ABSTRACT

Identifying Hepatic Genes Regulating the Ovine Response to Gastrointestinal Nematodes

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Gastrointestinal nematode (GIN) infections are considered the most important disease of grazing sheep. Due to increasing anthelmintic resistance, chemical control alone is inadequate. Resistance to GINs is a heritable trait, and many breeds of sheep throughout the world have naturally developed enhanced resistance. The study of the transcriptome from GIN-exposed and unexposed sheep using RNA-Sequencing technology can provide measurements of transcript levels associated with the host response to the infection. The objective of this study was to examine liver transcriptome from high and medium stress responding tracer sheep (naturally GIN-exposed) and control sheep (unexposed) in order to identify key regulator genes and biological processes associated with GIN infection.
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LIST OF ABBREVIATIONS

ABHD1: Abhydrolase Domain Containing 1
ACTH: adrenocorticotropic hormone
AKR1C3/4: Aldo-Keto Reductase Family 1 Member C3/4
APP: acute-phase protein
APR: acute-phase response
AR: anthelmintic resistance
bp: base pair
BP: Biological process
B2M: Beta-2-microglobulin
CC: Cellular component
CD74: CD 74 Molecule
CHP2: Calcineurin Like EF-Hand Protein 2
CREB3L3: CAMP Responsive Element Binding Protein 3 Like 3
CTSS: Cathepsin S
CXCL9: C-X-C Motif Chemokine Ligand 9
CYP51A1: Cytochrome P450 Family 51 Subfamily A Member 1
DC: dendritic cell
DEG: differentially expressed genes
EEF1A1: Eukaryotic Translation Elongation Factor 1 Alpha 1
ELOVL5: Elongation of Very Long Chain Fatty Acids Protein 5
FABP1: Fatty Acid Binding Protein 1
FC: Fold change
FDR: False Discovery Rate
FEC: fecal egg count
FECRT: Fecal Egg Count Reduction Test
FGF21: Fibroblast Growth Factor 21
GIN: Gastrointestinal nematode
GIT: Gastrointestinal tract
GO: Gene Ontology
GSTA1: Glutathione S-Transferase Alpha 1
H: High
HMOX1: Heme Oxygenase 1
HP: Haptoglobin
HPAA: hypothalamic–pituitary–adrenal axis
HSD17B7: 17-beta Hydroxysteroid Dehydrogenase Type 7
IEC: intestinal epithelial cell
IFI27: Interferon Alpha-inducible Protein 27
IFNγ: interferon gamma
Ig: immunoglobulin
IL: interleukin
INSIG: Insulin-induced Gene
IPA: Ingenuity Pathway Analysis
ISG15: ISG15 Ubiquitin-Like Modifier
L: Low
LDA: Larval Development Assay
LPS: Lipopolysaccharide
M: Medium
MF: Molecular function
MHC: major histocompatibility complex
NK: natural killer
OLA-I: Ovis aries OLA class I histocompatibility antigen, alpha chain BL3-7-like I
ORM1: Alpha-1-acid Glycoprotein
PAMP: pathogen-associated molecular patterns
PCA: principle component analysis
PCV: packed cell volume
PLAC8: Placenta-specific Gene 8 Protein
PLA2G7: Phospholipase A2 Group VII
PRR: pattern recognition receptors
PSMB9: Proteasome Subunit Beta 9
QTL: Quantitative Trait Loci
RNA-Seq: RNA Sequencing
RPKM: reads per kilo base per million mapped reads
SCD: Stearoyl-CoA Desaturase
SD: standard deviation
Th1: T-helper 1 cell
Th2: T-helper 2 cell
THRSP: Thyroid Hormone Responsive
TLR: Toll-like receptors
Treg: regulatory T cells
TXK: Tyrosine-protein kinase
Chapter I: Introduction
1.1 Introduction

Gastrointestinal nematode (GIN) infection is a common cause of morbidity and mortality in grazing sheep. Due to increasing anthelmintic resistance, chemical treatment alone is inadequate to control these infections. Understanding the genetics of the host response to GINs will be imperative to developing alternative strategies for Ontario sheep producers, which could include genetically selecting for animals more adept at managing GIN infection. This chapter will review the problem of GINs in the Ontario sheep industry, genetic resistance of sheep to GINs, and the ovine immune and stress responses to parasites.

1.1.1 Gastrointestinal nematode life cycle

The same life cycle generally applies to the typical GINs of Ontario sheep: Teladorsagia, Trichostrongylus and Haemonchus. For these three parasites, there is no intermediate host. In the GIN life cycle, the adult female parasites produce eggs that are passed in the manure of the host. Development occurs within the feces: the eggs hatch to larval stage L1 larvae (free-living), and then moult to larval stage L2 larvae (also free-living), these then moult to larval stage L3 larvae. The L3 are the infectious stage to sheep; the larvae migrate from the fecal matter into the forage where they can be ingested by sheep. The pre-patent period is the period from ingestion of the infective larval stage L3, to when the eggs are detected in feces, usually 15 to 21 days for most GINs. Once inside the gastrointestinal tract (GIT), the L3 larvae moult to the parasitic and feeding L4 stage larvae and subsequently moult to immature and then mature egg-laying adults (Zajac, 2006).
The timing of development from egg expulsion to L3 stage depends on the environmental conditions and species. With *Haemonchus contortus* (*H. contortus*), in hot summer weather the development of larvae can be as little as 5 days, whereas in the cooler weather of spring or fall, the development can take several months to the L3 stage; however the typical development time is 3 weeks (Zajac, 2006). During unfavourable environmental conditions, for example cold temperatures (no development occurs at <10°C), the L4 larvae will arrest development (hypobiosis) in the host and will not feed or develop into adults until conditions improve (Westers et al., 2016b).

### 1.1.2 Ovine gastrointestinal nematodes in Ontario

Gastrointestinal nematode infection is considered the most important disease of grazing sheep worldwide, causing a decrease in production traits such as growth, weight loss, diarrhea, anemia, and hypoproteinemia sometimes resulting in death (Falzon et al., 2013). The economic impact of GINs is related to increased drug and animal management costs, production losses – associated with decreased live carcass and fleece weight, inefficient feed conversion rates, and reduced lamb survival (Venturina et al., 2013).

In Ontario, the predominant parasites found in grazing sheep are *Teladorsagia*, *Trichostrongylus* and *Haemonchus*. Infection levels in lambs tend to peak in late July and August, and decline in the fall (Falzon et al., 2013). In adult ewes, the highest fecal egg count (**FEC**)s are seen in the spring (Mederos et al., 2010). On pasture, dry weather delays a rise in levels of free-living larvae (L1 and L2 stages), while wet weather increases these levels. In temperate colder climates such as Ontario, nearly all newly developed L4 larvae undergo hypobiosis starting mid fall, when conditions are not
ideal for survival of eggs, L1 and L2 on pasture and subsequent ability to infect the ovine host (Westers et al., 2016b). Lambs start to show immune responses at 2 to 3 months of age (Bishop et al., 1996; McRae et al., 2015), and after subsequent exposure to larval challenges, they develop a significant immune response that is fully developed and therefore protective by 10 to 12 months of age (Falzon et al., 2013; Seaton et al., 1989).

Among the GINs found in Ontario sheep, *H. contortus* is considered the most pathogenic, attaching to the abomasum and ingesting blood, causing anemia, edema, anorexia and death (Taylor et al., 2007; Westers et al., 2016b). In Ontario, there is a dramatic increase in *H. contortus* FEC during the summer months, with severe haemonchosis outbreaks associated with the periparturient and lactation periods in ewes, and during the grazing season in lambs (Westers et al., 2016b). *H. contortus* over-winter as hypobiotic larvae within their host, and periparturient ewes have been identified as the primary source of pasture contamination of *H. contortus* the following spring, when arrested larvae resume development due to improved environmental conditions for survival (Waller et al., 2006). The rise in egg counts during this time is also due to downregulated immunity in the periparturient animals, which results in increased survival of existing parasites and their egg production, and additionally increases susceptibility to further infection (Zajac, 2006). Falzon et al. (2014) investigated the over-wintering of free-living GIN larvae of sheep in Ontario; finding that *H. contortus* L3 larvae do not survive well on pasture over winter, while *Teladorsagia*, *Trichostrongylus* and *Nematodirus* do survive well. This has also been confirmed by van Dijk et al. (2010), who reported that -3°C was the lowest average temperature at which
*H. contortus* larvae could survive, while both *Teladorsagia circumcinta* (*T. circumcinta*) and *Trichostrongylus* could both survive at -10°C. With increasing average temperatures over winter associated with climate change (Rose et al., 2016), there is a concern that *H. contortus* may soon be able to survive on pasture over winter in Northern climates, increasing pasture contamination and subsequently host parasite load the following season.

### 1.1.3 Diagnosing gastrointestinal nematode infection

Clinical symptoms of GIN infection vary depending on severity of infection and species of parasite; symptoms can include weight loss, loss of appetite, and more severely, anemia, diarrhea and severe protein loss. GIN infection can also have indirect effects on metabolism, leading to an increased susceptibility to other pathogens (Falzon et al., 2013; Zajac, 2006).

The most common test for diagnosing GIN infection is a quantitative FEC, such as the modified McMaster count; this can be used to evaluate the efficacy of parasite control programs, monitor levels of pasture contamination and assess anthelmintic efficacy (Zajac, 2006). Other indicators of parasite burdens include the use of the FAMACHA© scoring that evaluates conjunctival mucous membrane colour, dag scores that evaluates fecal soiling of the breech, body condition scores and weight gain (Van Wyk & Bath, 2002; Westers et al., 2016b). FAMACHA© scores are used as a surrogate indicator of anemia in sheep, detected using the colour of the conjunctivae of the lower eyelid (Westers et al., 2016b). Dag scores are a surrogate measure of fecal consistency, with diarrhea causing wool contamination of the breech and may be an indicator of the severity of infection caused by internal parasites (Westers et al., 2016b).
These diagnostic tools are useful in determining the severity of clinical infection, and therefore when and which animals should be treated. They can also be used to select animals that may be more resistant to parasites; for example, animals that have consistently low FECs compared to others in the group (Zajac, 2006).

1.1.4 Anthelmintic resistance

Control of *H. contortus* is essential to ensure animal health and welfare and to reduce economic losses. Anthelmintics have been the main approach to controlling this problem, but GINs, such as *H. contortus*, are developing resistance to these drugs (Falzon et al., 2014; Westers et al., 2016a). Anthelmintic resistance (AR) is defined as the “heritable ability of the parasite to tolerate a normally effective dose of the anthelmintic” (Abbott et al., 2009), and when sufficiently prevalent in a parasite population, AR will result in treatment failure (Falzon et al., 2013).

The Fecal Egg Count Reduction Test (FECRT) is the standard test to determine AR in field conditions. This provides an indirect measurement of anthelmintic efficacy by determining the reduction in FECs after treatment (Falzon et al., 2013). The World Association for the Advancement of Veterinary Parasitology defines AR as a FECRT score of <95% and with the lower confidence interval >90% (Falzon et al., 2013).

An alternative test to determine AR is the Larval Development Assay (LDA), which is based on culturing a known number of GIN eggs in the presence of different anthelmintics, often at different concentrations (Taylor et al., 2002). This method is used less frequently, as the methodology requires a high level of technical expertise and some LDAs are unable to reliably detect resistance to avermectins (Kaplan & Vidyashankar, 2012; Taylor et al., 2002).
The first case of AR in Canada was identified in a sheep flock in Ontario, in 2007 (Glauser et al., 2007). More recent research in Ontario has determined widespread resistance of GINs to both ivermectin (macrocyclic lactone class) and fenbendazole (benzimidazole class) (Falzon et al., 2014; Westers et al., 2016a). Investigating an anthelmintic for sheep that had not yet been used in Canada, Westers et al. (2016a), determined that the efficacy of closantel (Flukiver® 55 oral suspension, Elanco Animal Health) against ivermectin and fenbendazole-resistant *H. contortus* infections was 99%-100% over a two-year study period. Although this new anthelmintic offered a promising solution to parasite management, a study by Oliveira et al. (2017) has recently determined AR to this drug in sheep in Brazil; as misuse of any anthelmintic can quickly lead to AR developing in single class anthelmintics. They also looked at the most recent anthelmintic commercially available for chemical control of GINs in sheep in Brazil: monepantel (Zolvix®, Novartis), and determined this drug was ineffective in 18% of tested flocks, confirming AR can be established very shortly after introduction into the market (Oliveira et al., 2017). Similarly, Mederos et al. (2014) showed monepantel resistance developed on a research farm after 2 years. In January 2018, Zoetis® released a new parasite control available for use in Canada; STARTECT™, which is a dual oral anthelmintic product containing abamectin and derquantel. No cases of AR to this product have been reported yet.

1.1.5 Managing anthelmintic resistance

Anthelmintic resistance can occur when all animals in the flock are repeatedly treated while grazing contaminated pasture, thus selecting overtime for resistant parasites. It can also occur with fewer treatments if there is a low level of refugia
(untreated populations of parasites) in the flock, i.e. low levels of pasture contamination and few to no infected animals excluded from treatment. In Ontario, because *H. contortus* L3 overwinters poorly, this can happen with that parasite when all sheep are treated while housed in the winter. The only *H. contortus* that remain in the flock are resistant parasites in the sheep that survive treatment to contaminate the pasture the following grazing season (Westers et al., 2016a). Therefore, anthelmintic use should be part of a sustainable integrated parasite management program that must include management practices that will delay the onset of AR, especially when introducing new anthelmintics into the flock. One of these practices is targeted selective treatment, which maintains refugia by treating only the animals with high parasite burdens in a flock. This selective treatment can delay the development of AR by maintaining populations of parasites that have not been exposed to the anthelmintic, while limiting disease within the flock (Leathwick et al., 2008; Westers et al., 2016b). Indicators of these high parasite burdens include FECs, FAMACHA© scores, body condition scores and weight gain (Van Wyk & Bath, 2002; Westers et al., 2016b). Particularly with *H. contortus* infection, the FAMACHA© score has been shown to be significantly associated with FEC (p=0.002) (Westers et al., 2016b), and therefore a viable indicator for targeted selective treatment on farms where that is the predominant parasite. Proper and limited use of anthelmintics will assist in the delay of AR, with targeted selective treatment being an effective part of a sustainable integrated parasite management program.
1.2 Genetic resistance of sheep to gastrointestinal nematodes

Because of increasing AR and consumer concerns of chemical-residues in food products, alternatives to anthelmintics to control GIN infections must be explored. Resistance to GINs is a heritable trait, and many breeds of sheep throughout the world have developed enhanced responses to manage them, therefore selecting for animals that are considered genetically resistant to GIN infections may be a strategy to decrease these infections.

Resistance to GINs is the ability of the host to mount an immune response that limits the establishment of the parasite and/or subsequent development of infection (Sweeney et al., 2016). Resistance includes both passive and active mechanisms to accomplish this. Passive resistance utilizes physical or chemical barriers (for example cuticle or integument) to deny the parasite entry into the body, or an altered pH of the gastrointestinal tract, providing an inadequate environment for parasite development (Saddiqi et al., 2011). Active resistance entails the innate and or adaptive immune responses produced in response to infection (Saddiqi et al., 2011). Resistance requires gene networks that are mainly associated with the innate immune system, supporting the GIT barrier to GINs, along with factors that elicit rapid inflammatory responses to accelerate pathogen clearance. Subsequent infections will then activate the acquired immune response, which then reduces parasite burden by reducing worm establishment, increasing adult worm mortality and/or reducing adult egg production (McManus et al., 2014; Sweeney et al., 2016). By selecting animals for this resistant trait, producers can limit anthelmintic use and more safely utilize pasture as a nutritional
source, which in turn will lower production costs, as well as improve the overall health and welfare of sheep.

Resistance differs from resilience, which is the ability of the host to maintain homeostasis despite the presence of replicating pathogens, while also limiting pathology (Sweeney et al., 2016). Resilience requires gene networks that suppress the immune response to parasite infection and/or prevent parasite-mediated toxicity, is associated with tissue repair and remodeling, and is typically measured by evaluating the individual’s performance relative to parasite burden (Sweeney et al., 2016). While resilience will maintain or increase the parasite load in the pasture, resistance will reduce the pasture contamination by decreasing the number of GnN eggs passed into the environment (Saddiqi et al., 2011). As such, selecting for resistance, rather than resilience, will be of more benefit to grazing sheep populations.

There have been reported studies of variations in resistance among breeds for many years. Gray (1991) and Baker et al. (1992) reviewed published studies on these variations, with reports beginning in the mid-1930s. The genetic resistance of breeds to *H. contortus* infection has been extensively studied in United States and Europe post World War II. The U.S. has found resistance associated with Florida Native, Saint Croix, and Gulf Coast Native breeds, compared to susceptible breeds of Suffolk and Dorper (Bradley et al., 1973; Burke & Miller, 2004; Miller et al., 1998). Similarly, studies out of Germany also found Suffolk to also be susceptible, along with Nill and Rhon breeds (Gauly et al., 2002; Gauly & Erhardt, 2001). A study by Kemper et al. (2010) found variation in resistance between individuals within the same breed - finding both susceptible and resistant Merino sheep. The results from these studies used
parameters of FEC, worm burden and/or packed cell volume (PCV), to determine resistance or susceptibility.

Determining resistance has been a challenge due to the extensive mechanisms of host responses to GINs. As a result, several different traits have been explored to determine their association with resistance to GIN infections. FEC is often used as an indicator trait of resistance, with a heritability of approximately 0.2 to 0.3, but this varies by breed, age, level of parasite challenge and the particular parasite being investigated (Karlsson & Greeff, 2012). Disadvantages of selection based on FEC include day-to-day variation, difficulty in determining parasite species, relying on the assumption that all animals are exposed to the same level of infection, gastrointestinal disturbances resulting in consequences for FEC interpretation, and variable correlated responses in production traits (Hunt et al., 2013; Sweeney et al., 2016). Bishop (2012) therefore recommended that FEC should not be the only measure when quantifying resistance. Alternative indicator traits of resistance include level of eosinophilia, and level of antibodies such as immunoglobulin (Ig) A, IgG and IgM. Haematological and biochemical measures of the impact of infection such as red blood cell parameters, gastrin, pepsinogen and fructosamine concentrations can also be used (McManus et al., 2014). Measuring anemia may provide a way to select for resistance to infection with *H. contortus* and other blood-feeding parasites; anemia can be measured by PCV (Saddiqi et al., 2011), and FAMACHA© scores (Westers et al., 2016b). Despite variation in heritability estimates for these mentioned phenotypes, it has been established that resistance to GIN is moderately heritable (Bishop, 2012), making genetic selection based on resistance a viable candidate for control of GIN infections.
Advances in genomic technologies have provided another strategy to select animals more resistant to GIN infection by identifying regions in the sheep genome that have undergone selection. This can be done using the SNP50 BeadChip which provides 54,241 SNPs across the sheep genome. This can allow for the identification of genes or genetic markers that have a significant association to GIN parasite resistance, accelerating the genetic improvement of GIN resistance through marker-assisted selection (or more likely genomic selection) (Benavides et al., 2016; McRae et al., 2014). Selection using these molecular markers, as opposed to phenotypic traits, will allow for simplified and more immediate selection, because there is no need for previous exposure to GIN to identify variation in responses to infection (McRae et al., 2016). Candidate genes through DNA analyses have been identified, with many studies focusing on chromosomes 3 and 20, which contain interferon gamma (IFNγ) and the major histocompatibility complex (MHC) region; all of which have alleles associated with resistance to GINs (Ahmed et al., 2015). However, the genetic control of parasite resistance in sheep remains a complex trait that is influenced by many genes with small effects (Atlija et al., 2016; Sweeney et al. 2016).

1.3 Ovine immune response to gastrointestinal nematodes

We have seen that resistance to GIN infection is established by interactions between the innate and acquired immune systems, although it can be noted that these immune mechanisms are still not fully understood (Saddiqi et al., 2010). It has been proposed that GIN resistance involves a balance between mounting an effective
immune response against invading GINs without overreacting, which can lead to chronic inflammation and subsequent tissue damage (Karrow et al., 2014).

1.3.1 Initiation of host defense to GINs

Physical barriers to GINs begin with the layer of mucus on the inner surface of the GIT. This mucus consists of mucin and various defensins and antimicrobial molecules (including natural IgM and IgA), along with pro-inflammatory molecules. This is the first-line of innate defense against pathogens in the GIT. Increased mucus production and inhibitory substances, such as histamine, in the mucus are also observed during the development of immunity to GIN (McRae et al., 2015). The host immune response to parasitic infection is also initiated when intestinal epithelial cell (IEC)s come into contact with the larvae and subsequently release cytokines and antimicrobial peptides. These IECs activate innate immune cells, such as dendritic cells (DC)s, macrophages, natural killer (NK) cells and mast cells (Karrow et al., 2014).

Pattern recognition receptors (PRR)s such as the Toll-like receptors (TLR)s family are expressed by many cell types, such as those cells on mucosal surfaces, and they work to identify GIN pathogen-associated molecular patterns (PAMP)s. Identification of GIN PAMPs by PRRs results in the initiation of an innate inflammatory response, which is characterized by the induction of cytokines and other signals that activate the adaptive immune system (Hansen et al., 2011; McRae et al., 2015). TLR genes, specifically TLR 2,4, and 9, have been found to be more highly expressed in the gut mucosa of genetically resistant sheep after a GIN challenge (Ingham et al., 2008), indicating they are likely an important player in the immunological host defense against GINs and may be used as an indicator of more resistant sheep.
1.3.2 Innate immune cells in host defense to GINs

Mast cells are inflammatory cells that respond directly to pathogens and also send signals to modulate the innate and adaptive immune responses (McRae et al., 2015). Activation of these mast cells is through Fc-receptor-IgE, or by interacting directly with PAMPs through PRRs. Mast cells work in GIN infections by releasing inflammatory mediators upon degranulation into infected tissues of the GIT. These mediators include histamine, leukotrienes and proteases, which result in smooth muscle contraction, increased vascular permeability and local blood flow, and enhanced mucus secretion in the GIT (McRae et al., 2015; Urb & Sheppard, 2012). During GIN infection, mast cells also produce Th2 cytokines IL-13, 4 and 5, along with chemotactic factors which recruit other cells such as eosinophils, NK cells and neutrophils to site of infection (Huntley et al., 1995).

After activation during GIN infection, eosinophils help to regulate the immune response, and resist parasitic invasion through degranulation and release of eosinophil secondary granule proteins, resulting in the damage and killing of larvae (Balic et al., 2006).

Macrophages are large, phagocytic leukocytes, which are activated through TLRs, and cytokines IFN-γ, interleukin (IL)-4 and IL-13 (McRae et al., 2015). Macrophages recognize and remove invading pathogens by processing and presenting these antigens through MHC molecules to T cells (Calcagni & Elenkov, 2006). During nematode infection, these macrophages work to regulate the immune response, heal damaged tissue, and prevent establishment of larvae (Bowdridge et al., 2015; McRae et al., 2015).
1.3.3 Acquired immune response in host defense against GINs

DCs and macrophages induce and maintain a Th2 acquired immune response by activating T helper-cells and inducing the differentiation of B-cells into plasma cells, which subsequently drive the production of antigen-specific antibody isotypes IgA and IgE (Karrow et al., 2014).

Immunoglobulin A is actively secreted across the mucosal epithelium of the abomasum and can control larval colonization and development, as well as egg production by specific binding to both larvae and adults (Venturina et al., 2013). It has also been proposed that IgA inhibits larval establishment by triggering eosinophil degranulation (Abu-Ghazaleh et al., 1989; Venturina et al., 2013), and also inactivates metabolic enzymes that results in feed-suppression by the host, which subsequently reduces adult worm length and fecundity (Craig et al., 2007; Stear et al., 2004). In support of IgA activity in reducing GIN infection, a negative correlation has been seen between serum IgA antibody levels and FEC (Beraldi et al., 2008).

Immunoglobulin E also plays a major role in parasite expulsion, with proposed mechanisms being through a Type 1 hypersensitivity reaction, mediated by mast cell proliferation and degranulation of IgE-sensitized mast cells. (Stear et al., 2004). Subsequent release of vasoactive mediators and cytokines leads to blood vessel and gut muscle contraction, increased mucus production, and an increase of interlectins, which may block larval colonization and development. IgE levels have also been seen to be inversely correlated with FEC (Huntley et al., 1995; Murphy et al., 2010; Venturina et al., 2013).
The Th2 response results in an increase of cytokines such as IL-4, 5, 9, 10 and 13. IL-4 plays an important role in the immunological control of GIN infection, as it largely promotes the maturation of naïve CD4+ T cells into the Th2 phenotype, promotes Ig class-switching from IgM to IgE and IgA, while also stimulating mast cell maturation and proliferation (Finkelman et al., 2004; Venturina et al., 2013). IL-13 stimulates IgE class switching, and enhances worm expulsion by increasing mucosal permeability, mucus production, and muscle contraction (Madden et al., 2002; Wynn, 2003). IL-10 has been shown to be an important immunomodulatory cytokine, and controls excessive inflammation at sites of infection and has been seen in higher levels in resistant animals (Li et al., 2006; Saddiqi et al., 2011). During GIN infection, regulatory T cells (Treg) are also promoted to regulate intestinal inflammation and the antibody-mediated immune response (Karrow et al., 2014).

### 1.3.4 Other host defense mechanisms against GINs

Along with the mechanisms mentioned, there are additional responses to GINs that play a role in pathogen clearance. Particularly with *H. contortus* infection, control over bleeding and blood vessel injury is of vast importance, as clotting at the infection site can severely reduce the blood supply to adult parasites, impairing parasite feeding and survival in the host, in addition to controlling host anemia (Benavides et al., 2016). GIN expulsion mechanisms remain unclear; however, it has been proposed that larvae may be damaged by the effector cells and molecules of the immune system (Saddiqi et al., 2011). Another hypothesis is the worms could be damaged by the physiological stress of their own efforts to resist attack (Amarante et al., 2005). The immune response to GINs is vastly complex, and there are still mechanisms to be explored in order to fully
understand how ovine hosts can be more adept at managing these infections than others.

1.4 Stress response in sheep and impact on immunity to gastrointestinal nematodes

Sheep have variable stress responses, and this variability is associated with immunity, due to the ability of immune function to be altered by stress hormones (Dhabhar, 2018). As seen, the host immune response is required to control GIN infections, therefore stress will impact the ability of the host to manage GINs.

1.4.1 Host stress response

Stress has been defined as a constellation of events, consisting of a stimulus (stressor), that precipitates a reaction in the brain (stress perception), that activates physiological fight-or-flight systems in the body (stress response) (Dhabhar & McEwan, 1997). Exposure to stressors results in the release of pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6, by liver cells (Li et al., 2006). These cytokines then activate the hypothalamic–pituitary–adrenal axis (HPAA) by inducing the secretion of corticotrophin-releasing factor and arginine vasopressin from the hypothalamus, which subsequently initiates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the circulation (You et al., 2008a). ACTH signals the secretion of glucocorticoids, such as cortisol from the adrenal cortex, which helps to control the potentially damaging host inflammatory response (Webster and Sternberg, 2004). Through this major endocrine axis, immune function is regulated and therefore any imbalance disrupting this neuroimmunoendocrine communication, such as stress, tends to result in pathological conditions (Jurberg et al., 2018).
The importance of macrophages in the immune response was discussed in the previous section, however stress-related mediators have been shown to affect monocyte and macrophage maturation, differentiation and migration (Spitsin et al., 2017). Additionally, macrophage-derived factors enhance glucocorticoid production through the stimulation of the HPAA (Jurberg et al., 2018). This is just one example of how stress can influence the immune response, and subsequently the host response to GINs.

Responses to stress involve both behavioral and nervous and/or endocrine adaptations in the host (You et al., 2008a). Cortisol is the primary corticosteroid released in sheep as a result of application of stressors – such as environmental temperature and physiological conditions (Fleming, 1997). Cortisol works to inhibit inflammation by blocking the transcription of pro-inflammatory cytokines and inducing the transcription of other anti-inflammatory molecules (Kabaroff et al., 2006). As such, plasma cortisol has been found to be elevated in certain disease states, including parasitism in sheep (Muehlenbein, 2006).

1.4.2 Stress and disease caused by GIN infection

Stressors have been shown to affect components of antibody and cell-mediated immune responses in livestock (Kelley, 1980). Research by Fleming (1997) demonstrated that parasitic burdens represent a chronic stressor to sheep, resulting in prolonged elevated cortisol levels. This stress from chronic nematode infection may also lead to susceptibility to other diseases, along with weakened immune responses to vaccinations, drugs, and nutritional supplements. Another study by Fleming (1997) found that sheep resistant to GINs were able to withstand stress which was induced by
warmer temperature, while susceptible sheep to GINs failed to withstand stressful conditions, resulting in higher parasitic infection indicated by higher FECs. Similarly, Sotiraki et al. (2013) found stress factors such as temperature and nutrition may increase the susceptibility of sheep to GINs.

**1.4.3 Variation in host stress response**

Sheep have shown considerable variation in the cortisol response to various types of stressors, with heritability estimates of this phenotype being moderate to high (Bartels et al., 2003; Federenko et al., 2004; Guimont & Wynne-Edwards, 2006). This variation in stress response was explored by You et al. (2008a), using lipopolysaccharide (LPS) endotoxin as an immune challenge. High (H), medium (M), and low (L) cortisol responsive sheep were identified based on their estimated breeding values for cortisol concentration measured 4 h post-systemic challenge with *E. coli* LPS endotoxin. This phenotype was seen to be stable over several years in all H, M, and L stress response animals (You et al., 2008a).

**1.4.4 Assessing stress response for controlling GIN infection**

Using the LPS model to stress phenotype (H, M, and L) animals will allow for further studies on the influence these phenotypes have on various host responses to disease. Using these phenotypes with sheep, You et al. (2008b) found a significant association between cortisol responsiveness to endotoxin and primary antibody-mediated immunity; the H and L cortisol responders exhibited an attenuated primary antibody response and stronger dermal hypersensitivity response when compared to the M cortisol-responding animals. These results can be used for further studies,
comparing M sheep to H or L responding sheep in their display of controlling GIN infection.

1.5 Immunological role of the liver in gastrointestinal nematode infection

The liver receives 80% of its hepatic blood supply from the gut (via the portal vein). This requires the liver to tolerate a blood supply consisting of dietary and environmental antigens, and molecules from the microflora of the gut, while at the same time surveilling for pathogen-associated antigens (Robinson et al., 2016). This requires exchange of molecules from blood to Kupffer cells, which are specialized macrophages that work to eliminate various bacteria, parasites, and cellular debris from the blood in order to protect the rest of the body from excessive immune activation (Fabbrini, Sullivan, & Klein, 2010; Rui, 2014).

Robinson et al. (2016) studied the immunological role of the liver, in summary they have identified the liver is the primary metabolic and detoxification organ in the body – functioning as an important buffer between contents of the gastrointestinal tract and systemic circulation. It also plays a very important role in the immune response, working to detect and respond to inflammatory signals from other sites in the body. This immunological role is mediated by a diverse immune cell repertoire, along with non-hematopoietic cell populations. The liver also produces acute-phase protein (APP)s, complement components, cytokines and chemokines – all of which are associated with directing the immune response.

The liver participates in the acute-phase response (APR) of the host, triggered by stimulation of the immune system and/or exposure to stressors (Colditz, 2003; Jakab &
Activation of macrophages and DCs through Kupffer cells and lymphocytes results in the secretion of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α during the APR (Kabaroff et al., 2006). During this time, hepatocytes will increase the production of APPs, while also synthesizing IL-6, to amplify the APR. These APPs are responsible for the systemic effects of inflammation on the host, which work to promote pathogen clearance (Robinson et al., 2016). The APR influences nutrient availability and uptake by reducing protein synthesis, decreasing fatty acid synthesis and increasing lipolysis in adipose tissue (Malmezat et al., 1998). This is due to the increase of nutrient requirements for the production of leukocytes, APPs, and Igs (Cheung & Morris, 1984). This was also proposed by Walkden-Brown and Eady (2003), reporting that metabolic costs of host defense and the decreased efficiency of nutrient utilization for growth, while pro-inflammatory cytokines influence the priorities for nutrient utilization, could contribute to the costs of immunity in sheep due to internal parasites. Additionally, Colditz (2003) found that changes in appetite, growth and nitrogen metabolism seen during GIN parasitism in sheep are associated with the systemic effects of the APR.

The liver also has a role in the adaptive immune response of the host. This is due to the presence of adaptive lymphocytes, such as MHC-restricted CD4+ and CD8+ T cells, and B cells. There is a large population of CD4+ T cells in the liver, along with activated T cells and memory T cells (Robinson et al., 2016). These T cells are mainly activated by hepatocytes and DCs acting as antigen presenting cells (Kubes & Jenne, 2018). Depending on the cytokine profile, different T cells will be activated, for example
DCs that produce large amounts of INF-γ promote the development and activation of cytotoxic T cells (Kubes & Jenne, 2018).

The liver will be an important participant in the host response to GINs due to its immunological role of inflammation, pathogen clearance, and the APR during GIN infection, however, there are few reports in literature on studies of the APR during GIN infection in sheep. Therefore, it would be warranted to explore the activation and metabolic costs of the APR during ovine GIN infection.
Chapter II: Objectives
Gastrointestinal nematode infection is a common cause of morbidity and mortality in grazing sheep. Due to increasing anthelmintic resistance, chemical treatment alone is inadequate to control these infections. Understanding the genetics of the host response to GINs will be imperative to developing alternative strategies for Ontario sheep producers, which could include genetic selection of animals more adept at managing GIN infection.

Resistance to GINs has been defined as the ability of the host to mount an immune response that limits the establishment of the parasite and/or subsequent development of infection (Sweeney et al., 2016). Determining resistance has been a challenge due to the extensive mechanisms of host response to GINs. As a result, several different traits have been explored to determine GIN resistance such as FEC, FAMACHA© score, and host antibody (IgA, IgG and IgM) responses to GIN. There is also evidence of activation of the APR during GIN infection (Colditz, 2003); therefore, studying the effects of the liver and initiation of APR with respect to resistance to GINs may also be beneficial.

Additionally, phenotyping sheep based on stress responsiveness may also be associated with resistance to GINs, as variation in stress response is associated with immunity. Parasite dormancy and FEC shedding appears to occur when ewes are most stressed- during parturition and lactation (Fleming, 1997). Sheep display considerable variation in the stress hormone response (cortisol) to various types of stressors, with heritability estimates of this phenotype ranging from moderate-to-high (Bartels et al., 2003; Federenko et al., 2004; Guimont & Wynne-Edwards, 2006). Variation in the ovine
stress response was explored by You et al. (2008a), using bacterial LPS endotoxin as an immune stressor. High (H), medium (M), and low (L) cortisol responsive sheep were identified based on their estimated breeding values for peak cortisol response measured 4 h post-systemic LPS challenge. This phenotype was shown to be stable over several years in all H, M, and L stress response animals (You et al., 2008a), telomere length - a biomarker of ageing (Yip et al. 2016) and was associated with immune response (You et al. 2008b). The You et al. (2008b) study found a significant association between cortisol responsiveness to LPS and cell-mediated immune response (CMIR) and antibody-mediated immune response (AMIR), the H and L cortisol responders exhibiting stronger CMIR and an attenuated primary AMIR when compared to the M cortisol-responding animals. This novel health phenotype can be used in future studies to determine if stress responsiveness in sheep contributes to resistance to GINs.

An additional tool for studying the host mechanisms involved in GIN resistance is transcriptomics, which provides genome-wide information about gene expression. Transcriptomics will be particularly important in capturing the dynamic, tissue-specific events occurring during the course of GIN infection (Sweeney et al., 2016; McRae et al., 2016). A number of studies have been performed to characterize the duodenal (Keane et al., 2006), abomasum mucosal (Knight et al., 2011) and lymph node transcriptomes (Gossner et al., 2013) in response to GIN infection. Although some studies have postulated pathways and biological processes associated with host response to GIN, no clear consensus has yet emerged (McRae et al., 2016). Continuing to explore biochemical pathways and gene regulatory networks associated with resistance will
provide a more solid foundation for the identification of genes associated with resistance to GIN infection and genetic variants that can be used as markers for the selection of GIN-resistant sheep. Because of the liver’s role in regulating the immune and stress responses, specifically with the initiation of the APR, it would be beneficial to investigate the transcriptome of this tissue in sheep during GIN infection. Additionally, using tissues from sheep displaying the novel health phenotype of varying stress responsiveness, will allow for further investigation of the contribution of these phenotypes to resistance to GINs.

Therefore, the overall objective of this study was to examine the liver transcriptome from H and M stress responding tracer sheep (exposed to GINs) and control (unexposed to GINs) sheep in order to identify key regulator genes associated with GIN infection. Specific objectives were:

- To identify differentially expressed genes (DEG) between i) H and M stress responding sheep, ii) H stress responding sheep and control, and iii) M stress responding sheep and control.

- To identify functional candidate genes and key regulator genes involved in metabolic pathways and biological functions associated with GIN infection.
Chapter III: Identifying hepatic genes regulating the ovine response to gastrointestinal nematodes
3.1 Abstract

Gastrointestinal nematode (GIN) infections are considered the most important disease of grazing sheep. Due to increasing anthelmintic resistance, chemical control alone is inadequate. Resistance to GINs is a heritable trait, and many breeds of sheep throughout the world have naturally developed enhanced resistance. The study of the transcriptome from GIN-exposed and unexposed sheep using RNA-Seq technology can provide measurements of transcript levels associated with the host response to the infection. The objective of this study was to examine liver transcriptomes from high and medium stress responding tracer sheep (GIN-exposed) and control sheep (unexposed). RNA-Seq was performed on liver tissue of five high stress responding, six medium stress responding, and four control sheep. There were no significant differentially expressed genes (DEG) between the high and medium stress responders, however, 146 significant DEG were found between the high stress and control groups, and 159 significant DEG were found between the medium stress and control groups (P-value ≤ 0.01; false discovery rate ≤ 0.05; and fold-change of >±2). In both stress groups in comparison to control group, functional analysis of the DEG revealed downregulation of lipid metabolic pathways and upregulation of immune response processes.

3.2 Introduction

Gastrointestinal nematode (GIN) infection is a common cause of morbidity and mortality in grazing sheep. Due to increasing anthelmintic resistance, chemical treatment alone is inadequate to control these infections. Understanding the genetics of
the host response to GINs will be imperative to developing alternative strategies for sheep producers, which could include genetically selecting for animals more resistant to GIN infection. Resistance to GINs has been defined as the ability of the host to mount an immune response that limits the establishment of the parasite and/or subsequent development of infection (Sweeney et al., 2016). Both the innate and acquired immune systems protect the host from GINs by supporting the gastrointestinal tract (GIT) barrier to GINs, and producing factors that elicit rapid inflammatory responses to accelerate pathogen clearance. Subsequent infections will then elicit an acquired immune response that can reduce parasite burden by reducing GIN establishment, increasing adult worm mortality and reducing adult egg production (McManus et al., 2014; Sweeney et al., 2016). However, complete knowledge of the extensive host mechanisms used to control GIN infection is unclear.

Several traits have been explored to determine GIN resistance, such as fecal egg count (FEC), FAMACHA© score, and host antibody (immunoglobulin (Ig)) responses to GIN (Hunt et al., 2013; McManus et al., 2014). Genomic technologies have also been explored to identify genes and genetic markers that have a significant association to parasite resistance (Benavides et al., 2016; McRae et al., 2014). However, because of the complex host response mechanisms to GINs, no clear consensus has yet emerged concerning the most effective strategy to determine resistance (McRae et al., 2016). A potentially powerful candidate tool for studying the host mechanisms involved in GIN resistance is transcriptomics, which provides information about gene expression of the whole transcriptome using high-throughput RNA-Seq technology (Cánovas et al., 2010; Wickramasinghe et al., 2014). Transcriptomics will be particularly important for
capturing the dynamic, tissue-specific events (Cánovas et al., 2014a and 2014b), specifically occurring during the course of GIN infection (Sweeney et al., 2016; McRae et al., 2016).

Additionally, phenotyping sheep based on stress responsiveness may also be associated with resistance to GINs, as variation in stress response is associated with immunity (You et al., 2008). Sheep display considerable variation in the stress hormone response (cortisol) to various types of stressors, with heritability estimates of this phenotype ranging from moderate-to-high for the immune stressor bacterial lipopolysaccharide (LPS) (You et al., 2008). Parasite emergence and increased FEC shedding appears to occur when ewes are most stressed during parturition and lactation (Fleming, 1997). Exposure to stressors triggers an acute-phase response (APR) that manifests as the release of pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6, that target many tissues, including the liver, to restore homeostasis (Li et al., 2006). Robinson et al. (2016) reviewed the immunological role of the liver; in summary, they identified the liver as the primary metabolic and detoxification organ in the body – functioning as an important buffer between contents of the GIT and systemic circulation. The liver also plays a very important role in the immune response, which is mediated by a diverse cell repertoire, such as Kupffer cells and hepatocytes and their acute-phase protein (APP)s, all of which are involved in directing the immune response (Crispe, 2009). As described previously by Colditz (2003), changes in appetite, growth and nitrogen metabolism seen during GIN parasitism in sheep are associated with the systemic effects of the APR.
Continuing to explore gene regulatory networks and biological pathways associated with resistance to GINs in sheep will provide a more solid foundation for the identification of genes associated with resistance to GIN infection and genetic variants that can be used as markers for the selection of GIN-resistant sheep. Given the liver's role in regulating the immune and stress responses, specifically with the initiation of the APR, it would be beneficial to investigate the transcriptome of this tissue in sheep during GIN infection. Additionally, using tissues from sheep displaying the novel health phenotype of varying stress responsiveness, will allow for further understanding of the contribution of these phenotypes to resistance to GINs. Therefore, the objective of this study was to examine liver transcriptomes of varying stress responsive sheep with GIN infection to identify differentially expressed genes (DEG) between i) high (H) and medium (M) stress responding sheep, ii) H stress responding sheep and control, and iii) M stress responding sheep and control, and to perform functional analysis on the list of DEG to identify biological process, molecular function, cellular component, and functional pathway analysis.

3.3 Materials and Methods

3.3.1 Animal and sample collection

The lambs described here were part of a larger study regarding the immune and stress response of Rideau X Dorset sheep naturally exposed to Haemonchus contortus, Teladorsagia circumcincta and Trichostrongylus GINs (Borkowski et al., 2018). All procedures were approved by the University of Guelph Institutional Animal Care and
Use Committee. Lambs, born and housed at the Ponsonby Sheep Research Facility, University of Guelph, Ponsonby, ON, were identified as H and M stress responding phenotypes from a population of 180 sheep based on peak cortisol levels 4 hours post 0.4 \( \mu \text{g/kg} \) *Escherichia coli* lipopolysaccharide (LPS) (O111:B4 E. coli_L2630_Sigma-Aldrich) immune challenge (You et al., 2008). Peak cortisol levels were graphed, and 15 lambs with cortisol as close to the population mean (117 nmol/L) as possible were chosen as M stress responders (mean=111.4 nmol/L, SD=27.0), and 15 lambs with cortisol levels greater than 1 standard deviation (SD) above mean (328.2 nmol/L) were chosen as H stress responders (mean=336.33 nmol/L, SD=82.9). The H and M stress responding phenotypes were chosen because You et al. (2008) found that H and low stress responders displayed no difference in their antibody and cutaneous delayed-type hypersensitivity immune responses, despite extreme differences in cortisol responsiveness. Male lambs were castrated after selection to reduce differences related to sex hormones (5 males in the H stress and 1 male in the M stress groups). At approximately one year of age (age range= 8-12months), these “tracer” sheep were sent to a commercial Ontario farm (Norwood, ON) where they spent a full grazing season (April 28, 2016 to November 10, 2016) with a flock with a known GIN infection problem caused mainly by *H. contortus*. This allowed for natural exposure to a mixed GIN infection. In August, one M stress responder was euthanized due to illness unrelated to parasitemia. A ration of shelled corn mixed with trace mineral salt was fed in metal troughs on pasture at the rate of approximately 0.23 kg per head per day while the lambs were grazing on pasture until slaughter in November, 2016, and access to water was provided ad libitum.
Eight control animals, which were all M stress responders (mean=111.38 nmol/L, SD=35.6), were born and housed at the Ponsonby Sheep Research Facility, University of Guelph, at the same time of tracer sheep selection (all male and castrated after selection), these sheep remained at the research facility for the grazing season and, although had free-access to pasture, were not exposed to GINs as this facility is free of these pathogens. This was confirmed with FECs and visual inspection of the GIT post-slaughter. These lambs were maintained on a first cut hay diet and allowed access to water ad libitum and were given approximately 0.23 kg of 80% whole barley/20% whole corn mix (per feeding) twice a week.

In November, the tracer sheep (n=29) and control sheep (n=8) were slaughtered by electrical stunning followed by exsanguination. Immediately after slaughter, animals were dissected and the GIT was stored for further analysis of parasite burden, while a sample of approximately 5g of the center of the right liver-lobe was removed with a sterile scalpel. The liver sample was then washed in phosphate buffer solution and placed immediately in liquid nitrogen for transportation. Samples were then stored in -80°C until RNA was isolated.

The most extreme animals in each group were selected for RNA-Seq by performing principle component analysis (PCA) using R software (https://www.r-project.org) based on cortisol level, and total immature and total mature abomasum GIN parasite count at slaughter. Animals chosen for RNA-Seq were: M stress responding sheep (n=6) with high parasite load (average GIN parasite count = 270, SD=240.4), H
stress responding sheep (n=5) with low parasite load (average GIN parasite count = 5, SD=9.8), and control sheep (n=4) with zero GIN parasite load (Figure 3.1).

3.3.2 RNA extraction, RNA-Seq library preparation and sequencing

A representative sample of liver tissue (30 mg) was ground using a mortar and pestle with liquid nitrogen and homogenized using a hand-held homogenizer. Total RNA was extracted following the manufacturer’s instructions using Qiagen RNeasy® Mini Prep Kit (Qiagen, Calif., USA). The quality and quantity of total RNA was assessed using a Nano drop spectrophotometer (Thermo Fisher Scientific, Mass., USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Calif., USA), respectively. All RNA samples used in this study had an RNA integrity value ≥ 8.0, indicating very good quality of the RNA.

The NEBNext® Ultra RNA Library Prep kit for Illumina® was used for library preparation with Poly-A selection, according to the manufacturer’s recommendations. Library quality and concentrations were assessed using a Qubit fluorometer (Thermo Fisher Scientific, Mass., USA) (Cánovas et al., 2013). Sequencing was completed with an Illumina HiSeq 2000 analyzer that yielded 150 base pair (bp) paired-end reads.

3.3.3 RNA-Seq Analysis

RNA-Seq analysis to obtain differentially expressed genes (DEG) was performed using the CLC genomics workbench software, version 12 (CLC Bio, Aarhus, Denmark). As described by Cánovas et al. (2014a), quality control analysis, including GC content, ambiguous base content, Phred score, base coverage, nucleotide
contributions and over-represented sequences parameters, was performed on fastq files. All the samples passed the quality control analysis showing same length (150 bp), 100% coverage of all bases, 25% of A, T, G and C nucleotide contributions, 50% GC base content and less than 0.1% over-represented sequences (Cánovas et al., 2014a). Sequence reads were aligned to the annotated Oar_v3.1 ovine reference genome (release 95). Counts per gene were transformed using a Log10 transformation and normalized to reads per kilo base per million mapped reads (RPKM) values. The DEG analysis was performed between control (n = 4) and M stress (n = 6) sheep; and control (n=4) and H stress (n=5) sheep; and M stress (n=6) and H stress (n=5) sheep (CLC Bio, Aarhus, Denmark). Genes were considered to be differentially expressed among groups when they had a p-value ≤ 0.01, a false discovery rate (FDR) ≤ 0.05, and a fold change (FC) of > ±2. Associated gene name annotation was performed using the Ensembl biomart tool (http://useast.ensembl.org/index.html). For the genes that had no associated gene name in Ensembl, cDNA sequences were retrieved from Ensembl using Biomart (http://useast.ensembl.org/index.html), and the National Center for Biotechnology Information (NCBI) nucleotide BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST) was used to perform blast searches with the following options: against the nucleotide collection, with no specified species, and optimize for highly similar sequences- megablast, E-value= ≤0.01, NCBI % alignment= 91%-100%, and query cover= 24%-100%.

3.3.4 Functional Analyses
Gene ontology (GO) enrichment analysis was performed using WEB-Based Gene Set AnaLysis Toolkit software (http://www.webgestalt.org) using the following parameters: \textit{minNum} (minimum number of genes per category) = 5 of the total genes in the input, \textit{fdrMethod} (FDR method) using BH (Benjamini–Hochberg), \textit{sigMethod} (Significant method) using FDR, and \textit{fdrThr} (FDR threshold) of 0.05. The GO terms associated with the three main GO categories (biological process, molecular function, and cellular component) were analyzed (Cánovas et al., 2012; Cardoso et al., 2018). These analyses were performed on the upregulated and downregulated lists of unique DEG from H stress versus control, M stress versus control, and the common DEG found between H and M stress versus control.

Functional pathway analyses and regulator effect analyses were performed on the full list of DEG in the M and H stress groups versus control using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Inc., http://www.ingenuity.com). Thresholds of \textit{P}-value \leq 0.01, FDR \leq 0.05, and \textit{FC} > \pm 2 were applied to filter significant genes for function, pathway, and network analyses effects (Cardoso et al., 2017).

3.4 Results and Discussion

3.4.1 Sequencing and alignment- basic statistics

Each of the 15 RNA-Seq samples generated an average 56,726,456 paired-end reads per sample (SD=5,260,581.8), with an average input read length of 150 bp. RNA-Seq analysis revealed 82.09\% (SD=4.5) of the reads were mapped to the annotated Oar_v3.1 ovine reference genome (release 95). The alignment rates were similar to others using ovine liver, with mapped reads averaging \~71\% to \~83\% (Alvarez Rojas et
al., 2015; Izadnia et al., 2018; Sabino et al., 2018). For both H stress versus control and M stress versus control, the transcriptomes of the samples were clustered completely by group (control, H and M) as illustrated in the heat maps (Figures 3.2a and 3.2b).

3.4.2 Differentially expressed genes between high and medium stress

No significant DEG were found between the H and M stress groups; however, when comparing the list of DEG between H and M stress groups and control, 86 DEG were common to both stress groups (52 downregulated, 34 upregulated). There were 60 genes found only in the H stress group when compared to control (30 downregulated, 30 upregulated), and 73 genes found only in the M stress group compared to control (50 downregulated, 23 upregulated) (Figure 3.3). Table 3.1 highlights the ten most highly expressed DEG (based on RPKM values), and ten most extreme up- and downregulated genes (based on FC values) found among the DEG that were common to both H and M stress groups when compared to control.

Among the common DEG in both stress groups, the most highly expressed was beta-2-microglobulin (B2M) with an RPKM value of 1904.48 in the H stress (P-value=8.4E-08, FC=2.25) and 1948.48 in the M stress groups (P-value=2.0E-05, FC=2.09). The B2M gene encodes a protein associated with the class I major histocompatibility complex (MHC) alpha chain, which is involved in presenting endogenous antigens to CD8+ T-cells that subsequently kill the cell (Liu et al., 2018). This is important in the immune response in order to ensure response to potential internal threats (Venturina et al., 2013). B2M has been found to be highly expressed in GIN-resistant sheep in several studies (Keane et al., 2007; Ahmed et al., 2015; Berton
et al., 2017). This complements the finding in the present study of the importance of this gene during GIN infection, as it was the most highly expressed gene in the GIN-exposed groups.

The most upregulated gene compared to control was Fibroblast Growth Factor 21 (FGF21), with FC=28.72 in the M stress (P-value=5.61E-17, RPKM=67.69) and FC=17.2 in the H stress groups (P-value=5.37E-07, RPKM=36.08). This protein is a secreted endocrine factor that functions as a major metabolic regulator. Its expression is altered by physiological, metabolic and environmental factors (Erickson & Moreau, 2016). Circulating levels of FGF21 are increased in response to fasting and environmental stress (Schaap et al., 2013). Because this gene is altered in response to many different factors, it cannot be concluded that the GIN infection is upregulating the expression of this gene in the GIN-exposed groups; factors such as the different environments and slightly different diets between the control and exposed groups could also be influencing its expression. Nonetheless, there is a connection between the immune and endocrine systems via the endocrine-immune axis (Dhabhar & McEwen, 1997).

The most downregulated gene was Thyroid Hormone Responsive (THRSP), with FC= -61.9 in the H stress (P-value=6.04E-53, RPKM=2.37); and FC= -28.9 in the M stress groups (P-value=2.97E-36, RPKM=5.59). The protein encoded by this gene is involved in lipid metabolism, specifically, cholesterol synthesis. Lipid metabolism is regulated during the host response to infection (Grunfield, 1996), however, parasites induce significant changes in lipid metabolism. Candidate mechanisms for such
changes include: altering gastrointestinal hormones, altering epithelial function and changing the amount and type of immune cells in metabolic tissues (Bansal et al., 2005). This gene, being the most downregulated in the GIN-exposed groups (H and M stress groups) compared to the unexposed control group, may support this evidence that GINs alter host lipid metabolism.

Interestingly, two other highly expressed genes associated with host response to GINs were also directly related to MHC; *Ovis aries OLA class I histocompatibility antigen, alpha chain BL3-7-like 1 (OLA-I)* with an RPKM value of 1055.40 in the H stress (P-value=3.6E-08, FC=2.77) and 1524.85 in the M stress groups (P-value=7.1E-08, FC=3.36), and *CD 74 Molecule (CD74)* with an RPKM value of 244.65 in the M stress (P-value=3.5E-06, FC=2.38) and 213.55 in the H stress groups (P-value=1.1E-12, FC=2.34). The *OLA-I* gene is associated with MHC I (Liu et al., 2018). Whereas, the CD74 protein encoded by *CD74* associates with MHC class II and regulates exogenous antigen presentation during the immune response (Schroder, 2016). Alleles OMHC1-188 and OLADRB2-282 of the ovine MHC have been associated with GIN resistance (Outteridge et al., 1996; Castillo et al., 2011), and quantitative triat loci (QTL) studies of the ovine MHC region, found on chromosome 20, have found a statistically significant association with GIN resistance (Outteridge et al., 1996; Keane et al., 2007; Stear et al., 2007). Our study reaffirms the importance of ovine MHC during GIN exposure, as the most highly expressed liver genes in the GIN-exposed groups were associated with MHC.

Another highly expressed gene in both groups was *alpha-1-acid glycoprotein*
ORM1, with RPKM values of 483.04 in the H stress (P-value=2.3E-13, FC=10.51) and 769.54 in the M stress groups (P-value=2.0E-06, FC=15.20). This gene encodes for the APP orosomucoid, and its expression is regulated by glucocorticoids, IL-1, TNF-α, and IL-6 (Luo et al., 2015). The ORM1 gene is an immunomodulator that is induced by stressful conditions, such as GIN infections. Receptors for ORM1 have been found on macrophages and neutrophils, and activation has been shown to suppress pro-inflammatory gene expression (Lee et al., 2010). Orosomucoid also aids in B- and T-cell maturation (Okumura et al, 1985; Fournier et al., 2000), and has been shown to participate in the innate immune response, binding directly with LPS and neutralizing its toxicity (Murata et al., 2004).

Another highly expressed gene in the exposed groups was Haptoglobin (HP) with RPKM values of 2496.80 in the H stress (P-value=7.7E-09, FC=18.23) and 959.43 in the M stress groups (P-value=1.2E-05, FC=6.44). Haptoglobin is another important APP, functioning to both inhibit mast cell proliferation and suppress T-cell proliferation, as part of its integral immunomodulatory and anti-inflammatory role, and also an important iron-binding protein (Murata et al., 2004). Along with being one of the most highly expressed genes in both stress groups, ORM1 was also the second most upregulated gene, with FC= 15.20 in the M stress and FC=10.51 in the H stress groups. Similarly, HP was also highly upregulated with FC=18.23 in H stress and FC=6.44 in M stress groups. This further contributes to the findings that the APR may be an important part of the host response to GINs, and warrants further investigation.
The *CAMP Responsive Element Binding Protein 3 Like 3 (CREB3L3)* gene is another noteworthy gene commonly upregulated in both GIN-exposed groups, with an RPKM value of 256.72 in the M stress (P-value=3.6E-06, FC=2.84) and 192.96 in the H stress groups (P-value=1.1E-04, FC=2.33); *CREB3L3* is associated with acute inflammatory responses and hepcidin expression (Canali et al., 2016). Hepcidin values increase in response to inflammatory cytokines and low iron levels (Abreu et al., 2018), and thus may be involved during *H. contortus* infection. Since the GIN-exposed groups were exposed to *H. contortus*, it is unsurprising to see an increase in the expression of *CREB3L3*.

### 3.4.3 Functional analysis of DEG between High and Medium stress

The GO terms associated with the 3 main GO categories (biological process (BP), molecular function (MF), and cellular component (CC)) were analyzed. The 52 common downregulated genes were clustered in 21 GO terms (11 in BP, 5 in MF, and 5 in CC categories; Table 3.2). The enriched terms in BP category were related to metabolic processes, with the most significant being “small molecule metabolic process” (P-Value=0, FDR=0). The “endoplasmic reticulum membrane” (P-Value=4.00E-11, FDR=3.25E-08) was the most enriched in CC category and “cofactor binding” (P-Value=1.11E-16, FDR=3.93E-13) was the most enriched in the MF category. The 34 common upregulated genes were clustered in 18 GO terms (10 in BP and 8 in CC) (Table 3.3). Among the BP terms, all were related to the immune response, with “acute inflammatory response” being the most enriched (P-Value=3.81E-09, FDR=3.47E-05). Related to the immune system, “MHC protein complex” (P-Value=3.81E-06, FDR=0.01) was also the most significant CC term.
3.4.3 Unique differentially expressed genes between medium stress and control

A total of 159 significant DEG were found between the M stress and control groups, with 73 unique to the M stress group (50 downregulated, 23 upregulated). Table 3.4 highlights the ten most highly expressed, and ten most extreme up- and downregulated genes found among the DE analysis of M stress versus control. Among the unique DEG in the M stress group, the most highly expressed gene was *Glutathione S-Transferase Alpha 1 (GSTA1)* with an RPKM value of 11280.82 (P-value=1.1E-08, FC=-2.66); GSTA1 is involved in detoxification of toxins and products of oxidative stress. Zhang et al. (2019) found GSTA1 to be upregulated in the abomasum of a line of sheep resistant to *H. contortus*, complementing the importance of this gene with the present study during exposure to *H. contortus*.

The most upregulated gene unique to the M stress group was *Abhydrolase Domain Containing 1 (ABHD1)*, FC=9.86 (P-value=1.2E-07, RPKM=5.38), which is involved in lipid metabolic processes such as medium chain fatty acid biosynthesis and catabolic processes (Edgar, 2003). In contrast, the most downregulated (FC=-6.08) gene was *Phospholipase A2 Group VII (PLA2G7)* (P-value=6.1E-06, RPKM=0.96), which is also involved in lipid metabolism, such as lipid catabolic and oxidation processes (Dyar et al., 2018). The involvement of these genes may be attributed to the fact that a substantial effect of GIN infection is a reduction in metabolic efficiency of the host, resulting in decreased available nutrients required for growth and production (Louie et al., 2007). This is likely due to the need for nutrient utilization during the host immune response to GINs, so any tissues not involved in the immune response may have a reduced capacity to utilize those nutrients (Yu et al., 2000), along with GIT tissue.
damage caused by GINs (Venturina et al., 2013). The altered gene expression of these metabolic genes could be attributed in part to the GINs altering the host lipid metabolism compared to the unexposed group; however, the aforementioned difference in diets could also contribute to the difference in gene expression.

Other genes that are worth noting in the list of highly expressed genes in the M stress group are two IFN-inducible genes; *Interferon Alpha-inducible Protein 27 (IFI27)* and *ISG15 Ubiquitin-Like Modifier (ISG15)*, with RPKM values of 712.01 (P-value=1.8E-04, FC=7.48) and 245.37 (P-value=1.6E-04, FC=6.31), respectively. IFI27 is upregulated by IFN-α and functions in the innate immune response and resisting cellular or environmental stress by sensitizing cells to apoptotic stimuli (Parker & Porter, 2004; Cheriyath et al., 2011). Similarly, the protein product of ISG15, activated by IFN-α, induces IFN-γ production by T cells, activates monocytes and DCs, and displays antiviral activity (Campbell and Lenschow, 2013). Li et al. (2011) found IFI27 to be more highly expressed in cows that were more resistant to GINs compared to susceptible cows (FC=2.99), and Bhuiyan et al. (2017) found ISG15 to be highly expressed in a GIN-resistant group of goats. These findings contribute to the present study, as GIN-exposure induces these IFN genes.

### 3.4.4 Functional analysis of unique DEG to medium stress

The 50 downregulated genes were clustered within 22 GO terms: 11 in BP, 10 in MF and 1 in CC categories (Table 3.5). In the BP category, most terms were related to metabolic processes; with "*carboxylic acid metabolic process*" being the most significant (P-Value=2.22E-12, FDR=2.02E-08). In the CC category, the only significant term was
“mitochondrion” (P-Value=3.97E-05, FDR=0.04), and in MF category, the most significant term was “oxidoreductase activity” (P-Value=4.04E-09, FDR=4.25E-06). The 23 upregulated genes were clustered into three GO terms, with the only significant terms found in the BP category (Table 3.6). The most enriched BP term was “type I interferon signaling pathway” (P-Value=3.37E-06, FDR=0.01). The three enriched terms were all associated with IFN processes and included the previously mentioned highly expressed genes in the M group, ISG15 and IFI27. A T-helper 1 cell (Th1) response depends on the production of IFN-γ, which inhibits the T-helper 2 cell (Th2) response, that is the response that mainly controls GIN infection. Studies have found an increase in IFN-γ is associated with sheep that are susceptible to GIN infection (Muñoz-Guzmán et al., 2006; Shakya et al., 2011). However, this contrasts with other studies that have found an increase in ISG15 and IFI27 are associated with GIN-resistant cattle (Li et al., 2011) and goats (Bhuiyan et al., 2017). The present study shows that GIN-exposure and a high parasite burden induces these genes.

Downregulation of metabolic processes and upregulation of interferon pathways was also seen in the IPA functional analysis. The top canonical pathways from IPA were cholesterol biosynthesis in the M stress group, along with the top molecular and cellular functions such as lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism, molecular transport and amino acid metabolism.

The regulator-effect function in IPA connects upstream regulators, dataset genes and downstream functions or diseases. In the M stress group, the first regulator effect network (Figure 3.4a) had notable regulators of Insulin-induced Gene (INSIG1/2) and
Cytochrome P450 Family 51 Subfamily A Member 1 (CYP51A1), which were common to both stress groups. These regulators affected multiple genes that were all downregulated, such as Aldo-Keto Reductase Family 1 Member C3/4 (AKR1C3/4), 17-beta Hydroxysteroid Dehydrogenase Type 7 (HSD17B7) and GSTA1, all of which were unique to the M stress group, along with Stearoyl-CoA Desaturase (SCD) and THRSP, which were the most highly downregulated genes common to both groups. These genes lead to the predicted inhibition of functions such as metabolism of cholesterol, synthesis of terpenoids, conversion of lipid, and concentration of colfosceril palmitate.

Another effect network (Figure 3.4b), regulated by IFN-related genes, resulted in the downstream effect of predicted activation of hypersensitivity reaction and cytotoxicity of cells. This was due to the increased measurements of highly expressed genes common to both groups such as CD74, B2M and OLA-I. The only downregulated gene, unique to the M group, was PLA2G7. IFI27 was also among this group and was the most highly expressed gene unique to the M stress group. This network may be related to GIN infection, because IgE and mast cells are associated with hypersensitivity reactions and these are also crucial in controlling GIN infection; mast cells release inflammatory mediators upon degranulation into infected tissues of the GIT which result in smooth muscle contraction, increased vascular permeability and local blood flow, and enhanced mucus secretion (McRae et al., 2015; Urb & Sheppard, 2012). Mast cells also produce Th2 cytokines IL-13, 4 and 5, along with chemotactic factors that recruit other immune cells such as eosinophils, NK cells and neutrophils to site of infection (Huntley et al., 1995). IgE results in the release of vasoactive mediators and cytokines that lead to blood vessel and gut muscle contraction, increased mucus production, and an
increase of interlectins, which may block larval colonization and development. IgE levels have also been seen to be inversely correlated with FEC (Huntley et al., 1995; Murphy et al., 2010; Venturina et al., 2013).

### 3.4.5 Unique differentially expressed genes between high stress and control

A total of 146 significant DEG were found between the H stress and control group, with 60 unique to the H stress group (30 downregulated, 30 upregulated). Table 3.7 highlights the ten most highly expressed and the ten most extreme up- and downregulated genes found among the DE analysis. Among the unique DEG in the H stress group, the most highly expressed gene was *Fatty Acid Binding Protein 1* (*FABP1*) with an RPKM value of 1055.68 (P-value=3.02E-06, FC=-2.05); as the name suggests, *FABP1* is involved in the metabolism of fatty acids (Chao et al., 2017). As previously mentioned, GINs can impact metabolism.

The most upregulated unique gene in the H stress group was *Eukaryotic Translation Elongation Factor 1 Alpha 1* (*EEF1A1*) with a FC of 251.39 (P-value=1.29E-04, RPKM=154.46); *EEF1A1* encodes an isoform of the alpha sub-unit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome. It also forms a complex with tyrosine-protein kinase (*TXK*) that acts as a Th1 cell transcription factor and regulates IFN-γ transcription; and is therefore an important player in Th1 cytokine production (Takeba et al., 2002). A possible reason for this upregulation is that control of GIN infection relies strongly on a Th2 response, but because gene expression was evaluated at the end of the grazing season (and months
after peak GIN-infection), there may have been a shift toward a Th1 response after an active Th2 response during peak infection.

The most downregulated gene was *Calcineurin Like EF-Hand Protein 2 (CHP2)* with a FC of -5.05 (P-value=1.20E-07, RPKM=3.81). The protein gene product of *CHP2* functions in the calcineurin/NFAT (nuclear factor of activated T cells) signaling pathway, which is crucial for T-cell activation (Daniel et al., 2014). The downregulation of this gene contrasts with many of the other highly upregulated or expressed genes in the present study that are known to promote T cell functions. For example, *C-X-C Motif Chemokine Ligand 9 (CXCL9)* was upregulated in the H stress group, with a FC= 6.5 (P-value=7.46E-07, RPKM=8.64); CXCL9, which is induced by IFN-γ, is a chemoattractant for T-cells and enhances immune responses following parasite infection (Trotta et al., 2009). Studies have found *CXCL9* to be upregulated in sheep resistant to GIN infections (Gosner et al., 2013; Hui et al., 2012). This finding contributes to the present study, as exposure to GINs (with a low parasite burden) resulted in the upregulation of *CXCL9* compared to the unexposed controls, suggesting this gene could be playing an important role in enhancing the host response to GIN infection.

### 3.4.6 Functional Analysis of unique DEG to high stress

The 30 downregulated genes were clustered in 11 GO terms, with the only significant terms being identified in the BP category (Table 3.8). Similar to the comparison between M stress and control sheep, the enriched terms were associated with metabolic processes; with “regulation of small molecule metabolic process” being
the most significant (P-Value=4.26E-09, FDR=3.88E-05). The 30 upregulated genes were clustered in 21 GO terms (10 in BP, 9 in CC and 1 in MF; Table 3.9). The most highly enriched terms in each of these categories were “immune response” (P-Value=1.61E-11, FDR=1.46E-07), “lysosome” (P-Value=1.40E-06, FDR=0.002) and “protein-containing complex binding” (P-Value=4.48E-06, FDR=0.003), respectively. A large number of enriched terms related with immune function were identified in the liver transcriptome of the H stress sheep, with genes such as Heme Oxygenase 1 (HMOX1), Placenta-specific Gene 8 Protein (PLAC8) and EEF1A1 involved in terms such as “leukocyte activation and degranulation”, “immune effector process”, and “cell activation”, to name a few. These processes are important in controlling GIN infection by activating lymphocytes and macrophages, and activating other cells and pathways involved in clearing GINs such those involved in a Th2 response.

Metabolic pathway analysis confirmed the findings in the GO functional analysis; the top canonical pathways were cholesterol biosynthesis in the H stress group, and the top molecular and cellular functions in the high stress group were lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism, molecular transport and carbohydrate metabolism. In the H stress group, the first regulator-effect network (Figure 3.5a) had a top regulator as Elongation of Very Long Chain Fatty Acids Protein 5 (ELOVL5), which was unique to the H group. The regulators effected genes that were all downregulated in the H stress group, with the exception of HMOX1, which was upregulated. Among these genes were again the most highly downregulated genes common to both groups; SCD and THRSP. These genes lead to the predicted inhibition of functions of steroid metabolism, fatty acid metabolism, conversion of fatty acid and
concentration of colfosceril palmitate.

Another effector network in the H stress group led to the predicted activation of functions such as activation of leukocytes, inflammatory response, cell movement of mononuclear leukocyte, and immune response of cells (Figure 3.5b). These functions were regulated by genes that were highly expressed among both groups, such as CD74, B2M and OLA-I, as well as Proteasome Subunit Beta 9 (PSMB9), a gene unique to the H stress group. A similar effector network in the H stress group lead to the predicted activation of immune functions such as chemotaxis of phagocytes and granulocytes, immune response of cells, response of antigen presenting cells, and cytotoxicity of cells (Figure 3.5c). Genes that lead to this activation that were unique to the H group were again PSMB9, along with Cathepsin S (CTSS) and HMOX1. The same genes common to both groups were also among these genes; CD74, B2M and OLA-I. These immune functions are necessary for controlling GIN infection; for example, chemotaxis of granulocytes involve neutrophils, which are part of the innate response to GINs. Antigen presentation, associated with the MHC complex, is required for T-cells to respond to GINs (Alba-Hurtado & Muñoz-Guzmán, 2012; Liu et al., 2018).

The overall findings from the GO functional analysis and the metabolic pathway analysis for each of the comparisons followed the trend of downregulation of metabolic processes and upregulation of immune processes. There is known cross-talk between immune cells and metabolic organ cells required for homeostasis. This is especially true with GINs that spend most of their life cycle within the GIT, resulting in enhanced cross-talk and even beneficial effects on host glucose and lipid metabolism (Shea-Donohue et
al., 2017). This is due to an increased Th2 response, activated by the presence of GINs, which upregulate genes that regulate glucose and lipid metabolism (Harnett, 2014). Parasitic infections, even those restricted to the intestine, increase circulating levels of IL-4, IL-5, and IL-13, which may act to either inhibit, or reverse the Th1-induced inflammation in metabolic tissues while also regulating metabolic homeostasis in the host (Madden et al., 2002; Harnett, 2014). There is continued evidence that GINs induce an effect on genes that modulate fat metabolism; for example N. brasiliensis infection downregulated genes encoding for main lipogenic enzymes in the liver (Yang et al., 2013). The present study also saw these effects, with the GIN-exposed group showing a difference in gene profile regarding lipid metabolism, along with an overall increase in genes associated with the immune response compared to the unexposed control group.

3.5 Conclusion

Several genes involved in lipid metabolism were downregulated in the GIN-exposed groups of sheep, such as PLA2G7 and GSTA1 in the M stress group, HMOX1 and ELOVL5 in the H stress group, and THRSP and SCD in both groups. Upregulation of genes involved in the immune response were also identified, such as ISG15 and IFI27 in the M stress group, EEF1A1 and CXCL9 in the H stress group, and B2M, CD74 and OLA-I in both groups. Additionally, ORM1 and HP genes involved in the APR were highly expressed in both groups. In summary, the sheep exposed to GINs (M and H stress groups) showed an overall decrease in metabolic processes with an increase in immune processes, compared to the unexposed control group. These results highlight
the host immune response to rid infection remains activated after peak infection, while the GINs themselves may be influencing metabolic genes making metabolic processes less efficient.
Table 3.1: Top common differentially expressed genes (DEG) between high and medium stress groups versus control

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<td>3.90E-32</td>
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<td>5.59</td>
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FC: Fold Change, FDR: False Discovery Rate, RPKM: reads per kilo base per million mapped reads
Table 3.2: Significant terms from the Gene Ontology (GO) enrichment analysis for downregulated genes in the comparison between medium stress and high stress sheep

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene Ontology ID</th>
<th>Gene Ontology Name</th>
<th>Number of genes in the GO term</th>
<th>P-Value</th>
<th>False Discovery Rate (FDR)</th>
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<tbody>
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Table 3.3: Significant terms from the Gene Ontology (GO) enrichment analysis for upregulated genes in the comparison between medium stress and high stress sheep

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<th>False Discovery Rate (FDR)</th>
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<td>3.47E-05</td>
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<td>2.92E-04</td>
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<tr>
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<td>GO:0033077</td>
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<tr>
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**Table 3.4:** Top ten unique DEG between medium stress and control

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<th>RPKM control</th>
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<td><strong>based on highest RPKM values</strong></td>
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<td>APOA5</td>
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<td>61.77</td>
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<td><strong>based on highest FC values</strong></td>
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Table 3.5: Significant terms from the Gene Ontology (GO) enrichment analysis for downregulated genes in the comparison between medium stress and control sheep

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<th>Gene Ontology Name</th>
<th>Number of genes in the GO term</th>
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Table 3.6: Significant terms from the Gene Ontology (GO) enrichment analysis for upregulated genes in the comparison between medium stress and control sheep

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<td>none significant (FDR &gt;0.05)</td>
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Table 3.8: Significant terms from the Gene Ontology (GO) enrichment analysis for downregulated genes in the comparison between high stress and control sheep

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Table 3.9: Significant terms from the Gene Ontology (GO) enrichment analysis for upregulated genes in the comparison between high stress and control sheep

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Figure 3.1: Principle component analysis (PCA) based on cortisol level, and total immature and total mature abomasum worm count at slaughter.
Figure 3.2a: Heat map of gene expression of the entire transcriptome for each sample (based on RPKM values) between control and medium stress group

Figure 3.2b: Heat map of gene expression of the entire transcriptome for each sample (based on RPKM values) between control and high stress group
Figure 3.3: Venn diagram representing number of differentially expressed genes (DEG) in the comparison between control and high stress, and control and medium stress.
Figure 3.4a: Functional network and metabolic pathway analysis showing inhibition of metabolic pathways in the medium stress group

Figure 3.4b: Functional network and metabolic pathway analysis showing activation of immune response processes in the medium stress group
Figure 3.5a: Functional network and metabolic pathway analysis showing inhibition of metabolic processes in the high stress group

Figure 3.5b: Functional network and metabolic pathway analysis showing activation of immune response processes in the high stress group
Figure 3.5c: Functional network and metabolic pathway analysis showing activation of immune response processes in the high stress group
Chapter IV: Discussion and Conclusions
4.1 Discussion and Conclusions

Gastrointestinal nematode (GIN) parasitism is considered the most important disease of grazing sheep. GIN infections are a cause of substantial morbidity, and economic and production losses (Falzon et al., 2013). Resistance to GINs is a heritable trait, and many breeds of sheep throughout the world have naturally developed enhanced resistance. Several studies have explored this resistant trait, however it is complex and encompasses several mechanisms. The study of the transcriptome from GIN-exposed and unexposed sheep using RNA-Sequencing (RNA-Seq) technology can provide measurements of transcript levels associated with the host response to GIN infection. This will allow for the exploration of biological pathways and gene networks associated with resistance in order to provide a more solid foundation for the identification of genes associated with resistance to GIN infection and genetic variants that can be used as markers for the selection of GIN-resistant sheep. Therefore, the overall objective of this thesis was to examine liver transcriptomes from high (H) and medium (M) stress responding tracer sheep (exposed to GINs) and control (unexposed to GINs) sheep in order to identify key regulator genes associated with GIN infection. Chapter III of this thesis provided the specific objectives of this thesis, which were to identify differentially expressed genes (DEG) between i) H and M stress responding sheep, ii) H stress responding sheep and control, and iii) M stress responding sheep and control. Additionally, to identify functional genes involved in metabolic pathways and biological functions associated with GIN infection.
Chapter III used RNA-Seq technology to examine the liver transcriptomes of varying stress responsive sheep that were exposed to GINs, and control sheep that were unexposed to GINs. DEG analysis revealed no significantly DEG between the H and M stress responding sheep. However, when comparing these stress sheep to control, there were 159 DEG between M stress and control (102 downregulated and 57 upregulated), and 146 DEG between H stress and control (82 downregulated and 64 upregulated). Among these two lists of DEG, 86 were common between the H and M stress group, 73 found only in the M stress group, and 60 found only in the H stress group. Exploring the 86 genes that were common among the stress groups is looking at the gene expression profile of DEG between GIN-exposed and unexposed sheep, regardless of stress phenotype or parasite burden. This is important in order to identify highly expressed genes as well as biological pathways and gene networks that are involved when the host is exposed to GINs.

Highly expressed genes identified among the list of 86 DEG between exposed and unexposed groups included \textit{B2M}, \textit{OLA-I} and \textit{CD74} – all of which are associated with the ovine major histocompatibility complex (MHC). MHC presents foreign antigen to T cells which will then subsequently remove the invading pathogens (Liu et al., 2018). The high expression of these three genes confirms the importance of ovine MHC as a mechanism to control GIN infection during exposure. There were also two acute-phase proteins that were found to be highly expressed among the GIN-exposed group; \textit{ORM1} and \textit{HP}. The high expression of these two genes confirm the acute phase response is activated during GIN exposure.
4.2 Limitations

There are a number of limitations to the current study, the first being in order to ensure the control group of sheep were not exposed to GINs, they were kept on the research facility where, although they had access to pasture, the main source of feeding was hay, as opposed to the H and M groups that were mainly supported by pasture as their feeding source. Both groups of animals were fed a corn mix that was similar in composition. It is possible that this difference in diet may have an effect on the liver, as one of the main metabolic processing organs. Therefore, when interpreting the results of metabolic genes that were found differentially expressed between the exposed and unexposed groups, it is important to consider the difference in diets and environments as being a contributor to the gene expression, along with the effect of GIN exposure. However, among the immune response genes found, they appear to be related to GIN exposure and can more confidently be considered as differentially expressed due to the exposure of GINs.

There were both male and female sheep in the study, and although males were castrated to reduce differences related to sex hormones, gender may still factor into differences seen between sheep and parasite loads.

The time of tissue collection was another limitation, as the liver samples were collected at the end of grazing season (in November) and when GIN infection is mainly resolved, and any newly ingested L3 are likely hypobiotic. The host immune response to GINs is most active during peak infection time (usually July-August in Ontario). Fecal egg counts of the exposed sheep confirmed peak infection time in August for the year.
the sheep were on pasture. The transcriptomic profile of the liver samples in this study therefore provide insight of the transcriptome during the resolution phase of GIN infection, as opposed to peak stage of GIN infection.

An additional limitation of the study was the tissue the transcriptomic analysis was performed on. The liver receives 80% of its blood supply from the gastrointestinal tract (**GIT**) (Robinson et al., 2016), therefore there will be indirect evidence of GIN infection as the immune cell population of the liver will be surveying for pathogens coming from the GIT. The liver is also where the acute phase response is initiated, and acute-phase proteins are produced (Colditz, 2003), providing insight into the role of the acute-phase response during GIN exposure. However, the liver transcriptome is not going to provide a direct gene profile associated with GIN infection compared to a tissue like the abomasum, as this is where the GINs actually attach and feed off the host. The liver provides a more systemic and indirect view into GIN infection, rather than a direct and localized view that the abomasum or GIT would provide.

### 4.3 Future research and implications

This thesis provided insight into the genes, biological pathways and gene networks associated with GIN exposure, however there is still more research to be done in determining the host response to GINs and the subsequent trait of GIN-resistance in sheep.

Using the results from this study, the key genes found in relation to immune response (**B2M, CD74, OLA-I, HP** and **ORM1**) and lipid metabolism (**FGF21** and **THRSP**) can be further explored for variation (identify single nucleotide polymorphisms,
insertions and deletions, and splice variants). Doing this will allow for the possible development of genetic tests for the resistant trait, and/or implementation into breeding programs.

A future direction of transcriptomic analysis of GIN-exposed and unexposed sheep can be performed on other tissues related to the host response to GIN infection; such as the abomasum, lymph nodes, caecum, and others that are directly related to GIN infection, in order to provide further insight on mechanisms activated due to GIN exposure.

In conclusion, this thesis provided insight into key genes, biological pathways and gene networks resulting from GIN exposure. Results can be used to further explore the immune mechanisms involved in GIN infection, as well as potential metabolic pathways that are altered due to GIN exposure. This may be beneficial in providing information required for the development of genetic selection programs for GIN-resistant sheep.
REFERENCES


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sheep genetically resistant and susceptible to gastrointestinal nematodes. 

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Kemper, K., Palmer, D., Liu, S., Greeff, J., Bishop, S., & Karlsson, L. (2010). Reduction of faecal worm egg count, worm number and worm fecundity in sheep selected for worm resistance following artificial infection with *Teladorsagia circumcinta* and *Trichostrongylus colubriformis.* *Veterinary Parasitology, 171,* 238-246.


APPENDICES

Appendix 1: Student progress report

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Conference posters:
- OSNA Ontario Sheep Convention
  - Oct. 26-27
- DMAFRA Climate Change Research Knowledge Exchange Day
  - Oct. 19
- FFT Research Integration Symposium
  - Jan. 10
- FFT Annual General Meeting
  - May 1
- DMAFRA Small Ruminant Research Day
  - May 14
- Arrell Food Summit
  - May 22
- ASAS-CSAS Annual Conference and Tradeshow
  - Oct. 31
  - Nov. 1
  - July 9
- OSF Annual general meeting
  - Oct. 31
- International Veterinary Immunology Symposium
  - Nov. 1
  - July 9

Conference talks:
- OSF Annual general meeting
  - May 1
- ON Small Ruminant Veterinary Conference
  - June 17

Articles in conference proceedings:
- ASAS-CSAS Annual Conference and Tradeshow
  - July 9

Industry extension posters:
- Introduction to Sheep Production manual; chapter 8: flock health and deadstock
  - Aug. 31
- 2018 Handbook for the control of internal parasites of sheep and goats
  - Aug. 31

Industry extension talks:
- DMAPFA and CGIL Workshop: Genetic improvement in the ON sheep industry - where are we going? Eiora, ON
  - Aug. 1

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<tr>
<td>1st place Graduate Student Poster Comp.</td>
<td>May 14</td>
</tr>
<tr>
<td>OMAFRA Small Ruminant Res. Day</td>
<td></td>
</tr>
<tr>
<td>3rd place Research Poster Comp. OSF</td>
<td>Oct. 31</td>
</tr>
<tr>
<td>Annual General Meeting</td>
<td>Nov. 1</td>
</tr>
<tr>
<td>Amos Kitchen Award</td>
<td>Jan. 15</td>
</tr>
</tbody>
</table>

**Extracurricular activities:**

<table>
<thead>
<tr>
<th>Activity</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Academic Student Council member</td>
<td>Sept. 2017-Aug. 2019</td>
</tr>
<tr>
<td>Volunteer with College Royal</td>
<td>March</td>
</tr>
<tr>
<td>CGIL Journal Club member</td>
<td>Sept 2017-Aug. 2019</td>
</tr>
<tr>
<td>Volunteer at Graduate Student University Teaching Conference</td>
<td>Aug. 31</td>
</tr>
<tr>
<td>Immunology Journal Club member</td>
<td>May 2018-Aug. 2019</td>
</tr>
<tr>
<td>Graduate Teaching Community member</td>
<td>Jan. 2018-Aug. 2019</td>
</tr>
</tbody>
</table>