages to inhibit the production of typical T helper 1 (Th1) pro-inflammatory cytokines, including IL-6 and TNFα (Couper et al., 2008). IL-10 can also target $CD4^+$ T cells to inhibit the production of T helper 2 cytokines (Th2), such as IL-4 (Joss et al., 2000). Furthermore, LPS is known for promoting Th1 responses (McAleer and Vella, 2008), consistent with the cytokine responses we observed in this study.

Serum chemokines were also measured in this study because they are directly responsible for mediating immune cell trafficking and positioning in tissues during the immune response (Sokol and Luster, 2015). All chemokines measured in this study, except IL-8, demonstrated an increase at 4 hrs post-LPS challenge, but differences among the stress response groups at 4 hrs were only evident for CCL2 (HSR, MSR > LSR). Distribution and numbers of different immune cells is achieved, in-part, by redundancy in the recruiting ability of chemokines, as immune cells express multiple chemokine receptors and chemokine receptors can bind multiple chemokines, albeit with variable affinity (Lu et al., 1998). For example, CCL3 and CCL4 both act on CCR1, CCR2, CCR4, and CCR5 receptors that are found on numerous immune cells, including monocytes, T cells, dendritic cells, neutrophils, and macrophages (Barmania & Pepper, 2013; Gilliland et al., 2013; Jing et al., 2003). By acting through the CCR5 receptor, CCL3 and CCL4 recruit $CD4^+$ and $CD8^+$ T cells (Wang et al., 2013). These two chemokines have also been known to increase the infiltration of natural killer cells, a major source of IFNγ (Allen et al., 2017; Megjugorac et al., 2004). However, CCL3 has been demonstrated to additionally promote the

infiltration of neutrophils (Ramos et al., 2005). Again, this ability is not unique to CCL3, as it is shared with both CCL2 (Reichel et al., 2009) and IL-8 (de Oliveira et al., 2013; Gangur et al., 2002). CCL2 binds CCR2 with high affinity, leading to the recruitment of macrophages and monocytes (Deshmane et al., 2009), but it has also been found to enhance the inflammatory response of macrophages (Carson et al., 2017). In this study, HSR and MSR had greater production of CCL2 and inflammatory responses than LSR. Whether this is partially a result of higher serum CCL2 concentrations augmenting the inflammatory responses of specific immune cells in these sheep was not established in this study. The last chemokine measured in this study was CXCL10, that acts through the CXCR3 receptor known to promote differentiation of $CD4^+$ T cells to Th1 (Groom and Luster, 2011) and enhance the migration of activated lymphocytes to sites of inflammation (Bondar et al., 2014). Consistent with this, T lymphocyte infiltration was associated with upregulated CXCL10 in numerous tissues in dogs with endotoxemia (Frangogiannis et al., 2000).

Based on the above-mentioned changes in chemokine concentrations induced by LPS, it is not surprising that changes in immune cell populations were also observed. Neutrophils involved in early inflammation (Butterfield et al., 2006), for example, increased by 4 hrs, whereas lymphocytes and monocytes decreased and eosinophil populations did not change over time. An ovine study utilizing an i.v. LPS challenge, found that lymphocyte populations increased by 2 hrs, then decreased by 4 hrs, and an opposite profile was observed in the neutrophils (Yates et al., 2011). Furthermore, no changes in eosinophil populations were observed. Consistently, for the sheep in the current study, circulating neutrophils were the only subset of leukocytes that increased in number by 4 hrs, and no changes were observed in eosinophil populations. During inflammation there is a rapid mobilization of neutrophils from the bone marrow into the circulation that is

regulated by the cytokine G-CSF and chemokine CXCL12 (Furze and Rankin, 2008); unfortunately these were not monitored in the current study. There is, however, evidence that neutrophil egression from the bone marrow is also stimulated by cortisol. Olnes et al. (2016) for example, demonstrated that exogenous administration of cortisol in humans increased neutrophils and decreased monocyte and lymphocyte numbers in the circulation. Despite no differences in circulatory neutrophil numbers between low and high doses of cortisol, neutrophils were elevated for longer with the high dose. In the present study, this effect would have been missed in the variable stress-responding sheep by measuring at a single timepoint post-LPS challenge. The reduction of monocytes and lymphocytes in the circulation could potentially be explained by elevated CCL2, important for recruiting monocytes to inflamed tissue (Shi and Pamer, 2011), and CXCL10, that increases T cell adhesion to endothelial cells, also facilitating homing to inflamed tissues (Lloyd et al., 1996). Moreover, another study found that activation of T cells in response to systemic LPS challenge in humans was highly selective among T cell subsets. For example, Th1 cells expressing CXCR3 and CCR5 had a pronounced decrease in the circulation in response to LPS that paralleled the increase of their chemokine ligands, CXCL10 and CCL3, respectively, in the circulation (De et al., 2005). Ultimately, chemokine targets are specific to the level of immune cell subsets and how these more specific populations are differentially altered in response to LPS in the variable stress responders remains to be explored.

Not only chemokines, but also cortisol can influence specific immune cell subpopulations. For example, You et al. (2008b) assessed delayed-type hypersensitivity reactions (DTH)—a T cell mediated immune response—to several antigens in High (HCR), Middle (MCR), and Low (LCR) cortisol responding sheep. The HCR and LCR had exacerbated DTH responses compared to the MCR. Because the different test antigens used to elicit the DTH response involve different T cell

subsets, the variable cortisol-responders may have had differences in their Th1 and Th2 populations. Ultimately, the cortisol phenotype was contributing to differential T cell mediated immunity, which would not be reflected by examining general leukocyte populations. Supporting cortisol's influence on specific T cell subsets has also been demonstrated by cortisol-dependant upregulation of CXCR4 on $CD4^+$ and $CD8^+$ T cells (Besedovsky et al., 2014). Upregulation of this chemokine receptor on these T cells consequently redirects them to the bone marrow from the circulation. It could be speculated that the different cortisol production of the variable stress-responders differentially influences their T cell subpopulations. Again, this supports using more refined methodologies, such as flow cytometry, for examining immune cell populations in variable stress-responders in future studies.

The NLR was calculated in this study, as it is frequently used clinically as a measure of subclinical inflammation and to monitor stress. In many animal species, high levels of glucocorticoids correspond to an increased NLR due to the hormone's ability to increase neutrophils and decrease lymphocytes (Davis et al., 2008). Similarly, a higher NLR has been found in alpacas infused with LPS for 96 hrs and during bacterial infections in humans (Naess et al., 2017; Passler et al., 2013). This same phenomenon has also been observed in dogs and cattle in response to transportation stress (Bergeron et al., 2002; Hong et al., 2019). Due to the differences in the cortisol responsiveness of our stress phenotyped sheep, it would be expected that different NLR would exist among the groups—a difference not detected in this study despite an increase at 4 hrs. Again, this warrants more refined cell quantification methods and sampling at more timepoints post-challenge.

4.6 Conclusion

Changes in pro- and anti-inflammatory cytokines, chemokines, and white blood cell populations have functional consequences in regard to immune competence and may influence the resiliency of an animal to stress challenges. Results from this study indicate that variable stress-responding sheep have distinct fever, CCL2 cell recruitment signalling, and pro- and anti-inflammatory cytokine responses during an LPS immune challenge. Overall, HSR had the most pronounced responses, and the LSR had more modest responses. At this time, it is unclear what an optimal phenotype would be for improved stress resilience; a strong, protective immune response is beneficial for recognition and resolution of the stress challenge, but also can lead to tissue damage. Additionally, different stress challenges may elicit different immune response profiles, and it is unclear how the different phenotypes would respond to these other stressors and how their varied immune function would influence long-term health. Because differences were detected among the groups for CCL2, but no differences were observed in immune cell populations, future studies could quantify immune cell subsets using more refined methods and over more timepoints, including the resolution of acute inflammatory stress. Further, to help identify the optimal phenotype in a practical setting, the phenotyped sheep should be evaluated during environmental stress (i.e. heat waves), and whether phenotypes are stable over time should be confirmed. Answering these questions are necessary to justify inclusion of the stress response phenotype into breeding programs.

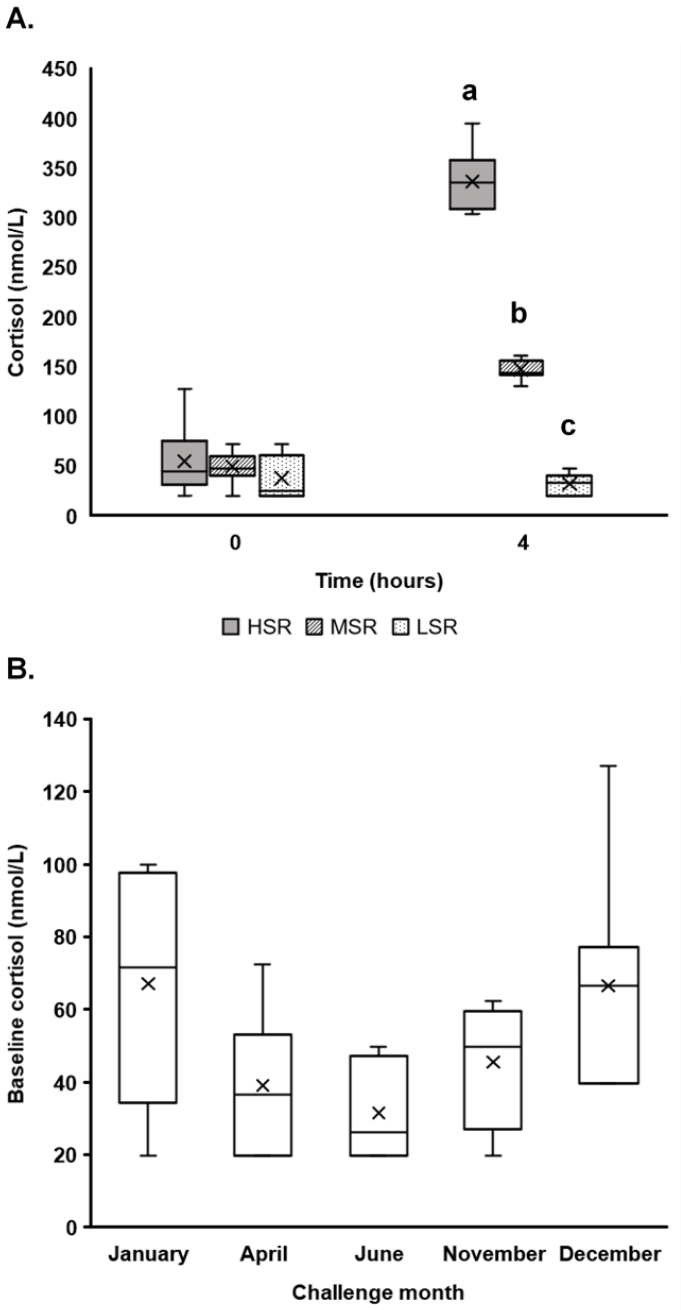

Figure 4.1: Serum cortisol at baseline (0 hr) and 4 hrs after i.v. LPS (400 ng/kg) challenge of HSR (n = 12), MSR (n = 12), and LSR (n = 12) sheep (A) and serum cortisol at baseline for phenotyped sheep (n = 36) according to month of challenge (B). Box-plots were generated from raw data. 'X' represents the mean and the line inside the box represents the median. The letters (a, b, and c) above the plots denote differences between the groups within each time ($P < 0.01$).

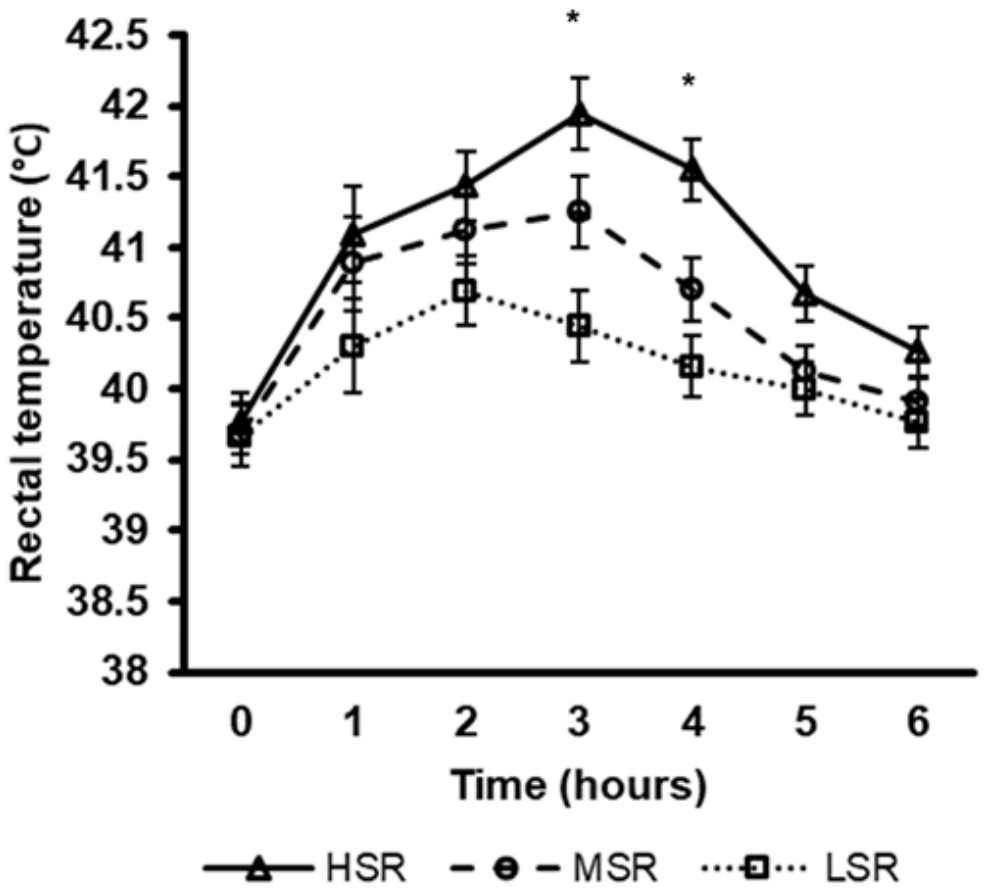

Figure 4.2: Rectal temperature responses in HSR (n = 12), MSR (n = 12), and LSR (n = 12) sheep following i.v. LPS (400 ng/kg) challenge. Temperature is reported as the mean with upper and lower 95% confidence limits at 0, 1, 2, 3, 4, 5, and 6 hr. Distinct temperatures for HSR, MSR, and LSR are denoted by an asterisk (*) representing $P < 0.01$.

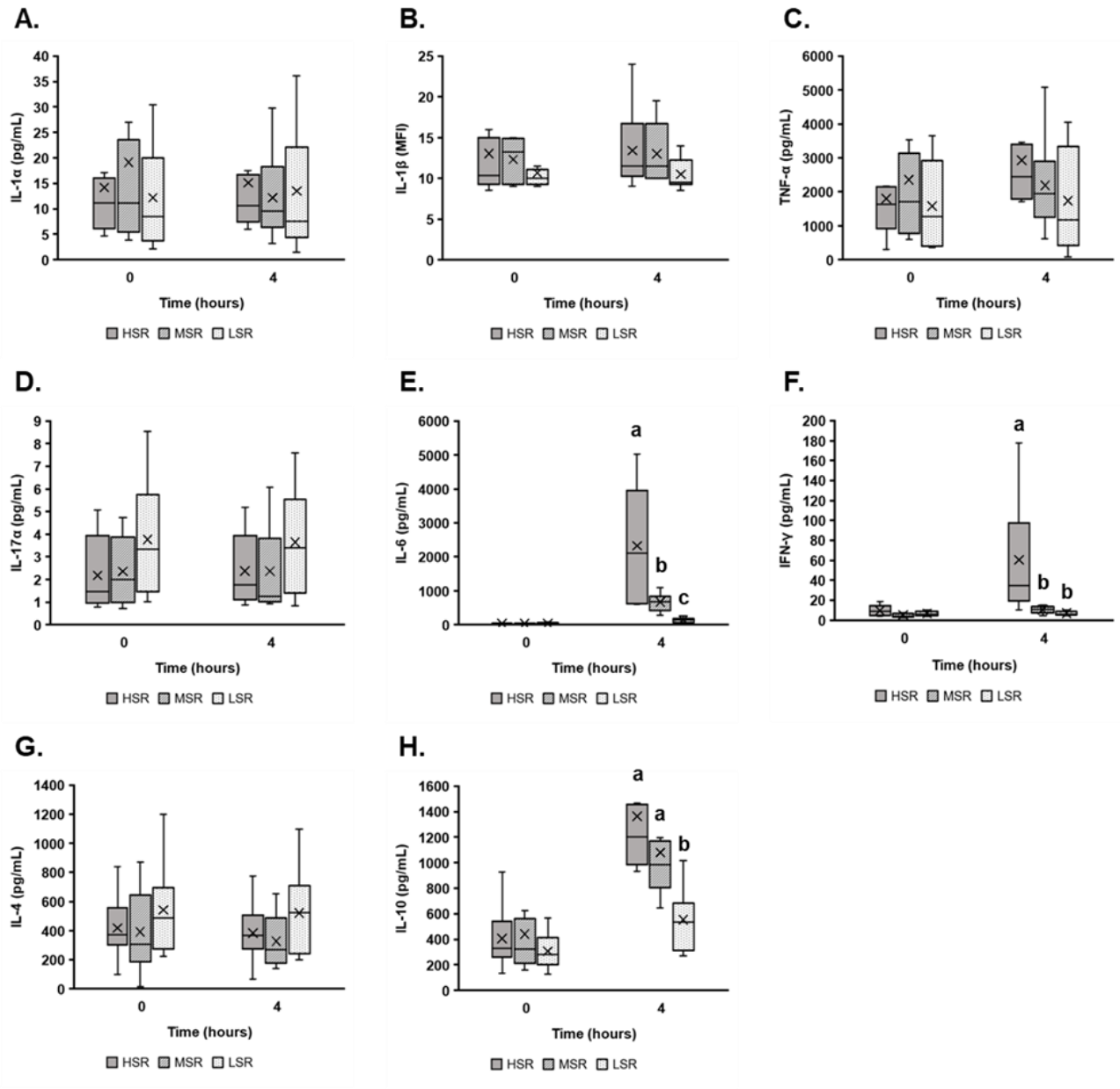

Figure 4.3: Serum IL-1α (A), IL-1β (B), TNF-α (C), IL-17α (D), IL-6 (E), IFN-γ (F), IL-10 (G), and IL-4 (H) at baseline (0 hr) and 4 hrs after i.v. LPS (400 ng/kg) challenge of HSR (n = 8), MSR (n = 8), and LSR (n = 9) sheep. Box-plots were generated from raw data. 'X' represents the mean and the line inside the box represents the median. The letters (a, b, and c) above the plots denote differences between the groups within each time ($P < 0.05$).

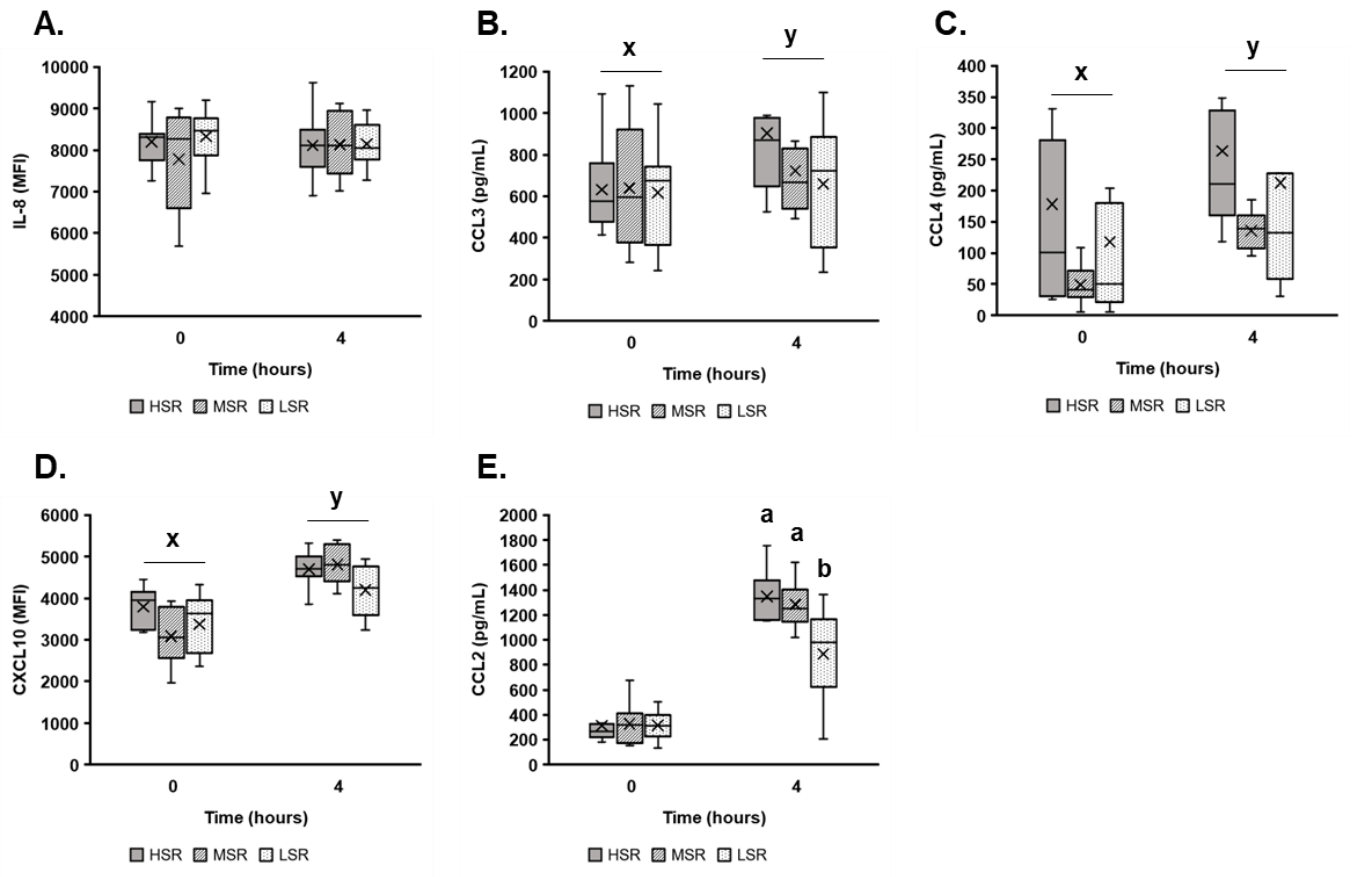

Figure 4.4: Serum IL-8 (A), CCL3 (B), CCL4 (C), CXCL10 (D), and CCL2 (E) at baseline (0 hr) and 4 hrs after i.v. LPS (400 ng/kg) challenge of HSR (n = 8), MSR (n = 8), and LSR (n = 9) sheep. Box-plots were generated from raw data. 'X' represents the mean and the line inside the box represents the median. The letters (a, b, and c) above the plots denote differences between the groups within each time ($P < 0.05$), and 'x' and 'y' denote differences between baseline and 4 hrs ($P < 0.05$).

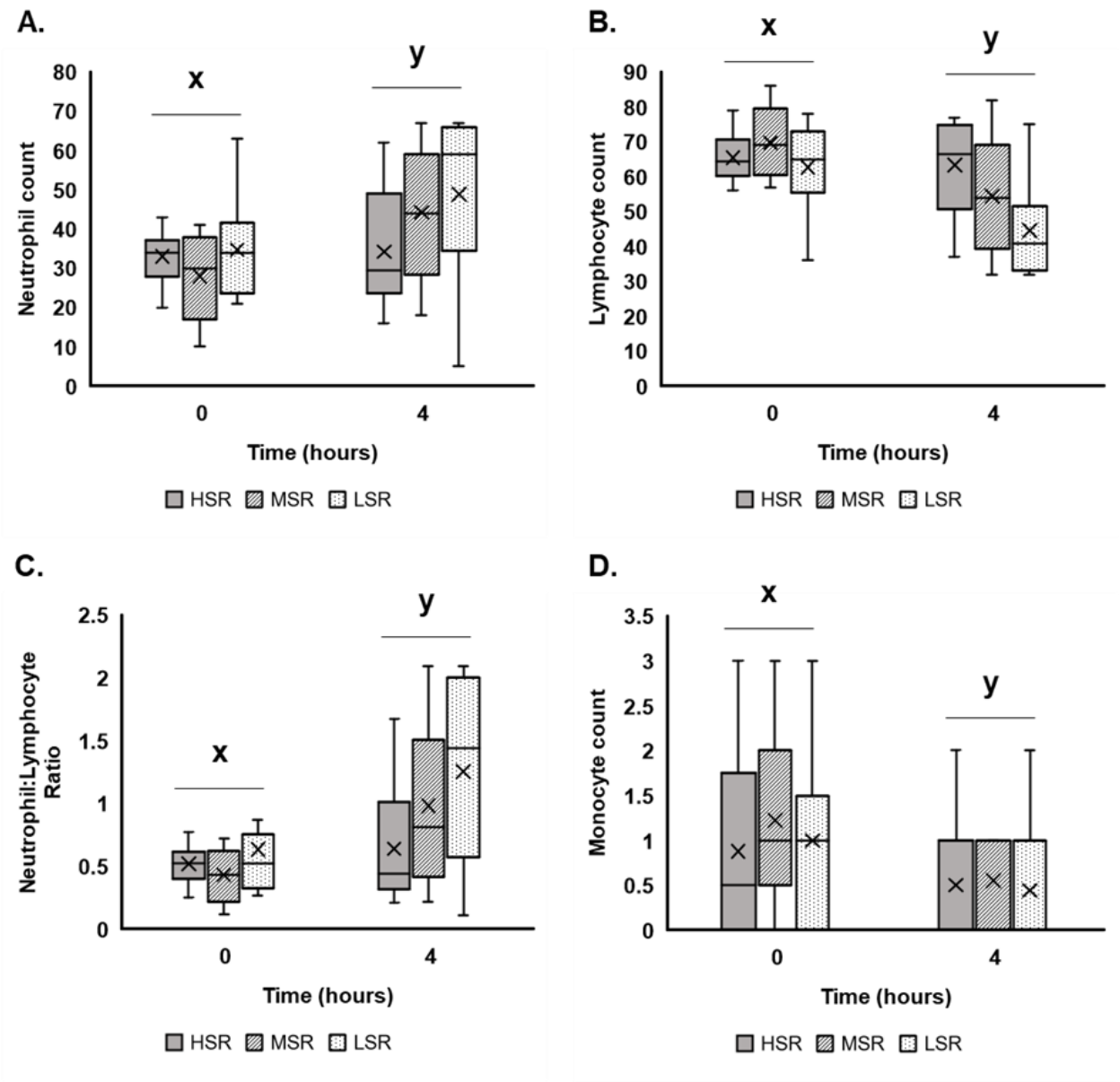

Figure 4.5 Whole blood white blood cell counts for neutrophils (A), lymphocytes (B), NLR (C), and monocytes (D) at baseline (0 hr) and 4 hrs after i.v. LPS (400 ng/kg) challenge of HSR (n = 8), MSR (n = 9), and LSR (n = 9) sheep. Box-plots were generated from raw data. 'X' represents the mean and the line inside the box represents the median. The letters (x and y) above the plots denote differences between baseline and 4 hrs ($P < 0.05$).

Chapter V: General Discussion and Conclusions

5.1 General Discussion

The sustainability of livestock production is in question partly due to the anticipated increases in the severity and variety of stressors resulting from global climate change (Rojas-Downing et al., 2017). Activation of the HPA axis occurs in response to various stressors and the end-product, cortisol, influences numerous cells and tissues, and therefore, many physiological and biological systems of the body. Of these systems affected, is the immune system, which is relevant for combating several of the stressors that are expected to worsen due to climate change; this includes, systemic endotoxin from heat stress-related gut leakage (Atrian and Shahryar, 2012; Vargas and Marino, 2016), disease and parasite challenges (Rojas-Downing et al., 2017), and mycotoxin exposure (Mannaa and Kim, 2017). Since the stress response is a heritable trait (Pant et al., 2016) and has wide-ranging effects on physiology, and particularly the immune system (Karrow, 2006), selecting for this trait could contribute to improvements in the stress resiliency of livestock.

High (HCR) and low cortisol responding (LCR) sheep have been identified by using a systemic LPS challenge and measuring peak cortisol production (You et al., 2008a). The biological processes and pathways activated during this acute inflammatory stress challenge are complex and involve a large array of mediators (Karrow, 2006). The LPS challenge model can therefore be used to more completely understand the immune mediators involved in acute inflammation and how they vary based on an animal's stress response phenotype. Therefore, the overall objective of this thesis was to comprehensively characterize the circulatory mediators involved during LPS-induced acute systemic inflammation in sheep (chapter III) and how the immune response to LPS varies depending on an animal's stress response phenotype (chapter IV).

Chapter III utilized a systemic LPS challenge in sheep to identify relevant immune related stress biomarkers in the blood by measuring pro- and anti-inflammatory cytokines and chemokines, cortisol, and miRNA. All sheep had an acute fever response to LPS challenge, and their serum cortisol increased and resolved within the 6-hr monitoring period. A specific profile of pro- and anti-inflammatory cytokines was induced including TNF-α, IL-6, and IFN-γ (pro-inflammatory), and IL-10 (anti-inflammatory); however, only TNF-α returned to its baseline value by the end of the monitoring period. Surprisingly, concentrations of the pro-inflammatory cytokines IL-1α and IL-1β remained constant over time, in addition to IL-17α and the anti-inflammatory cytokine IL-4. Conversely, all chemokine concentrations increased after LPS challenge, but only CCL3, CCL4, and CXCL10 returned to baseline levels by 6 hrs. Finally, expression of miRNA was assessed, and all were elevated by the end of the monitoring period, but miR-223 and miR-1246 had a more rapid increase in expression than miR-145. Overall, a systemic LPS challenge in the sheep elicited a strong innate immune response that could be monitored by the production and expression of several immune and stress hormone biomarkers in the blood.

LPS is well-known to drive a Th1-type response in humans (Netea et al., 2005), and this seems to be the case in sheep as well, based on the pro- and anti-inflammatory cytokine profile post-LPS challenge in chapter III. Due to some overlap in cytokine production, the most concise definition of a Th1 or Th2 cell is based on secretion of IFN-γ or IL-4. Specifically, Th1 cells produce IFN-γ and Th2 cells produce IL-4 (Spellberg and Edwards, 2001). Type 1 immune responses are considered protective during infection for enhanced phagocytosis and upregulation of MHC class I and II molecules to increase antigen presentation to T cells (Spellberg and Edwards, 2001). Furthermore, the strong fever response in the sheep would help to promote a Th1 cell phenotype (Evans et al., 2015).

The strong chemokine response implies there was a dynamic change in circulating white blood cell populations over the study period. Early neutropenia followed by neutrophilia for example, has been reported following LPS challenge (Wagner and Roth, 1999; Yates et al., 2011). Yates et al. (2011) challenged sheep systemically with LPS and found that neutrophil counts were decreased in the circulation at 2 hrs post-challenge, but by 4 hrs were elevated compared to baseline. Additionally, lymphocytes had an opposite response profile, and together demonstrate the rapid changes in immune cell populations during acute LPS induced inflammation. Similarly, in chapter IV, neutrophils were increased at 4 hrs post-LPS challenge and lymphocytes decreased. Curiously, IL-8 (CXCL8), which is known for recruiting neutrophils, was not elevated in the studies from either chapter III or IV. This suggests that other chemokines or chemoattractants could be regulating neutrophil populations during systemic LPS challenge. For example, CCL3 and CCL2, that responded strongly to LPS in chapter III, promote the infiltration of neutrophils (Ramos et al., 2005; Reichel et al., 2009). Additional chemokines that were not part of the commercial panel used in chapter III can also be involved in neutrophil movement. For example, the chemokines CXCL1 and CXCL2 support neutrophil chemotaxis (Girbl et al., 2018), and granulocyte colony-stimulating factor (G-CSF) and CXCL12 regulate the rapid mobilization of neutrophils into the circulation from bone marrow during inflammation (Furze and Rankin, 2008). Even cortisol has been shown to increase neutrophils and decrease lymphocytes and monocytes in the circulation (Olnes et al., 2016).

Although the changes observed in chapter IV's immune cell counts matched the chemokine and cortisol profile in chapter III, it should be considered that chemokines have a high level of specificity for immune cells, which is largely determined by the expression of numerous chemokine receptors. For example, activation of T cells in response to systemic LPS challenge in

humans was highly selective among T cell subsets. For example, Th1 cells expressing CXCR3 and CCR5 had a pronounced decrease in the circulation in response to LPS that paralleled the increase of their chemokine ligands, CXCL10 and CCL3, respectively, in the circulation (De et al., 2005). Although CXCL10 and CCL3 were measured in chapter III, lymphocyte subsets were not evaluated in chapter IV. In this study, a manual WBC differential technique was used to quantify changes in WBCs, but unfortunately, this only allows for cell counting at a more general phenotypic level and cannot differentiate among immune cell subsets. Ultimately, to get a thorough understanding of how the chemokines measured in this study influence immune cell populations, more detailed cell quantification methods, such as flow cytometry, are required.

Although inflammation is an important component of the immune response to clear bacterial infections, excessive tissue damage can occur if it is not adequately regulated. Cortisol and IL-10 are well-known regulators of inflammation, and in the present studies they consistently responded strongly during LPS-induced inflammation. Additionally, the expression of miR-1246, -223, and -145 were found to be elevated at the end of the monitoring period and could also be involved in regulating inflammation. Although miR-1246 is a well characterized tumour promoter that targets tumour suppressors (Bott et al., 2017; Sakha et al., 2016), it also has been implicated in promoting inflammation. For example, LPS exposure increased miR-1246 expression in pulmonary microvascular endothelial cells (PMVECs) and promoted inflammatory cytokine production and apoptosis that was mediated by its target angiotensin-converting enzyme 2 (ACE2) (Fang et al., 2017). Similarly, miR-223 was upregulated in bovine endometrial epithelial cells after LPS challenge through the NF-κB pathway, but acted as a negative regulator of inflammation by targeting the NLPR3 inflammasome, which contributes to initiating and perpetuating inflammation (Zhao et al., 2018). MiR-145 has also been demonstrated as a negative regulator of

macrophage-induced inflammation, by targeting ADP ribosylation factor 6 (Arf6) and subsequently inhibiting NF-κB signalling and pro-inflammatory cytokine production (Li et al., 2018b). The upregulation of all these miRNA indicate they could be contributing to the fine-tuning and balance of pro- and anti-inflammatory conditions and more detailed examination of miRNA profiles in ovine serum may help to better understand the regulatory role of miRNA during LPS-induced inflammation.

Regarding all the variables measured in chapter III, there are some that would be more useful as biomarkers of inflammation and potentially stress resiliency. The variables to consider are those that responded to LPS challenge and had large variation in production or expression that could aid in a more defined ranking of responses. Potential candidate biomarkers would then be IL-6, TNF-α, IFN-γ, and miR-1246 for pro-inflammatory biomarkers, and cortisol, IL-10, miR-223 and miR-145 for anti-inflammatory biomarkers. The chemokines CCL2 and CCL3 could be considered as well, although it would be beneficial to better understand how they are impacting immune cell populations over the course of acute inflammation. Further, to simplify sampling, it would be beneficial to select a single time point. For example, at 4 hrs post-LPS challenge, cortisol demonstrated the greatest variation, and IL-6, IFN-γ, IL-10, and miR-223 were at or near their peak response and expression. Therefore, this time point would be suitable for further exploring individual variation during stress and acute inflammation.

In chapter IV, the immune response of variable cortisol responding sheep was assessed by measuring the cytokine and chemokine biomarkers from chapter III in HSR, MSR, and LSR sheep that were classified according to their cortisol production post LPS challenge, in addition to immune cell counts. The HSR had the strongest inflammatory response indicated by the most pronounced fever and production of pro-inflammatory cytokines IL-6 and IFN-y. The pro-

inflammatory cytokines from chapter III that did not respond to LPS were also unaltered in this study, but TNF-α, which was elevated previously, was unchanged in this study, due to measuring at 0 and 4 hrs only, when TNF-α had a very transient response peaking at 2 hrs and returning to baselines levels by 4 hrs post-LPS challenge. The HSR also had a stronger anti-inflammatory response than LSR as indicated by the increased production of IL-10. No differences among the variable cortisol responders were detected for the cell counts, even though lymphocyte, monocyte, and neutrophil counts were altered post-LPS challenge. However, following the previously mentioned rationale surrounding the level of specificity that chemokines have on immune cells, exploring immune cell population dynamics in more detail using flow cytometry is warranted. Unfortunately, miRNA could not be assessed as part of this chapter due to a lack of sufficient serum samples. Considering their upregulation during LPS challenge and large variation, these miRNA would be great candidates to follow-up on in future studies with variable stress responding sheep. Despite not being able to evaluate miRNA, accounting for all the other immune-stress biomarkers measured in chapter IV, the stress response phenotype was associated with the immune response in the context of a systemic LPS challenge.

The association of the stress response phenotype and immune function has been briefly explored in HCR and LCR sheep (You et al., 2008a; You et al., 2008b). Cell- and antibody-mediated immune responses were found to be associated with this phenotype (You et al., 2008b), and although there was an increase in production for IL-6, IL-1, and IFN-γ, in addition to a fever response during an LPS challenge, there were no differences detected between HCR and LCR (You 2006; You et al., 2008a; You et al., 2008b). The main discrepancies between the study in chapter IV and You et al. (2008a), You et al. (2008b), and You (2006) studies, are a lack of differences between the groups for IL-6, IFN-γ, and fever response, as well as the increase in IL-

1. This could potentially be from differences in the age of the sheep during LPS challenge and the cortisol concentrations that define the stress/cortisol response phenotype. In this thesis the sheep were 2.5 months old at LPS challenge and classified as HSR (336.2 ±27.9 nmol/L cortisol concentration (mean ± SD mean)), MSR (147.3 ±9.5 nmol/L cortisol concentration), and LSR (32.1 ±10.4 nmol/L cortisol concentration), whereas the sheep in You et al. (2008a), You et al. (2008b), and You (2006) were 6 months old and classified as HCR (512.4±68.2 nmol/L cortisol concentration) and LCR (138.7±46.0 nmol/L cortisol concentration). Despite the LCR and MSR having similar cortisol response ranges and the HCR having a higher cortisol response range than HSR, differences were still detected in chapter IV between the MSR and HSR for IL-6 and fever. Therefore, the reason why differences were detected in chapter IV but not in You et al. (2008a and 2008b) and You (2006) is not due to the differences in the cortisol concentrations that define the groups. Age is the other possibility, but in the study by Hadfield et al. (2018) using 3 to 5-year-old sheep, Suffolk sheep had a stronger cortisol response as well as fever and IL-6 expression compared to Dorset, providing evidence that these parameters are linked. Regarding the discrepancies in IL-1 responses, it is surprising that no change over time was seen in chapters III or IV considering that a study by Skipor et al. (2017), also utilizing a 400 ng/kg LPS dose but in adult sheep, found a significant increase in plasma IL-1β at 3 hrs post-LPS challenge. It is possible that the lack of IL-1β or IL-1α increase in this thesis could be due to the differences in detection kits used. Considering the results on IL-1 responses in the You et al. (2008a) and Skipor et al. (2017) studies and the general paucity of literature on IL-1 responses in sheep during bacterial infection, confirming the IL-1 results in chapter III and IV is warranted.

A strong inflammatory response is important for initiating, perpetuating, and resolving the source of stress, but can also result in excessive tissue damage and chronic inflammation if not

tightly regulated. Although the HSR had the strongest inflammatory response, their anti-inflammatory response (IL-10) was indistinguishable from MSR but was higher than LSR. It is possible that HSR tend to be more pro-inflammatory and can more efficiently clear a bacterial stressor, and MSR more anti-inflammatory and less likely to develop chronic inflammatory pathologies. The minimal, or lack of, cortisol and pro- and anti-inflammatory responses in LSR sheep could indicate a lack of ability to recognize, deal with, and subsequently adapt to, a bacterial stressor. This could result in LSR sheep being more susceptible to Gram-negative bacterial infections. The disproportional pro- and anti-inflammatory responses between the HSR, MSR, and LSR could be indicative of variable capacity to handle different stress challenges and therefore resiliency.

5.2 Potential limitations

In chapter III, a mock challenged group of sheep was not used. A concern that may arise with this methodology, is not being able to compare challenged to mock challenged sheep to demonstrate that all sheep responded to the LPS challenge. However, a study by Kabaroff et al. (2006) that used the same LPS challenge model in sheep and also included a mock challenged group using phosphate buffered saline, found that temperature and IL-6 concentrations did not change over time in the mock challenged animals. Also, since several of the biomarkers measured in chapter III follow a circadian pattern, it could be that changes in their circulating levels are attributed to circadian regulation rather than the LPS challenge. However, the circadian pattern of cortisol and cytokines in the blood follows a peak within an hour of waking and in the early morning, respectively, before dropping (Elverson and Wilson, 2005; Yoshida et al., 2014). Consistently, in the Kabaroff et al. (2006) study, where sheep were challenged in the morning,

cortisol concentrations dropped in the mock challenged sheep, corresponding to cortisol's circadian dynamic. Further, all LPS challenges conducted in this thesis also begun at 8 am, so considering the increases seen in the responding biomarkers, any circadian effects would have been over-ridden by the LPS challenge. Overall, the changes in the biomarkers in chapter III can be confidently considered as a result of the LPS challenge.

5.3 Future research

This thesis revealed a panel of immune and stress biomarkers that are induced during acute inflammation and provided evidence that the stress response phenotype is associated with immune function in sheep. More research however, needs to be done to further refine important biomarkers of inflammatory stress and most importantly, how, or if, the altered immune function in variable stress responders reflects stress resiliency on the farm. This will contribute to identifying an optimal stress response phenotype.

Despite the comprehensive assessment of blood biomarkers in sheep during LPS challenge in chapter III and IV, there are still many more biomarkers that could be assessed. The three miRNA examined in chapter III for example, could be expanded to a larger panel since the miRNA transcriptome of sheep includes nearly 2000 unique miRNA (Zhang et al., 2013). It would also be prudent to follow-up with assessing the expression of miR-145, -223, and -1246 during acute inflammation in the variable stress responding sheep since they had very high levels of variation and could be important regulators of inflammation. Additionally, flow cytometry could be utilized to achieve better resolution of dynamic shifts in immune cell populations during LPS challenge.

To justify the inclusion of the stress response phenotype into breeding programs, two outstanding questions need to be answered. First, it is clear the stress response phenotype is

associated to immune function during stress, but does this affect resiliency? This question needs to be explored in a field setting by evaluating how the variable stress responding sheep respond and overcome different stressors, like heat, disease, and parasite challenges. Borkowski et al. (2018) assessed the relationship between the stress response phenotype and gastrointestinal nematode (GIN) resistance but did not find differences between HSR and MSR for either fecal egg counts, or GIN burden at the end of the grazing season; but LSR were not assessed in their study. In chapter IV, the greatest differences in immune and stress biomarkers were between HSR and LSR. Further, sometimes HSR and MSR had no distinguishable differences for some biomarkers, thus, including the range of phenotypes in future research is advised.

The ability of an animal to mount a protective immune response to vaccination can also improve resiliency, and some research suggests that vaccination could also be influenced by cortisol reactivity or the stress response phenotype. A study by Phillips et al. (2005) found that young adults who had a low cortisol response to acute mental stress also had an attenuated antibody response 5 months after influenza vaccination. You et al. (2008b) also evaluated HCR, MCR, and LCR sheep primary and secondary serum IgG responses to ovalbumin immunization. Interestingly, the primary IgG response was greater in MCR compared to HCR and LCR sheep, but the secondary response was not different among the groups. Since there appears to be a relationship between the cortisol response phenotype and antibody responses to vaccination, investigating the stress response phenotype in sheep to other relevant immunizations should be explored further.

Second, it is important to assess how the stress response phenotype influences the long-term health and productive capacity of the sheep (i.e. trade-offs between health and production). This would entail evaluating and performing a cost-benefit analysis on various health, functional, and productive traits including longevity, absence of disease, fertility, milk production, carcass

quality, among others. It has been demonstrated in rams that produce higher amounts of cortisol to an injection of ACTH also have worse feed efficiency (Knott et al., 2008), but in a Merino sheep breeding program, positive selection for multiple rearing ability, including increased lamb survival and number of lambs reared, also inadvertently resulted in the selection for increased HPA axis responsiveness (Hough et al., 2013b). Considering the differential effect cortisol reactivity appears to have on different animal traits, it will be important to assess the stress response phenotype in the context of different sheep production systems that emphasize different attributes.

Ultimately this thesis provides evidence of differential immune regulation in variable stress responding sheep by monitoring key acute inflammatory biomarkers. This supports exploring this phenotype further to assess its relevancy to resilience on-farm and in different production systems. This would aid in revealing an optimal stress response phenotype and potential inclusion into breeding programs to help achieve sustainable livestock production.

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