

**Investigation of the immune response of sheep to gastrointestinal
nematode infection under Ontario grazing conditions to identify
genetically resistant animals**

by

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A Thesis

Presented to

The University of Guelph

In partial fulfillment of requirements

for the degree of

Doctor of Philosophy

in

Pathobiology

Guelph, Ontario, Canada

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ABSTRACT

INVESTIGATION OF THE IMMUNE RESPONSE OF SHEEP TO GASTROINTESTINAL NEMATODE INFECTION UNDER ONTARIO GRAZING CONDITIONS TO IDENTIFY GENETICALLY RESISTANT ANIMALS

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Gastrointestinal nematodes (GINs) cause significant morbidity and financial losses to sheep enterprises. An appealing control strategy is to reduce GIN parasitism by identifying and breeding sheep with a superior immune response to GINs. However, most screening methods for GIN immunity were developed in warm temperate climates with year-round GIN exposure. Regions with long, cold winters that interrupt GIN exposure, such as Ontario, Canada, are under-represented in studies of GIN immunity. Moreover, limitations of existing GIN speciation methods preclude characterization of species-specific immunity. Therefore, the objectives of this thesis were to: 1) validate GIN speciation using deep amplicon sequencing; 2) assess effects of GIN parasitism on productivity of sheep grazing in Ontario; 3) evaluate salivary antibody to carbohydrate larval antigen (sCarLA) as a method of assessing GIN immunity in Ontario sheep; and 4) generate preliminary information on the relationship between acute stress responsiveness, cell- and antibody-mediated immune responses, and GIN parasitism. Deep amplicon sequencing of first-stage larvae (L₁s) generated comparable results to traditional morphologic speciation of third-stage larvae (L₃s). Furthermore, significantly higher proportions of eggs developed to L₁s

than to L₃s. Productivity and sCarLA were monitored in 140 replacement ewe lambs for two years in a commercial flock in central Ontario. Subclinical GIN infection had minimal effect on productivity of those ewe lambs. Even so, most of the animals developed detectable sCarLA by the end of their first grazing season; these levels waned over winter but reached higher levels in the second grazing season, suggestive of an anamnestic response. Additionally, sCarLA was always negatively associated with GIN fecal egg counts, and levels in individual animals were generally consistent over time. No significant differences in GIN parasitism were identified between lambs with high and medium acute stress responsiveness, or between lambs with immune responses biased towards antibody- or cell-mediated immunity. The data presented in this thesis will be used to direct wider-scale validation of sCarLA in selection of Ontario sheep with optimal GIN immunity.

DEDICATION

For my parents.

Who always knew this would happen,
even when I was unsure.

ACKNOWLEDGEMENTS

The work in this thesis would not have been possible without the contributions of many brilliant people dedicated to making the project a success. Though words are insufficient to express my gratitude, I wish to thank all who have helped along the way.

I must first recognize the contributions of my advisory committee. Thank you to my co-advisors, Andrew Peregrine and Niel Karrow, for their guidance throughout this project. I have learned so much from Andrew's attention to detail, and Niel can always be counted on for great new ideas. Paula Menzies is a fount of wisdom, and I have been lucky to benefit from her comprehensive knowledge of small ruminants. Brandon Lillie always supported me through my concurrent Anatomic Pathology training, and offered a voice of reason with dry wit and good humour.

I have also been fortunate to work with two incredible and talented technicians, Jacob Avula and Ziwei Li. Jacob's wide-ranging appreciation and understanding of parasites is amazing, and his curry dishes kept us all going through long days. I have always been in awe of Ziwei's ability to be equally at home working sheep in the field and running precise ELISAs in the laboratory.

No research is possible without funding, and several agencies deserve recognition. This project received support from the federal government through the Canadian Agricultural Adaptation Program, from the provincial government through the Ontario Agri-Food Innovation Alliance, and from Ontario Sheep Farmers. Jennifer MacTavish and Delma Kennedy were indispensable in helping move mountains of reports to sustain the project's funding. The University of Guelph

and the Ontario Ministry of Agriculture, Food, and Rural Affairs also supported my work through scholarships, allowing me to focus my effort on research. I am grateful for their financial support.

I was lucky to find many passionate helpers with inexhaustible energy. Rebecca Chant and Stéphanie Bourgon spent their summers working long hours on farm visits and even longer hours in the lab processing fecal and pasture samples, ever cheerful and ready for more. I am proud of all you accomplished and know that you will be wonderful veterinarians. Leah Lourenco was an attentive assistant during her undergraduate research project. Thank you to Pam Hasson and Jen Vervoort, from the Ponsonby Sheep Research Facility, for all your help. I am happy we did not end up taking away too many of your favourites for my study. Tracy To, Courtney Lun, Alaina Macdonald, Tori Brown and Peyton Tam were an eager ‘lamb watch’ team, and cared deeply for their tiny charges. Thanks also to Rebecca Fisher and Kevin Barbosa, who helped get the project started while I was still finding my footing, and to Mike Alcorn for stepping in last minute to help with a sweltering field visit. I am also very grateful to my pathology colleagues Amanda Mansz and Karen Carlton for being so willing to spend their free time working with sheep guts!

During this study, I had numerous fruitful collaborative opportunities. My thanks to John Gilleard and Elizabeth Redman at the University of Calgary for involving me in their exciting larval PCR research. Thanks also to Richard Shaw at AgResearch Inc. in New Zealand for coordinating our trans-Pacific saliva tests. None of the statistical analyses would have been possible without William Sears’ guidance and patience. It was a pleasure to work with Angela Cánovas and her students, who were always ready and willing to help when I needed an army,

even well into the night. In particular, Samantha Dixon was a rallying force and huge help with my field work.

I am immensely grateful to my parents, Kazi and Zorika, who have supported me unconditionally. They have been my sounding-board, a source of ideas, have stepped in to assist with fieldwork on multiple occasions, and have often been the first reviewers of my writing (though I know my father feels it was not often enough). None of my accomplishments would have been possible without you.

Lastly, I offer my deepest gratitude to the sheep producers (you know who you are) who agreed to allow me to work with their wonderful flock. Thank you for your patience with my frequent disruptions to your daily routine. I could not have done any of this without your commitment and ingenuity in helping the project run smoothly.

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LIST OF SYMBOLS, ABBREVIATIONS AND NOMENCLATURE

<i>Abbreviation of symbol</i>	<i>Definition</i>
AbMIR	Antibody-mediated immune response
ACTH	Adrenocorticotrophic hormone
AVP	Arginine vasopressin
BCS	Body condition score
CAA	<i>Candida albicans</i> antigen
CarLA	Carbohydrate larval antigen
CD	Cluster of differentiation
CHO	Carbohydrate
CI	Confidence interval
CMIR	Cell-mediated immune response
COD	Corrected optical density
CRF	Corticotrophin releasing factor
DAMP	Damage associated molecular pattern
DDF	Denominator degrees of freedom
DM	Dry matter
epg	Eggs per gram
FAMACHA©	Faffa Malan Chart
F _c	Fraction crystallizable
F _c εR	Fraction crystallizable epsilon receptor
FEC	Fecal egg count
Fizz	Found in inflammatory zone

Foxp3	Forkhead box p3
GABA	Gamma-aminobutyric acid
GATA3	Guanine-adenine-thymine-adenine binding protein 3
GFI1	Growth factor independent 1
GI	Gastrointestinal
GIN	Gastrointestinal nematode
HCT	Hematocrit
HEWL	Hen egg white lysozyme
HIR TM	High immune response
HPAA	Hypothalamic-pituitary-adrenal axis
HSR	High stress response phenotype
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNK	Invariant natural killer
iTCR	Invariant T cell receptor
ITS-2	Internal transcribed spacer-2
KCl	Potassium chloride
kDa	KiloDalton
L _n	Larval stage <i>n</i>
Ln	Natural logarithm
LPS	Lipopolysaccharide
MgCl ₂	Magnesium chloride

MHC	Major histocompatibility complex
MPTL	Monepantel
MSR	Medium stress response phenotype
NA	Not applicable
NaCl	Sodium chloride
NC	Noncollagenous
NDF	Numerator degrees of freedom
NK	Natural killer
NS	Not specified
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
POMC	Pro-opiomelanocortin
PPER	Periparturient egg rise
PRR	Pattern recognition receptor
QTL	Quantitative trait loci
RBC	Total erythrocyte count
rDNA	Ribosomal deoxyribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SA	Serum albumin
sCarLA	Salivary antibody to carbohydrate larval antigen
SG	Serum globulin
SNP	Single nucleotide polymorphism

STAT6	Signal transducer and activator of transcription 6
TGF	Transforming growth factor
Th	T helper
TNF	Tumour necrosis factor
Treg	T regulatory
WBC	Total leukocyte count

CHAPTER ONE:

INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 Epidemiology of gastrointestinal nematodes in sheep

On Ontario sheep farms, the four most prevalent and pathogenic species of gastrointestinal nematodes (GIN) are *Trichostrongylus axei*, *Teladorsagia circumcincta*, *Haemonchus contortus* and *Trichostrongylus colubriformis*, and their relative prevalence fluctuates depending on the time of the year (Mederos et al., 2010). All four species are in the superfamily *Trichostrongyloidea*, and their life cycles are similar (see Figure 1.1) (Bowman, 2014; Taylor et al., 2016). Morulated ova are shed in feces, and the hatched larvae (L₁s) moult twice to develop into infective third-stage larvae (L₃s) over 5-14 days under ideal environmental conditions (Bowman, 2014; Karrow et al., 2014; Taylor et al., 2016). The cuticle of the second stage larva (L₂) is not shed during the moult to L₃, acting as a protective layer but preventing the L₃s from feeding (Bowman, 2014; Taylor et al., 2016). As a result, survival of L₃s outside the host is restricted by the rate of consumption of stored nutrients, with more rapid metabolism and therefore shorter survival time at temperatures above 26 °C (Taylor et al., 2016). In order to facilitate ingestion by the host, L₃s move from feces into nearby herbage (Bowman, 2014; Karrow et al., 2014; Taylor et al., 2016). Following ingestion, L₃s shed the retained outer cuticle and moult twice more to develop into immature adults (L₅) (Bowman, 2014; Karrow et al., 2014; Taylor et al., 2016). In ruminants, maturation of *Haemonchus contortus* L₃s occurs adjacent to the abomasal glands, within abomasal glands for *Teladorsagia* sp. and between abomasal

glands for *Trichostrongylus axei*, and within the proximal small intestinal mucosa for *Trichostrongylus colubriformis* (Taylor et al., 2016). Shedding of ova, which are indistinguishable between the four species, usually commences approximately 3 weeks post-infection, however, L₃s of *Trichostrongylus* spp. and fourth stage larvae (L₄s) of *Haemonchus contortus* and *Teladorsagia* sp. can undergo developmental arrest (hypobiosis) within the host following exposure to environmental stress, such as cold or drought, during the L₃ stage (Miller and Horohov, 2006; Bowman, 2014; Karrow et al., 2014; Taylor et al., 2016). Should hypobiosis occur, resumption of development is triggered by an as yet unknown stimulus, typically when environmental conditions are more conducive to survival of larvae on pasture (Miller and Horohov, 2006; Bowman, 2014; Karrow et al., 2014; Taylor et al., 2016).

Pathology associated with GINs results in considerable morbidity and production loss, particularly in previously unexposed (naïve) lambs (Miller and Horohov, 2006; Saddiqi et al., 2011; Bowman, 2014; Karrow et al., 2014). A recent meta-analysis of 88 studies on the production impact of GINs found that infection with GINs causes a 10 % reduction in wool growth, 15 % reduction in weight gain in lambs and 22 % reduction in milk production by ewes compared with uninfected sheep (Mavrot et al., 2015). Effects of GIN infection on reproductive performance are less clearly defined. Loss of productivity occurs despite the fact that in general, high burdens of GINs are seen in a small proportion of the flock, with most animals carrying relatively light burdens (Miller and Horohov, 2006; Kemper et al., 2009). *Haemonchus contortus*, the most pathogenic GIN, feeds on erythrocytes; a single worm results in daily loss of 0.05 mL of blood, both

through ingestion and leakage from feeding sites (O'Connor et al., 2006; Bowman, 2014; Taylor et al., 2016). Heavy infestations can drain up to 20 % of a lamb's erythrocytes each day, and anemia can develop rapidly (Bowman, 2014; Taylor et al., 2016).

Inflammation and distention of abomasal glands adjacent to sites of *Trichostrongylus axei* penetration or containing developing *Teladorsagia* sp. larvae lead to mucosal hyperplasia and reduced secretion of hydrochloric acid, and consequently increased abomasal pH (McNeilly et al., 2009; Venturina et al., 2013; Karrow et al., 2014; Taylor et al., 2016). Protein digestion is impaired by high pH, as pepsinogen cannot be cleaved to form pepsin (Karrow et al., 2014; Taylor et al., 2016). The hyperplastic epithelial cells also have impaired cell-to-cell adhesion and therefore increased permeability to large molecules and pathogens, leading to loss of protein into the gastrointestinal tract (McNeilly et al., 2009; Venturina et al., 2013; Bowman, 2014; Taylor et al., 2016).

Increased permeability also permits inactive pepsinogen to enter the bloodstream, which suppresses appetite (Craig et al., 2014; Taylor et al., 2016). Weight loss is the most consistently seen clinical correlate to *Teladorsagia* sp. or *Trichostrongylus axei* infection; diarrhea occurs intermittently but evidence of breech soiling (increased dags) is common (McNeilly et al., 2009; Venturina et al., 2013; Taylor et al., 2016). Larvae of *Trichostrongylus colubriformis* cause direct epithelial damage and protein loss as they burrow in and emerge from the intestinal mucosa, and may also impair absorption by damaging villi (Karrow et al., 2014; Taylor et al., 2016). Clinical signs are similar to those of *Teladorsagia* sp. and *Trichostrongylus axei*, however diarrhea tends to be more pronounced (Bowman, 2014; Taylor et al., 2016).

Patterns of GIN infestation vary depending on the local environment (O'Connor et al., 2006; Falzon et al., 2014; Taylor et al., 2016). In regions with cold winters, for example Ontario, fecal shedding and clinical disease show two distinct peaks (O'Connor et al., 2006; Mederos et al., 2010; Bowman, 2014; Taylor et al., 2016). Larvae accumulated the previous grazing season emerge from hypobiosis and mature in early spring (April to June) (O'Connor et al., 2006; Mederos et al., 2010; Taylor et al., 2016). This usually occurs during or immediately following lambing, at which time ewes experience a periparturient egg rise (O'Connor et al., 2006; Mederos et al., 2010; Sargison 2011; Bowman, 2014; Taylor et al., 2016). The cause of this periparturient egg rise is not fully understood, though relaxation of immunity in the face of increased physiologic demand from the late-gestation fetus and mammary tissue has been postulated (Williams et al., 2010; Taylor et al., 2016). Fecal egg counts (FECs) of ewes increase, and occasionally clinical disease is observed in ewes, particularly the primiparous or nutritionally stressed (O'Connor et al., 2006; Mederos et al., 2010; Taylor et al., 2016). This syndrome is often referred to as type II disease (Bowman, 2014; Taylor et al., 2016). Shedding ewes contaminate pastures grazed by their naïve lambs, resulting in a second peak of FECs and clinical signs in lambs during summer (July to August), also referred to as type I disease (Mederos et al., 2010; Bowman, 2014; Taylor et al., 2016). L₃s may not survive prolonged freezing temperatures during winter on pasture, although *Teladorsagia sp.* and *Trichostrongylus* spp. are more resistant to cold than other GIN species, and *Trichostrongylus* spp. larvae have been recovered from Ontario pastures in March (Bowman, 2014; Falzon et al., 2014; Taylor et al., 2016). By contrast, in temperate regions that do not experience cold winters, such as Australia and New Zealand, ingested

larvae continue to accumulate during winter grazing resulting in type I disease in lambs in late winter to early spring (O'Connor et al., 2006; Taylor et al., 2016). In tropical climates, GIN parasitism correlates with rainfall, with peaks of type I disease in young lambs following periods of humid weather and larvae undergoing hypobiosis during dry periods (O'Connor et al., 2006; Taylor et al., 2016).

When subjected to pasture challenge from infective L₃s, naïve lambs gradually develop the ability to control GIN burdens, leading to a reduction in clinical signs and impaired productivity as well as parasite loads (Emery et al., 1999; Saddiqi et al., 2011; Falzon et al., 2013a). Studies on sheep in New Zealand and Australia have found that protective immunity begins as early as 4 months of age, and is well established in most lambs by 9 months of age (Douch et al., 1986; Douch and Morum, 1993; Saddiqi et al., 2011; Falzon et al., 2013a; Venturina et al., 2013; Craig et al., 2014). In Ontario, meat lambs are generally marketed before 6 months of age, in theory prior to the onset of their ability to control GIN burdens (Schallig and van Leeuwen, 1997; Emery et al., 1999; Schallig, 2000; Pettit et al., 2005; Mederos et al., 2010; Williams et al., 2010; Williams, 2011; Alba-Hurtado and Muñoz-Guzmán, 2013). Although clinical disease is common and requires intervention, there is variability in susceptibility to the clinical effects of GINs among lambs. As yet, limited data exist on how lambs develop immunity to GINs under Ontario grazing conditions. Moreover, the immune response to GINs is dependent on continued exposure to infective L₃s and wanes rapidly in the absence of exposure (McNeilly et al., 2009). Maintenance of the immune response is interrupted for 3-6 months during winter in Ontario in both sheep housed indoors and those with pasture

access, due to snow cover and reduced number of infective L₃s on pasture (Sykes et al., 2007; Mederos et al., 2010; Falzon et al., 2014). The reduced frequency and severity of clinical GIN parasitism in ewes compared with lambs indicates that adult sheep are capable of maintaining some degree of immunity to GINs between grazing seasons in northern temperate climates such as in Ontario. However, it is unclear what effect, if any, variation in development of immunity to GINs in one grazing season may have on the kinetics of the response following subsequent exposure.

1.2 Control of gastrointestinal nematodes in sheep

Administration of anthelmintics has long been the primary means of managing GINs and their impact on flock productivity (Jackson et al., 2009; Saddiqi et al., 2011; Falzon et al., 2013a; Shaw et al., 2013; Karrow et al., 2014). The three most widely used classes of anthelmintics are broad-spectrum macrocyclic lactones (ivermectin, moxidectin, abamectin), benzimidazoles (fenbendazole and albendazole), and imidazothiazoles (levamisole) (Hosking et al., 2009; Falzon et al., 2013a; Shaw et al., 2013; Taylor et al., 2016). Each class exerts its nematocidal effect via a different mechanism. Macrocyclic lactones act on glutamate-gated chloride channels, causing an influx of chloride that prevents depolarization of motor neurons and paralyzes the nematode (Plumb, 2011; Abbott et al., 2012; Taylor et al., 2016). Benzimidazoles cause starvation of GINs by disrupting polymerization of cellular tubulin, particularly in the GIN's gastrointestinal tract, and thus impairing uptake and intracellular movement of glucose, proteins and waste products (Plumb, 2011; Abbott et al., 2012; Taylor et al., 2016). Like macrocyclic lactones, the imidathiazole levamisole causes paralysis of GINs, but does so via agonism

of nicotinic acetylcholine receptors, leading to transient muscle spasm followed by depolarization and flaccidity (Plumb, 2011; Abbott et al., 2012; Taylor et al., 2016). Less widely used classes of anthelmintics include broad-spectrum amino-acetonitrile derivatives (monepantel) and spiroindoles (derquantel), and the narrow-spectrum salicylanilide derivative closantel, which has a specific effect on *Haemonchus contortus* but not non-blood feeding nematodes (Hosking et al., 2009; Jackson et al., 2009; Abbott et al., 2012; Shaw et al., 2012; Shaw et al., 2013). Amino-acetonitrile derivatives and spiroindoles also cause paralysis through agonism of monepantel (MPTL)-1, a nicotinic acetylcholine receptor specific to nematodes, and β -nicotinic cholinergic antagonism, respectively (Abbott et al., 2012; Taylor et al., 2016). Closantel causes cellular starvation in blood-feeding nematodes by uncoupling mitochondrial oxidative phosphorylation (Abbott et al., 2012; Taylor et al., 2016). Of these compounds, only ivermectin, closantel and a combination product containing abamectin and derquantel are licensed for use in sheep in Canada, though off-label use of fenbendazole and albendazole is relatively common (Falzon et al., 2013a). Levamisole is used infrequently in Canada, as no commercial product has been available for more than a decade and own use importation of this product is no longer legal in Canada (Falzon et al., 2013a).

As a result of the diversity in mechanisms of action and target receptors between different classes of anthelmintics, mutations conferring anthelmintic resistance in GINs are generally class-specific and occur independently (Bartram et al., 2012). Nevertheless, over-reliance on anthelmintics has led to a high prevalence of multi-anthelmintic resistance among GINs and is progressively limiting utility of available classes (Hosking

et al., 2009; Jackson et al., 2009; Saddiqi et al., 2011; Sargison, 2011; Shaw et al., 2012; Shaw et al., 2013; Karrow et al., 2014). In a recent study of Ontario sheep farms reporting anthelmintic failure, fecal egg count reduction tests conducted in lambs demonstrated resistance of nematodes to a common macrocyclic lactone (ivermectin) in 97 % of farms (28/29 farms), resistance to a common benzimidazole (fenbendazole) in 95 % (19/20 farms), and resistance to levamisole, which at the time of the study was not used in Canada, on one farm (1/17 farms) but the level of resistance was very low on that farm (Falzon et al., 2013a). Resistance to monepantel and early evidence of reduced efficacy of the abamectin/derquantel combination product has already been reported outside of Canada (Andronicos et al., 2012; McRae et al., 2015; Sales and Love, 2016; Lamb et al., 2017). Considerable effort has gone into promoting management practices that prolong the efficacy of available anthelmintics, as well as investigating alternative approaches to managing the impact of GINs (Shaw et al., 2012; Shaw et al., 2013; Karrow et al., 2014). Selective anthelmintic treatment of at-risk sheep aims to maintain a population of GINs within the flock that is not exposed to selection pressure for anthelmintic resistance, known as *refugia* (Sargison, 2011; Jackson et al., 2014). Rotation of pasture such that flocks graze uncontaminated herbage or a different livestock species grazes pasture contaminated by sheep to eliminate GIN larvae is an effective strategy to limit GIN burdens, but is often not practical (Bowman, 1995; Jackson et al., 2009). Certain species of soil fungi are larvicidal and promoting growth of these fungi may help reduce pasture contamination; however, the fungi do not migrate readily from the environment into fecal material and must be either directly applied to feces or given as a feed additive in a spore form that survives gastrointestinal passage (Jackson and Miller,

2006; Kearney et al., 2016). Nutrition is also an important tool in management of GINs; optimizing nutritional status by separate feeding of ewes with large litters or poor body condition can mitigate the periparturient egg rise and reduce risk of type II disease (Jackson et al., 2009). Condensed tannins in forages such as sainfoin inhibit larval motility and establishment, reducing GIN burdens and fecal shedding (Jackson and Miller, 2006; Iqbal et al., 2007; Werne et al., 2013a, Werne et al., 2013b). In addition to these direct effects on GINs, condensed tannins also improve nutrient availability by reducing rumen fermentation and protein degradation, but it is challenging to ensure adequate intake across a flock (Jackson and Miller, 2006; Iqbal et al., 2007; Werne et al., 2013a, Werne et al., 2013b; Ahmed et al., 2014). Two additional alternatives to anthelmintics that have been subject to extensive research are development of GIN vaccines and selection of breeding stock with genetic resistance to GIN parasitism (see section 1.6) (Bowman, 1995; Shaw et al., 2012; Shaw et al., 2013; Karrow et al., 2014; Taylor et al., 2016). Continuously improving understanding of mechanisms involved in the immune response to GINs is of great benefit in the practical application of both of these strategies.

1.3 Mechanisms of the innate immune response to gastrointestinal nematodes

Following ingestion, infective L₃s encounter mucus that lines the gastrointestinal tract (De Veer et al., 2007; McNeilly et al., 2008; Hoang et al., 2010; McRae et al., 2015).

Several substances released by mucosal cells into gastrointestinal mucus are indirectly associated with reduction in the number of larvae encountering the epithelium and establishing infection. Histamine, leukotrienes and bradykinin mediate vasodilation,

mucus production and contractility of smooth muscle, and are inversely correlated with *Teladorsagia* sp. burdens (Jones et al., 1994; Harrison et al., 1999; Hoang et al., 2010; Williams, 2012; Alba-Hurtado and Muñoz-Guzmán, 2013; Venturina et al., 2013; McRae et al., 2015). Increased peristaltic movement and mucus production associated with these mediators is believed to contribute to the phenomenon of immune rejection, wherein sheep previously exposed to GINs rapidly expel ingested L₃s (Harrison et al., 1999; McNeilly et al., 2008; Kemp et al., 2009; Williams, 2012; Alba-Hurtado and Muñoz-Guzmán, 2013; McRae et al., 2015). Moreover, histamine and leukotrienes impair larval movement *in vitro*, and may play a role in preventing L₃s from reaching their predilection sites (Alba-Hurtado and Muñoz-Guzmán, 2013). The role of galectins (β -galactoside binding lectins) in innate immunity is incompletely elucidated; however, they are believed to attach to surface carbohydrates of GINs and alter the viscosity of mucus through crosslinking of glycans (De Veer et al., 2007; Vasta, 2009; Paraná da Silva Souza et al., 2015; Preston et al., 2015). In particular, galectin-11 produced by intestinal epithelial cells binds specifically to L₄ and adults of *Haemonchus contortus*, and is associated with their reduced growth (Preston et al., 2015).

Teladorsagia sp. and *Trichostrongylus* spp. larvae that breach the mucus barrier penetrate and/or feed on the epithelium, causing damage to gastrointestinal epithelial cells (De Veer et al., 2007; Andronicos et al., 2012; Karrow et al., 2014; McRae et al., 2015). Following this, the innate immune system is exposed to pathogen-associated molecular patterns (PAMPs), of which the best characterized are carbohydrates present on the surface of GINs and in their secretions (De Veer et al., 2007; McNeilly et al., 2008;

Karrow et al., 2014; McRae et al., 2015). This allows activation of the complement cascade via two different pathways. Mucosal collectins (a group of lectins capable of recognizing PAMPs) can bind to these PAMPs, activating the lectin pathway, or, more commonly, complement proteins present in the mucosa can bind directly to larvae, activating the alternative pathway (De Veer et al., 2007; Alba-Hurtado and Muñoz-Guzmán, 2013; Paraná da Silva Souza et al., 2015). The products of complement activation have multiple effects, among them promotion of mast cell degranulation and granulocyte chemotaxis, particularly of eosinophils via component C5a (De Veer et al., 2007; Alba-Hurtado and Muñoz-Guzmán, 2013). In addition to GIN PAMPs, damaged gastrointestinal epithelial cells release damage-associated molecular patterns (DAMPs), such as heat shock protein (De Veer et al., 2007; Andronicos et al., 2012; McRae et al., 2015). Both PAMPs and DAMPs are recognized by pattern recognition receptors (PRRs) on a variety of resident mucosal cells, which initiate a range of complementary and interconnected physical and chemical responses to the nematode infection (see Figure 1.2) (McNeilly et al., 2008; Karrow et al., 2014; McRae et al., 2015).

Recognition of DAMPs or GIN PAMPs by gastrointestinal epithelial cells stimulates production of interleukins (ILs), including IL-33 and IL-25, a relative of IL-17 (Paul and Zhu, 2010; Andronicos et al., 2012; Karrow et al., 2014). Tuft cells, an epithelial cell subtype that comprises a small proportion of the abomasal and small intestinal epithelium in uninfected animals, proliferate following GIN infection and secrete IL-25, which is critical to initiating the subsequent type 2 adaptive response and altering mucus production (Gerbe et al., 2012; Karrow et al., 2014; Gerbe et al., 2016). Interleukins 25

and 33 from the epithelial cells, and direct activation by GIN PAMPs, increase production of the type 2 interleukins IL-4, IL-5 and IL-13 by mucosal dendritic cells, macrophages, and mast cells (Miller and Horohov, 2006; De Veer et al., 2007; Saddiqi et al., 2011; Andronicos et al., 2012; Venturina et al., 2013; Karrow et al., 2014; McRae et al., 2015). Natural killer (NK) cells also produce IL-4 and IL-13, as well as interferon gamma (IFN- γ), which downregulate further production of type 2 cytokines (Coltman et al., 2001; De Veer et al., 2007; McNeilly et al., 2008; Rowe et al., 2009; Paul and Zhu, 2010; Karrow et al., 2014; McRae et al., 2015). $\gamma\delta$ T cells are also believed to produce type 2 cytokines, and depletion of $\gamma\delta$ T cells is associated with increased worm length and egg production (Paul and Zhu, 2010; Karrow et al., 2014; Hernández et al., 2017). In response to IL-4 and IL-13, goblet cells proliferate and increase mucus production to facilitate expulsion of the nematodes (Venturina et al., 2013; Karrow et al., 2014; Gerbe et al., 2016). Tuft cell proliferation is also promoted by IL-4, creating a positive feedback loop on secretion of IL-25 and downstream interleukins (Gerbe et al., 2016).

Increased production of IL-4, IL-5 and IL-13, products of complement activation and PAMPs, stimulate degranulation of mucosal mast cells (De Veer et al., 2007; Karrow et al., 2014; McRae et al., 2015). Release of reactive nitrogen and oxygen species causes direct damage to invading GINs, and additional histamine release by mast cells promotes increased peristaltic movement (Menzies et al., 2010; Saddiqi et al., 2011; Venturina et al., 2013; Karrow et al., 2014; McRae et al., 2015). Degranulation also releases chemotactic mediators that act in combination with IL-4, IL-5 and IL-14 to attract other granulocytes (De Veer et al., 2007; Saddiqi et al., 2011; Venturina et al., 2013; Karrow et

al., 2014; McRae et al., 2015). Eosinophils, and to a lesser extent neutrophils, migrate to sites of GIN invasion, degranulate, and contribute to pathogen killing (Miller and Horohov, 2006; De Veer et al., 2007; Saddiqi et al., 2011; Saddiqi et al., 2012; Venturina et al., 2013; Karrow et al., 2014; McRae et al., 2015). Galectin-14 is produced by eosinophils during GIN infection and while its role in the innate response is not fully understood, it is believed to exert effects on both mucus viscosity and GINs (De Veer et al., 2007; Paraná da Silva Souza et al., 2015; Hernández et al., 2017). Following the early increase in mucosal mast cell activity, there is a second expansion of a population of intraepithelial mast cells, also called globule leukocytes (Kemp et al., 2009; Alba-Hurtado and Muñoz-Guzmán, 2013; Karrow et al., 2014). Mucosal mast cells are involved in the rapid immune rejection of GINs, and have been found in increased numbers in some resistant breeds such as Gulf Coast Native sheep, in Romney sheep selectively bred for reduced FEC, and following repeated experimental infection with live *Haemonchus contortus*, *Trichostrongylus colubriformis* and/or *Teladorsagia* sp. L₃s (Stankiewicz et al., 1995; Bisset et al., 1996; Miller and Horohov, 2006; Shakya et al., 2009; Hein et al., 2010; Robinson et al., 2010). Conversely, studies of resistant Florida Native sheep compared with susceptible Rambouillet sheep, and resistant St. Croix sheep compared with susceptible crossbred sheep, found no significant difference in numbers of mucosal mast cells between breeds following challenge with *Haemonchus contortus* (Amarante et al., 1999; Bowdridge et al., 2015). Thus, the contribution of mucosal mast cells to innate immunity appears to vary with breed and possibly with GIN species.

In addition to granulocytes, IL-4, IL-5, IL-13, PAMPs and DAMPs also recruit macrophages and dendritic cells to the sites of GIN invasion (Karrow et al., 2014; McRae et al., 2015). In response to IL-4 and IL-13, tissue macrophages undergo alternative activation to the M2 phenotype (De Veer et al., 2007; McRae et al., 2015). M2 macrophages produce proteins in the found in inflammatory zone (Fizz) family and chitinases, which have three incompletely defined roles (Nair et al., 2005; De Veer et al., 2007; McRae et al., 2015). Both chitinases and Fizz proteins are associated with modeling of the extracellular matrix, and are believed to be involved in wound healing (Nair et al., 2005; McRae et al., 2015). Increased expression of these proteins has also been observed in lymph nodes draining sites of GIN infection, so an immune regulatory function has been speculated (Nair et al., 2005; McRae et al., 2015). Lastly, chitinases may have direct antiparasitic activity by damaging the cuticle of GINs (McRae et al., 2015). Although macrophages participate in antigen presentation, their capacity to present antigens is eclipsed by that of dendritic cells (De Veer et al., 2007; McNeilly et al., 2009; Karrow et al., 2014). Activated dendritic cells take up GIN antigens, travel to mucosal lymphoid follicles and local lymph nodes, and interact with naïve T and B cells to initiate the acquired immune response (De Veer et al., 2007; Karrow et al., 2014; McNeilly et al., 2009; McRae et al., 2015; Jaurigue and Seeberger, 2017).

1.4 Mechanisms of the acquired immune response to gastrointestinal nematodes

1.4.1 Target antigens

Development of the acquired immune response to GINs is complex due to frequent changes in antigen expression associated with multiple moults occurring within the host

(Maass et al., 2009; Hein et al., 2010; Alba-Hurtado and Muñoz-Guzmán, 2013; McRae et al., 2015). One of the best-characterized antigens is a 20-70 kDa glycolipid specific to the epicuticle of the L₃ stage, referred to as carbohydrate larval antigen (CarLA), which has been identified on the surface of all strongylid nematode larvae examined thus far and is believed to be involved in rejection of invasion (Harrison et al., 2003a; Harrison et al., 2003b; Maass et al., 2007; Harrison et al., 2008; Maass et al., 2009; Hein et al., 2010; Pernthaner et al., 2012; Shaw et al., 2012; Alba-Hurtado and Muñoz-Guzmán, 2013; Shaw et al., 2013). The degree of protection resulting from the response to CarLA varies depending on GIN species, with a greater reduction in worm count observed for *Trichostrongylus* spp. than *Haemonchus* sp. or *Teladorsagia* sp. (Harrison et al., 2008; Hein et al., 2010; Alba-Hurtado and Muñoz-Guzmán, 2013). Further, although each L₃ only expresses one epitope of CarLA, these epitopes are not uniform between GIN species and there is also within-species variation in *Trichostrongylus* spp. and *Teladorsagia* sp., postulated to be an adaptation to the immune response (Maass et al., 2007; Maass et al., 2009; Hein et al., 2010; Pernthaner et al., 2012). Two other L₃ surface antigens, a high molecular weight glycoprotein and a poorly characterized low molecular weight antigen, also have variable contributions to the acquired immune response (Balic et al., 2003; Maass et al., 2007; Hein et al., 2010).

Differences in feeding behavior between GIN species also affect which antigens stimulate an effective response. Since *Haemonchus* sp. feeds on blood, its gastrointestinal antigens are particularly susceptible to targeting by circulating antibody (Smith et al., 2003a; Smith et al., 2003b; Alba-Hurtado and Muñoz-Guzmán, 2013). Exposure to these

antigens requires regurgitation of gastrointestinal contents by feeding worms and was not believed to occur in natural infection; however, *Haemonchus* sp. intestinal antigens have been demonstrated at the host's mucosal surface and can stimulate immune cell activation (Knox et al., 2003; Smith et al., 2003a; Smith et al., 2003b; Jasmer et al., 2007; Alba-Hurtado and Muñoz-Guzmán, 2013). Two integral membrane glycoproteins isolated from the intestines of adult *Haemonchus* sp. have shown promise in attempts to create vaccines: H-gal-GP, a complex of aspartyl- and metalloprotease, and H11, a microsomal aminopeptidase (Andrews et al., 1995; Andrews et al., 1997; Knox et al., 1999; Smith et al., 2001; Knox et al., 2003; Smith et al., 2003a; Smith et al., 2003b; Ekoja and Smith, 2010; Alba-Hurtado and Muñoz-Guzmán, 2013; Matthews et al., 2016). Antibody binding of H-gal-GP blocks digestion of blood, leading to starvation of the worm; antibody to H11 is believed to exert a similar effect (Knox et al., 1999; Knox et al., 2003; Smith et al., 2003b; Ekoja and Smith, 2010; Alba-Hurtado and Muñoz-Guzmán, 2013). A variety of excretory/secretory antigens, especially low molecular weight glycoproteins, have also been implicated in the immune response to both *Haemonchus* sp. and the non-blood feeding GINs (Schallig and van Leeuwen, 1997; Miller and Horohov, 2006; De Veer et al., 2007; McNeilly et al., 2009; Smith et al., 2009; Pernthaner et al., 2012; McRae et al., 2015). The nature of the subsequent acquired immune response varies depending on the type of inciting GIN antigen.

1.4.2 Acquired response to protein antigens

Protein-containing GIN antigens, such as H-gal-GP and H11, initiate a T cell-mediated adaptive response (Jaurigue and Seeberger, 2017). Dendritic cells, and to a lesser extent

macrophages, internalize and process these antigens to short peptides, migrate to mucosal lymphoid tissue and local lymph nodes, and present them on major histocompatibility complex class II (MHC II) (McNeilly et al., 2009; Pernthaner et al., 2012; Karrow et al., 2014; McRae et al., 2015; Jaurigue and Seeberger, 2017). Naïve CD4⁺ T cells interact exclusively with dendritic cells presenting antigen on MHC II, and products of the dendritic cells direct T cell differentiation (McNeilly et al., 2009; Karrow et al., 2014; McRae et al., 2015; Jaurigue and Seeberger, 2017). Interleukin-4 inhibits differentiation of naïve T cells into T helper 1 and 17 (Th1 and Th17) cells, which promote a cell-mediated cytotoxic response and a granulomatous response, respectively (Paul and Zhu, 2010; McRae et al., 2015). IL-4 does this by inducing growth factor independent 1 (GFI1), a transcription repressor that prevents transcription of IFN- γ and IL-17 by naïve T cells (Paul and Zhu, 2010; McRae et al., 2015). Dendritic cells also produce transforming growth factor- β (TGF- β) to up-regulate forkhead box P3 (Foxp3), directing other naïve T cells to differentiate into T regulatory (Treg) cells (McNeilly et al., 2009; Taylor et al., 2012; Venturina et al., 2013; Karrow et al., 2014; McRae et al., 2015). Treg cells produce IL-10 and TGF- β to modulate the immune response, suppressing production of cytokines by other immune cells and reducing dendritic cell activity (McNeilly et al., 2009; Taylor et al., 2012; Venturina et al., 2013; Karrow et al., 2014; McRae et al., 2015).

Differentiation of naïve T cells into Th2 cells is central to initiation of the antibody-mediated immune response to protein antigens (Paul and Zhu, 2010; Venturina et al., 2013; Karrow et al., 2014; McRae et al., 2015). The critical protein for Th2

differentiation is GATA-binding protein 3 (GATA3), which is induced by IL-4 mediated activation of signal transducer and activator of transcription 6 (STAT6) (Paul and Zhu, 2010; Venturina et al., 2013; McRae et al., 2015). Activated Th2 cells produce additional IL-4, IL-5, IL-9, IL-13 and IL-25, providing positive feedback on further Th2 differentiation (Paul and Zhu, 2010; Alba-Hurtado and Muñoz-Guzmán, 2013; McRae et al., 2015). These cytokines, in particular IL-4, also influence antibody production by B cells (Paul and Zhu, 2010; Venturina et al., 2013; McRae et al., 2015). B cells that have encountered GIN antigen and been exposed to IL-4 from activated Th2 cells will then proliferate and mature into plasma cells that produce antibodies (Paul and Zhu, 2010; Venturina et al., 2013; McRae et al., 2015). Multiple immunoglobulin (Ig) subtypes are produced by plasma cells, with IgM the predominant immunoglobulin produced immediately after infection (Douch et al., 1994; Venturina et al., 2013; McRae et al., 2015). Later in the course of the immune response, IL-4 stimulates plasma cells to switch immunoglobulin class and produce IgG1, IgE and IgA (Paul and Zhu, 2010; Venturina et al., 2013).

IgE is found circulating in blood but is most closely associated with granulocytes including mast cells and eosinophils, stimulating degranulation by binding antigens and cross-linking fraction crystallizable (Fc) receptors found on the membrane of granulocytes (McNeilly et al., 2008; Paul and Zhu, 2010; Saddiqi et al., 2011; Venturina et al., 2013; Karrow et al., 2014; McRae et al., 2015). Cross-linking of Fc receptors also produces positive feedback on production of Fc receptors, increasing the sensitivity of the cells to GIN antigen (Paul and Zhu, 2010). Increased IgE, both total and GIN antigen-

specific, is associated with reduced FEC but also with increased breech soiling, speculated to be due to increased granulocyte-mediated mucosal inflammation (Shaw et al., 1999; McNeilly et al., 2009; Shakya et al., 2009; Venturina et al., 2013; Karrow et al., 2014; McRae et al., 2015). IgA2 is the best-known isoform of IgA, and is secreted as a dimer at mucosal surfaces (Macpherson et al., 2008; Karrow et al., 2014; McRae et al., 2015). There is also a circulating isoform of this immunoglobulin, IgA1 (Macpherson et al., 2008; Karrow et al., 2014; McRae et al., 2015). Both isoforms also occur as membrane-bound monomers on plasma cells (Hung et al., 2008). Mucosal IgA2 specific to GINs binds invading larvae, impairing their maturation and ability to interact with the mucosa (Schallig, 2000; Shaw et al., 2012; Karrow et al., 2014; McRae et al., 2015). Increased levels of both circulating and mucosal IgA lead to smaller adult worms, reduced egg production and therefore reduced FEC (Strain et al., 2002; Martínez-Valladeres et al., 2005; Halliday et al., 2007; Shaw et al., 2012; Shaw et al., 2013; Karrow et al., 2014; McRae et al., 2015). Conversely, IgG1 is found primarily as a circulating immunoglobulin, but elevated IgG1 also reduces GIN burdens and FECs (Douch et al., 1994; Bisset et al., 1996; Karrow et al., 2014; McRae et al., 2015). Circulating IgG1, and to a lesser extent IgA1, target gastrointestinal antigens of *Haemonchus* following ingestion of blood by the nematode (Knox et al., 2003; Ekoja and Smith, 2010). Since the adaptive immune response to GINs is Th2-driven, nematode-specific antibodies rather than effector cells dominate the adaptive response (see Figure 1.3) (Paul and Zhu, 2010; Saddiqi et al., 2011; Andronicos et al., 2012; Williams, 2012; Karrow et al., 2014; McRae et al., 2015). Indeed, development of a Th1 or Th17-dominated response favours establishment and maintenance of GIN infection (Venturina

et al., 2013; McRae et al., 2015). Nevertheless, some Th1 cytokines such as IL-12 may be expressed during the Th2 response to GIN infection, suggesting the possibility of a minor cell-mediated component to the adaptive response (Venturina et al., 2013).

1.4.3 Acquired response to non-protein antigens

The acquired response to non-protein GIN antigens, such as the glycolipid CarLA antigen, was traditionally believed to develop independently of T cell regulation, as antigens lacking protein cannot be presented on MHC II (Avci et al., 2013; Jaurigue and Seeberger, 2017). As with protein antigens, dendritic cells recognize and take up these antigens via PRRs (Avci et al., 2013; Jaurigue and Seeberger, 2017). Pure carbohydrates are converted to oligosaccharides, presented directly on the cell membrane, and are recognized by carbohydrate antigen-specific naïve B cells (Jaurigue and Seeberger, 2017). B cells activated via the T cell-independent pathway do not undergo immunoglobulin class switching or affinity maturation, and as a result the antibody response is weaker than for protein antigens (Jaurigue and Seeberger, 2017). Conversely, the response to glycolipids is not truly T cell-independent. Glycolipids are presented on the MHC I-like molecule CD1d (Avci et al., 2013). Invariant natural killer T (iNKT) cells, a subset of T cells with invariant T cell receptors, recognize glycolipid antigens presented on CD1d, and initiate a type 2 cytokine response similar to that for GIN protein antigens (Avci et al., 2013). Production of large amounts of IL-4 by iNKT cells activates additional dendritic cells, which present the antigen to naïve B cells (Avci et al., 2013). Activated B cells can switch immunoglobulin class when stimulated by activated iNKT cells, leading to production of higher levels of IgA, IgE and IgG1 than with T cell-

independent responses (see Figure 1.4) (Avci et al., 2013; Jaurigue and Seeberger, 2017). However, development of immunologic memory to non-peptide antigens is weak and responses wane in the absence of ongoing antigen exposure (Jaurigue and Seeberger, 2017). Conjugation of carbohydrates to protein carriers allows presentation of glycolipids on MHC II and development of more lasting T cell-dependent memory, a strategy under investigation in vaccine development (Avci et al., 2013; Jaurigue and Seeberger, 2017).

1.5 Effect of stress on immunity to gastrointestinal nematodes

The immunomodulatory effect of the hypothalamic-pituitary-adrenal axis (HPAA) is activated concurrently with the immune response to GINs. Production of type 1 cytokines IL-1, tumour necrosis factor alpha (TNF- α) and IL-6 stimulates release of corticotrophin releasing factor (CRF) and arginine vasopressin (AVP) from the hypothalamus (Karrow, 2006; You et al., 2008a). These in turn cause the anterior pituitary gland to convert pro-opiomelanocortin (POMC) to adrenocorticotrophic hormone (ACTH) (Karrow, 2006; You et al., 2008a). In response to ACTH, the adrenal cortex produces glucocorticoids, which feed back on the hypothalamus to reduce further synthesis of CRF and AVP (Karrow, 2006; You et al., 2008a). Glucocorticoids also affect immune cells, though the nature of their effect varies depending on the duration of glucocorticoid release (Dhabhar, 2009; Demas et al., 2011) and cell type (Hopkin et al., 2019). During acute stress, glucocorticoid release inhibits synthesis of type 1 cytokines (TNF- α , IFN- γ , IL-12), and promotes synthesis of type 2 cytokines (IL-4, IL-13, IL-10) (Karrow, 2006). This bias towards a type 2 cytokine milieu directs the subsequent antibody-mediated adaptive response (Karrow, 2006; Dhabhar 2009). However, development of Th1 memory cells is

also enhanced when antigen exposure is coupled with an acute stress response (Dhabhar, 2009). Thus, both the antibody- and cell-mediated response to subsequent antigen exposure is heightened during acute stress (Dhabhar, 2009). Conversely, chronic glucocorticoid elevation causes suppression of both cell- and antibody-mediated immunity (Dhabhar, 2009; Demas et al., 2011). Immunosuppressive effects of glucocorticoids are mediated through genomic and nongenomic pathways (Löwenberg et al., 2007). In the genomic pathway, glucocorticoids bind glucocorticoid nuclear receptors that inhibit transcription of inflammatory cytokines such as IL-1, and promote transcription of anti-inflammatory IL-10 (Löwenberg et al., 2007; Dhabhar, 2009). Attenuation of the cellular response takes hours to days due to the requirement for transcription of new anti-inflammatory proteins and degradation of existing inflammatory mediators (Löwenberg et al., 2007). Non-genomic effects occur more rapidly, and include reduction in activity of phagocytic and B cells via altered signal transduction and cytosolic calcium (Löwenberg et al., 2007). In addition, glucocorticoids generally exert a stimulatory effect on the early immune response, but play a more inhibitory role later in developed immune responses (Dhabhar, 2009), and also promote tissue repair during resolution of the inflammatory response (Hopkin et al., 2019; Jurberg et al., 2018).

Sheep display considerable variation in reactivity of the HPA axis to inflammatory stimuli, demonstrable when challenged with lipopolysaccharide (LPS), a bacterial PAMP (You et al., 2008a). This variation in HPA axis response remains relatively constant for at least 3 years and has been shown to affect acquired immunity, with both extremely high and extremely low cortisol responsiveness being associated with increased cutaneous

hypersensitivity response and decreased primary antibody response (Karrow, 2006; You et al., 2008a; You et al., 2008b). Glucocorticoid release in sheep is affected by many factors, such as increased metabolic stress during gestation, parturition and lactation, inherent to reproduction, environmental stressors such as climactic extremes, and nutritional stress including that resulting from parasite infestation (Karrow, 2006). It is unclear how HPAА reactivity affects immunity to GINs, or whether it may be a basis for identifying sheep that are more or less able to mount an immune response to GINs.

1.6 Efforts to improve immunity of sheep to gastrointestinal nematodes

1.6.1 Vaccines

The complementary and interconnected effects exerted by the innate and adaptive immune responses on the GIN life cycle have been extensively researched in an attempt to improve productivity of sheep in the face of GIN parasitism. One approach is development of GIN vaccines. A simple form of vaccine involves oral administration of irradiated, nonviable larvae, but this approach has limited efficacy in lambs (Urquhart et al., 1966; Miller and Horohov, 2006; Kemper et al., 2009; Saddiqi et al, 2011; Andronicos et al., 2012). Efforts to develop more refined and effective vaccines have improved understanding of specific GIN antigens that stimulate the immune response, particularly CarLA and *Haemonchus* sp. gastrointestinal antigens (Andrews et al., 1995; Andrews et al., 1997; Knox et al., 1999; Smith et al., 2001; Knox et al., 2003; Smith et al., 2003b; Miller and Horohov, 2006; Ekoja and Smith, 2010; Matthews et al., 2016). A vaccine using H-gal-GP gastrointestinal antigen purified from *Haemonchus contortus* has been approved for use in Australia since 2014 (Bassetto and Amarante, 2015; Matthews

et al., 2016). However, production of this vaccine requires isolation from adult *Haemonchus* sp., as all attempts to date at generating recombinant antigen have failed to elicit immunity in vaccine trials (Emery et al., 2016; Matthews et al., 2016). Moreover, repeated vaccination at 4-5 week intervals is necessary to maintain robust immunity, likely owing to the reduced memory response to the carbohydrate component of glycoprotein antigens (Emery et al., 2016). The labour and cost associated with both production and delivery of this vaccine limits its practicality in the sheep industry (Emery et al., 2016). More recently, a vaccine incorporating eight recombinant larval protein antigens derived from *Teladorsagia circumcincta* has proven successful at reducing FECs and burdens following experimental challenge with *T. circumcincta* L₃s (Nisbet et al., 2013; Nisbet et al., 2016). It is not known how the vaccine achieves these reductions, however, as levels of antibody to the recombinant antigens were not associated with FEC (Nisbet et al., 2016). This vaccine is subject to other limitations shared with the *H. contortus* vaccine, namely the requirement for repeated vaccinations and protection being limited to one GIN species (Nisbet et al., 2013; Nisbet et al., 2016). Few other effective vaccines have been identified due to a combination of the complexity of the immune response to GINs, differences in GIN behavior, and difficulties in initiating mucosal immunity and generating recombinant antigens (Knox et al., 1999; Smith et al., 2001; Knox et al., 2003; Smith et al., 2003b; Miller and Horohov, 2006; Maass et al., 2007; McNeilly et al., 2008; Jackson et al., 2009; Hein et al., 2010; Shaw et al., 2013; Matthews et al., 2016; Emery et al., 2016). Selection of breeding animals with genetic resistance to GINs likely offers farmers a practical, economical alternative, and has received much attention.

1.6.2 *Selecting for genetic resistance versus resilience*

An important distinction must be made between sheep that are genetically resistant to GINs and sheep that are genetically resilient to GINs (Albers et al., 1987; Bisset and Morris, 1996; Woolaston and Baker, 1996; Jasmer et al., 2007; Colditz, 2008; Jackson et al., 2009; Sargison, 2011; Saddiqi et al., 2011; Alba-Hurtado and Muñoz-Guzmán, 2013). Sheep are considered resilient to GINs if they maintain productive parameters despite high GIN burdens, and resistant if their immunological response to GINs constrains infective nematode burdens to low levels (Albers et al., 1987; Bisset and Morris, 1996; Douch et al., 1996; Woolaston and Baker, 1996; Colditz, 2008; Jackson et al., 2009; Saddiqi et al., 2011; Saddiqi et al., 2012; Alba-Hurtado and Muñoz-Guzmán, 2013). Both phenotypes have positive and negative attributes from a production standpoint, and there remains debate as to which is most desirable with respect to addressing the problem posed by GINs (Albers et al., 1987; Bisset and Morris, 1996; Woolaston and Baker, 1996; Colditz, 2008). Since resistant sheep generally carry lower GIN burdens they shed fewer ova, thus reducing pasture contamination and protecting more susceptible flockmates – a benefit not shared by the resilient phenotype (Albers et al., 1987; Woolaston and Baker, 1996; Sayers and Sweeney, 2005). One concern of selection for resistant animals is increased selection pressure on GINs to increase their pathogenicity in an attempt to overcome increased host defences (Woolaston et al., 1992; Kemper et al., 2009; Kemper et al., 2013). However, multiple trials have found no evidence of adaptation to host resistance following up to 30 generations of serial infection (Adams, 1988; Kemper et al., 2009; Kemper et al., 2013). In an experiment by Kemper et al, strains of *Haemonchus contortus* and *Trichostrongylus colubriformis* passaged for

multiple generations through resistant or susceptible lambs were used to infect susceptible lambs, and no significant difference was found in FEC or, in the case of *Haemonchus contortus*, packed cell volume, between the strain passaged through resistant lambs and the strain passaged through susceptible lambs (Kemper et al., 2009). Moreover, a computer model of the probability of single locus variation causing GINs to adapt to host resistance indicated low overall likelihood of GINs undergoing genetic adaptation to altered host resistance (Kemper et al., 2013). Given these results, adaptation of GINs to changing host resistance appears to occur slowly, if at all (Adams, 1988; Woolaston et al., 1992; Kemper et al., 2009; Kemper et al., 2013).

Another key criticism of the resistant phenotype is the greater tendency in such sheep towards developing diarrhea and wool soiling following initial exposure to GIN challenge, believed to be due to an enhanced inflammatory response (Bisset and Morris, 1996; Colditz et al., 1996; Douch et al., 1996; Bisset et al., 1997; Shaw et al., 1999; Bisset et al., 2001; Greer, 2008; Williams, 2011; Williams and Palmer, 2012; Shaw et al., 2013). Breech soiling increases the risk of fly larvae infestation of the wool and underlying skin (myiasis) and, in wool-producing breeds, increased dags result in financial loss due to loss of wool (Adams, 1988; Woolaston and Baker, 1996; Williams, 2011; Williams and Palmer, 2012). However, numerous studies have indicated that the relationship between dag score and resistance varies depending on the immunological mechanisms conferring resistance (Bisset and Morris, 1996; Colditz et al., 1996; Bisset et al., 2001; Williams and Palmer, 2012). Specific immune responses associated with increased fecal soiling include elevated IgE, elevated IgG1, and increased eosinophil

infiltration of the abomasal and/or intestinal mucosa (Albers et al., 1987; Douch et al., 1996; Shaw et al., 1999; Williams, 2011). Conversely, although elevated GIN-specific IgA is associated with reduced FEC, no relationship between IgA and dag score has been identified. Therefore, selection based on IgA response may mitigate the risk of dag-associated myiasis and wool loss (Strain et al., 2002; Shaw et al., 2012; Shaw et al., 2013). The impact of the resistant phenotype on productivity parameters such as weight gain, wool growth and milk production is similarly complex. Reduced milk production by resistant phenotype ewes is believed to contribute to reduced rate of gain in their lambs (Greer, 2008). In the case of weaned lambs classified as resistant based on low FEC or high IgE, numerous studies report reduced growth and wool production compared with resilient or random bred lambs when grazed together under GIN challenge (Bisset and Morris, 1996; Douch et al., 1996; Bisset et al., 1997; Shaw et al., 1999; Colditz, 2008; Greer, 2008). However, lambs selected for low FEC may display improved wool growth and weight gain when grazed separately from lambs with higher FEC, speculated to be due to lower exposure to GINs (Bisset et al., 1997). Moreover, lambs with high GIN-specific IgA also display improved growth, further supporting the use of IgA in selection programs (Shaw et al., 2012; Shaw et al., 2013).

Despite the association between increased fecal soiling, reduced productivity and resistance, efforts to improve immunity in sheep have focused more on selection for resistance than resilience (Woolaston and Baker, 1996; Bisset and Morris, 1996; Bisset et al., 2001; Williams, 2011). An important reason is the difference in heritability of these phenotypes. Low FEC, the most commonly assessed indicator of resistance, has moderate

heritability, whilst resilience, as measured by maintenance of growth rate under challenge, has low heritability (Albers et al., 1987; Bisset and Morris, 1996; Woolaston and Baker, 1996; Bisset et al., 2001; Vagenas et al., 2007). Given that resistant sheep shed fewer GIN ova, it is likely that concerted efforts to increase resistance in a flock will gradually increase productivity by reducing overall exposure to GINs (Albers et al., 1987; Woolaston and Baker, 1996; Bisset et al., 2001; Saddiqi et al., 2012). The higher heritability of resistance also implies that the progress of genetic selection will occur more rapidly than would selection for resilience (Albers et al., 1987; Bisset et al., 2001).

1.6.3 Screening for genetic resistance

A number of parameters have been used to screen flocks and identify resistant animals. The best characterized and most widely used is FEC (Woolaston, 1992; Woolaston and Baker, 1996; Sayers and Sweeney, 2005; Kemper et al., 2009; Menzies et al., 2010; Alba-Hurtado and Muñoz-Guzmán, 2013; Venturina et al., 2013; Karrow et al., 2014). The McMaster method is the most commonly used technique for assessing FECs, and involves suspension of a predetermined amount of feces in a set volume of diluent (Gordon and Whitlock, 1939; Whitlock, 1948; Pereckienė et al., 2007; Vadlejch et al., 2011; Taylor et al., 2016). The solution is strained to remove large particulates and used to fill grid chambers on a glass slide (Gordon and Whitlock, 1939; Whitlock, 1948; Pereckienė et al., 2007; Vadlejch et al., 2011; Taylor et al., 2016). GIN ova within the grid lines are counted and a correction coefficient applied to yield FEC in eggs/gram (epg), accounting for the volume of diluent and degree of dilution of the fecal material (Gordon and Whitlock, 1939; Whitlock, 1948; Pereckienė et al., 2007; Vadlejch et al.,

2011; Taylor et al., 2016). However, there are numerous modifications to the amount of fecal material used, type and volume of solution used for dilution, and the number and volume of the grid chambers used to count collected ova, each of which have different correction coefficients and therefore varying sensitivity (Pereckienė et al., 2007; Vadlejch et al., 2011). The most commonly used modified McMaster method mixes 4 g of feces in 56 mL of saturated NaCl solution, does not include a centrifugation step, and reads FEC from two grid chambers, each with a volume of 0.15 mL, yielding a minimum level of sensitivity of 50 epg (Taylor et al., 2016). This method is relatively quick and easy to perform, but its low sensitivity prevents comparison of FECs from resistant animals with low egg counts. A higher sensitivity of 8.33 epg can be obtained with the newer triple-chamber modified McMaster technique, in which 4 g of feces are mixed in 26 mL of saturated NaCl solution and the FEC is determined from three 0.3 mL grid chambers (Pereckienė et al., 2007). The highest sensitivity is obtained through double centrifugation, in which 3 g of feces are mixed in 42 mL of water (Taylor et al., 2016). A 15 mL aliquot of the diluent is then centrifuged, the supernatant poured off, and the residue suspended in 15 mL saturated NaCl solution before a second centrifugation step to collect the GIN ova on a coverslip (Taylor et al., 2016). This method typically collects all GIN ova in the aliquot, yielding a sensitivity of 1 epg (Taylor et al., 2016). Despite its high sensitivity, repeated centrifugation makes this the most costly and time-consuming method, and it is not widely used outside of research facilities.

FEC strongly reflects GIN burdens and therefore selection for resistance can be accomplished by selecting individuals with low FEC (Stear et al., 1995; Douch et al.,

1996; Woolaston and Baker, 1996; Sayers and Sweeney, 2005; Saddiqi et al., 2012). However, regardless of method, there are several limitations inherent with FEC. Multiple species of GIN shed indistinguishable ova, including GINs with minimal to no pathologic effect; consequently, non-pathologic GINs can inflate FEC (Saddiqi et al., 2012; Bowman, 2014; Taylor et al., 2016). For the same reason, when used alone FEC cannot provide information on the variety of GIN species present. Identification and quantification of GIN species in sheep feces requires culture to the larval stage. The current standard for GIN speciation is morphologic identification of L₃s isolated from fecal culture (MAFF, 1986; van Wyk and Mayhew, 2013). This method involves culturing feces in an incubator at 26 °C for 7 days, followed by isolation of larvae using a Baermann apparatus and evaluation of microscopic morphologic characteristics (MAFF, 1986; van Wyk and Mayhew, 2013). However, morphologic speciation is rarely performed due to the extensive labour and expertise required, and the subtlety of morphologic differences between some GIN species make this method prone to error (Avramenko et al., 2015). Moreover, estimation of species composition requires morphologic identification of a minimum of 100 larvae, and high larval attrition during development to L₃s necessitates culture of large volumes of feces or smaller volumes with high FEC (MAFF, 1986; van Wyk and Mayhew, 2013). In sheep, fecal pellet volumes are rarely high enough to isolate sufficient L₃s from an individual fecal sample. Pooling samples to increase fecal mass overcomes this limitation, but prevents identification of individual animals with GIN resistance.

A novel GIN speciation method using deep amplicon sequencing of the ITS-2 rDNA locus has recently been described and validated for L₃ GINs of cattle (Avramenko et al., 2015; Avramenko et al., 2017). Use of PCR reduces the risk of error, allows concurrent speciation of thousands of larvae, increasing sensitivity, and requires considerably less labour than morphologic speciation (Avramenko et al., 2015; Avramenko et al., 2017). Both labour and required fecal volumes are reduced further when PCR is performed on L₁s, requiring culture for 48 hours at 20 °C (Burgess et al., 2012; Avramenko et al., 2017). Variation in rate of mitosis between ova leads to variable numbers of cells, and therefore DNA copy numbers, between eggs, and renders PCR of ova impractical due to a high probability of bias in species proportions (Avramenko et al., 2017). There is less within- and between-species variation in stage of development following culture to L₁s, and species estimates can be corrected for differences in size and cell numbers between larvae of different GIN species (Burgess et al., 2012; Avramenko et al., 2015; Avramenko et al., 2017). Despite its advantages relative to traditional morphologic speciation of L₃s, use of larval PCR has yet to be validated for GINs of sheep.

Using FEC to estimate resistance requires anthelmintic treatment to be withheld, which may impact animal welfare and productivity (Douch et al., 1996). Time of year also has an effect on mean FECs, which, in lambs, tend to peak by the middle of the grazing season and decrease afterwards (Mederos et al., 2010; Singleton et al., 2011; Karrow et al., 2014). For lambs born during the winter that do not graze throughout the typical grazing season, there may be insufficient GIN exposure to determine resistance based on FEC. In addition to seasonal fluctuation in mean FEC, individual FECs vary between

samplings due to a variety of factors (Douch et al., 1996; Sayers and Sweeney, 2005; Mederos et al., 2010; Singleton et al., 2011; Saddiqi et al., 2012; Falzon et al., 2013b). Stress due to nutrition or weather conditions can affect the interaction between GINs and their host, and therefore alter FECs (Douch et al., 1996; Sayers and Sweeney, 2005; Falzon et al., 2013b). Other environmental factors, including stocking density, density of pasture herbage, and individual grazing preferences, can alter degree of exposure to infective GIN larvae, and therefore GIN burdens (Singleton et al., 2011). Sampling individual FECs on multiple occasions facilitates assessment of resistance under varying environmental conditions, although the added cost and effort required to collect and process more fecal samples renders this not always feasible, particularly with very large flocks (Douch et al., 1996; Woolaston and Baker, 1996; Sayers and Sweeney, 2005; Saddiqi et al., 2012).

Recognition of the limitations of FEC has encouraged the investigation of several clinical parameters as potential markers of resistance. One well-validated method already used in targeted selective treatment protocols is the Faffa Malan Chart (FAMACHA®), which assesses ocular mucous membrane colour as an indicator of the degree of anemia secondary to parasitism by *Haemonchus contortus* (Jackson et al., 2009; Saddiqi et al., 2012; Venturina et al., 2013; Bath, 2014; Mederos et al., 2014; Pereira et al., 2016). Like FEC, FAMACHA® scores have moderate heritability and the added benefit that minimal training and equipment is required to perform scoring (Jackson et al., 2009; Saddiqi et al., 2012; Venturina et al., 2013; Bath, 2014; Pereira et al., 2016). However, as FAMACHA® is dependent on anemia, it has less utility when *Haemonchus contortus* is

not the primary GIN involved, as other GINs do not cause anemia (Jackson et al., 2009; Mederos et al., 2014). This renders FAMACHA© a more practical tool for selection of resistant animals in regions where *Haemonchus contortus* is known to be the predominant GIN (Mederos et al., 2014). Moreover, FAMACHA© does not distinguish between *Haemonchus* and other causes of anemia, such as fascioliasis or coccidiosis, and does not clearly differentiate between resistant and resilient animals (Saddiqi et al., 2012; Taylor et al., 2016). Selecting sheep with low dag scores, a measure of breech soiling by fecal material due to diarrhea, is another method that requires minimal training and has moderate heritability (Bisset and Morris, 1996; Colditz et al., 1996; Douch et al., 1996; Shaw et al., 1999; Williams, 2011; Pickering et al., 2012; Williams and Palmer, 2012; Shaw et al., 2013; Pickering et al., 2015). However, as with FAMACHA©, GINs are not the only cause of increased dag score. Indeed, other common causes of scouring include coccidial or bacterial enteritis (Taylor et al., 2016). Further, since higher dag scores are associated with some mechanisms of phenotypic resistance to GINs, and selection solely on the basis of low dag score will inevitably lead to culling of highly resistant sheep, dag score utility is improved when combined with FEC (Bisset and Morris, 1996; Colditz et al., 1996; Douch et al., 1996; Shaw et al., 1999; Bisset et al., 2001; Williams, 2011; Williams and Palmer, 2012).

Assessment of GIN-specific immunoglobulins offers a more precise method of screening for resistant animals. As with phenotypic markers of resistance such as FEC and FAMACHA©, circulating IgG1 and IgE have moderate heritability (Douch et al., 1996; Shaw et al., 1999). However, the association of these immunoglobulins with increased

dags and reduced lamb growth reduces their appeal as criteria for selection of resistant sheep (Douch et al., 1996; Shaw et al., 1999; Williams, 2011). The heritability of circulating IgA is higher than the other circulating immunoglobulin isoforms, though a relatively small proportion of lambs have high circulating IgA (Strain et al., 2002; Shaw et al., 2012). The low prevalence of elevated circulating IgA renders selection of lambs with high circulating IgA less practical than selection based on mucosal IgA, and serum IgA is not necessarily reflective of mucosal IgA levels at the site of GIN infection (Strain et al., 2002; Shaw et al., 2012; Shaw et al., 2013). Mucosal IgA offers several advantages compared with circulating immunoglobulins. Unlike circulating IgG1 and IgE, mucosal IgA is not associated with reduced growth or increased dags (Shaw et al., 2012; Shaw et al., 2013). Moreover, sampling is less invasive than for circulating immunoglobulins, as it requires a saliva sample rather than serum, and can be performed in lambs as early as 2 months of age (Shaw et al., 2012; Shaw et al., 2013). Salivary IgA originates primarily from plasma cells within the salivary glands and is reflective of, but not equivalent to, concentrations of IgA in the abomasum and small intestine (Shaw et al., 2012). B cells specific to GIN antigens reach the salivary glands and mature to plasma cells following priming by gut-derived dendritic cells in lymphoid tissues, a mechanism that allows antigen-specific lymphocytes to migrate to mucosal tissues distant to the site of initial infection (Samuelson et al., 2015). The best-validated assay for mucosal IgA quantifies IgA specific to CarLA, and therefore reflects mucosal immunity to all GINs, whereas existing assays of circulating immunoglobulins are generally species specific (Douch et al., 1996; Shaw et al., 1999; Harrison et al., 2003; Shaw et al., 2012; Shaw et al., 2013). This mucosal IgA test has shown promise in genetic screening programs in New Zealand,

but has yet to be assessed under Ontario grazing conditions (Shaw et al., 2012; Shaw et al., 2013).

An alternative to screening for resistance specific to GINs could be to select sheep based on overall health resilience, as measured by immune competence and stress responsiveness (Thompson-Crispi et al., 2013; Aleri et al., 2019). A method for evaluation of cell- and antibody-mediated immune responses to vaccination with *Candida albicans* and hen egg white lysozyme, respectively, has been patented and used successfully in selective breeding of dairy cattle (Heriazon et al., 2009; Thompson-Crispi et al., 2013). Following challenge, overall immune competence can be classified as below average, average, or high (Heriazon et al., 2009; Thompson-Crispi et al., 2013; Cartwright et al., 2017; Aleri et al., 2019). Cattle classified as high immune responders (HIRTM) have lower incidence of numerous common diseases, including digital dermatitis, ketosis, mastitis, and metritis (Thompson-Crispi et al., 2013; Cartwright et al., 2017). Recently, HIRTM cattle have also been found to shed lower numbers of GIN ova, and to be more resilient to stresses associated with handling (Aleri et al., 2019). However, to date there are no other reports of associations between immune responses to *Candida albicans* and hen egg white lysozyme and nematode parasitism in cattle, and it is not known whether these immune response phenotypes are also associated with overall health in sheep. As discussed in Section 1.5, sheep display variability in stress response to challenge with LPS, and this variation in stress response is associated with variable cell- and antibody-mediated immune responses (Karrow, 2006; You et al., 2008a; You et al., 2008b). As individual stress responses to LPS in sheep are consistent over time

(Karrow, 2006; You et al., 2008a; You et al., 2008b), selection based on stress response is possible but the relationship between stress response and GIN immunity is unknown. In light of the positive effects of selection for high immune competence and ability to cope with stress on health in dairy cattle, these phenotypes merit investigation in sheep.

1.6.4 Genetic markers of resistance

With the advent of high throughput genetic sequencing technology, it is now possible to identify variation in genetic sequences of sheep that are associated with GIN resistance (Jenko et al., 2015; Jonas and De Koning, 2015). Single nucleotide polymorphisms (SNPs) now form the basis of current genomic selection because of their high frequency and ease of genotyping (Jenko et al., 2015; Jonas and De Koning, 2015). Two groups of sheep with differential GIN resistance, for example, can be genotyped using these genetic markers, and SNPs that are found to be associated with resistance can then be used to breed for increased GIN resistance using genomic selection strategies (Diez-Tascón et al., 2005; Knight et al., 2010). This approach can involve comparisons between susceptible and resistant breeds, such as the Red Maasai and Barbados Blackbelly (Diez-Tascón et al., 2005; Dominik, 2005; Crawford et al., 2006; Knight et al., 2010; Kemper et al., 2011; Benavides et al., 2015), or it can involve comparisons within breeds that have resistant and susceptible lines (Coltman et al., 2001; Diez-Tascón et al., 2005; Dominik, 2005; Crawford et al., 2006; Knight et al., 2010). These comparisons have identified a wide variety of polymorphisms associated with GIN resistance on multiple chromosomes, but the significance of these resistance genes is often specific to the breed(s) examined, indicating that resistance to GINs is a complex polygenic trait (Coltman et al., 2001;

Diez-Tascón et al., 2005; Dominik, 2005; Sayers et al, 2005; Crawford et al., 2006; Knight et al., 2010; Kemper et al., 2011; Benavides et al., 2015). A summary of quantitative trait loci (QTLs) associated with GIN resistance is presented in Table 1.1. Genetic screening faces other challenges, among them the cost and effort required to genotype an entire flock, and the low frequency with which multiple favourable alleles occur in the same individual (Jenko et al., 2015). Moreover, the ovine genome is not completely annotated, thus the function(s) of many genes containing polymorphisms associated with resistance are currently unknown (Diez-Tascón et al., 2005; Benavides et al., 2015). As such, more investigation of the effects of ovine GIN resistance-associated genes is required before a screening program for resistance alleles can be implemented. Nevertheless, ongoing investigation of SNPs underlying both GIN resistance and productivity in sheep will eventually enable selection of breeding stock on the basis of favourable genetic polymorphisms rather than less sensitive phenotypic markers such as FEC and CarLA (Jonas and De Koning, 2015).

Novel genome editing technology, which enables precise cleavage and alteration of a target genetic sequence, offers a method to speed up the rate of genetic progress for traits such as GIN resistance (Jenko et al., 2015). This technology could be used to generate embryos carrying large numbers of favourable alleles, which could then be used as breeding stock to disseminate those alleles and increase their frequency within a population (Jenko et al., 2015). At present, the success rate of genome editing is very low, with poor survival of edited embryos and low frequency of edited alleles in surviving embryos (Jenko et al., 2015). In addition, models of genetic progress with

genome editing indicate an increased rate of inbreeding, given the genetic superiority of progeny of edited animals relative to unedited animals (Jenko et al., 2015). This may lead to reduced diversity in traits unrelated to immunity to GINs and have unintended detrimental effects on animal health. This effect can be mitigated if inbreeding is monitored through collation of pedigree and genotype information and efforts made to control such inbreeding (Jenko et al., 2015). However, both genomic selection and genome editing may be challenging to implement in the Canadian sheep industry due to the small overall flock size and lack of centralized genetic stock suppliers (Jenko et al., 2015; Jonas and De Koning, 2015). Consumer wariness of genetic manipulation may also prove an additional barrier to genome editing. Nevertheless, further development of these technologies offers immense potential for improvement in GIN resistance.

1.7 Thesis rationale and objectives

Gastrointestinal nematodes (GINs) are a significant problem in sheep both within Canada and globally, adversely affecting health and productivity. However, the impact of GIN infection on reproductive performance has not been clearly elucidated. In Ontario, resistance to commonly used anthelmintic drugs, the traditional means of managing the impact of GINs, is widespread. Strategies that exploit enhanced immune response to GINs in some sheep may lead to improved productivity and health under Ontario grazing conditions. The immune response to GINs involves complex interplay between stress and the innate and adaptive branches of the immune system. A Th2 antibody-mediated adaptive response to carbohydrate-containing parasite antigens appears to be favoured, although this response wanes rapidly in the absence of ongoing GIN exposure. Thus far,

extensive research has failed to generate an effective GIN vaccine that can be easily mass-produced and generates a protective immune response that lasts more than a few weeks. Therefore, selective breeding of animals that can generate a superior immune response to GINs appears to be a more attractive solution.

Numerous criteria for selection of resistant sheep including FEC, dag score and CarLA salivary antibody have demonstrated moderate heritability and have been applied successfully to genetic improvement programs in other countries, particularly in New Zealand and Australia. Although lambs are generally believed to develop resistance to GINs by 4-9 months of age, it is unclear whether this is also the case under Ontario grazing conditions where lambs are typically marketed at less than 6 months of age. Moreover, the epidemiology of GINs under Ontario grazing conditions differs considerably, with a prolonged period during winter with minimal to no exposure to infective larvae. To the authors' knowledge, there are no data regarding how immunity to GINs develops in lambs in temperate grazing conditions with cold winters (as in Ontario), whether the immune response develops differentially to different species of GIN under such conditions, or the efficacy of criteria for selection of GIN-resistant sheep under Ontario grazing conditions.

The traditional means of determining the composition of mixed GIN infections, which is typical on most sheep farms in Ontario, is through morphologic speciation of L₃s. However, this method is highly labour-intensive and prone to error. Deep amplicon sequencing of the ITS-2 rDNA locus in L₁s overcomes many limitations of morphologic

speciation of L₃s and may offer a more practical and accurate alternative, though validation of this method in GINs of small ruminants is needed.

The primary goal of this thesis is to investigate the immune response of sheep to GINs under Ontario grazing conditions in order to identify criteria that can be used in genetic selection for GIN resistance. To address this goal, the primary objectives of this thesis are to:

- 1) Compare deep amplicon sequencing of the ITS-2 rDNA locus in L₁s with traditional morphologic speciation of L₃s small ruminant GINs (Chapter Two).
- 2) Evaluate the association between GIN parasitism and growth and reproductive performance of Ontario ewe lambs (Chapter Three).
- 3) Determine the correlation of salivary anti-CarLA antibody with GIN parasitism and performance in Ontario ewe lambs (Chapter Four).
- 4) Determine whether sheep with different acute stress responsiveness display differences in GIN parasitism (Chapter Five).
- 5) Generate preliminary information on the effect of variable cell- and antibody-mediated immune responses on GIN parasitism in sheep (Chapter Six).

The findings described in these chapters may be used to direct efforts at genetic selection of sheep for resistance to GINs under Ontario grazing conditions. A summary of key messages, limitations, and directions for future research is discussed in Chapter Seven.

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1.9 Table

Table 1.1. Selected quantitative trait loci associated with gastrointestinal nematode (GIN) resistance in sheep.

Breed	GIN	Phenotype	Chromosome	Related product	Function	Reference
Mixed, Merino predominate	<i>T. colubriformis</i>	FEC	15	NS	NS	Kemper et al., 2011
			20	NS	NS	
			26	NS	NS	
	<i>H. contortus</i>	FEC	1	NS	NS	
			17	NS	NS	
			18	NS	NS	
Red Maasai X Dorper	Mixed	FEC	2	NS	NS	Benavides et al., 2015
			6	NS	NS	
			11	SOX9	Transcription factor	
			12	LAMC1	Cytokine	
			15	MUC15	Mucus synthesis	
Soay	<i>T. circumcincta</i>	FEC	3	IFN- γ	Cytokine	Coltman et al., 2000
Romney X Coopworth	<i>T. colubriformis</i>	GIN burden	8	NS	NS	Crawford et al., 2006
Suffolk	Mixed	FEC	20	DRB1	MHCII antigen binding cleft	Sayers et al., 2005

FEC = fecal egg count; NS = not specified.

1.10 Figures

Figure 1.1. Prototypical trichostrongylid nematode life cycle. Derived from Bowman, 2014.

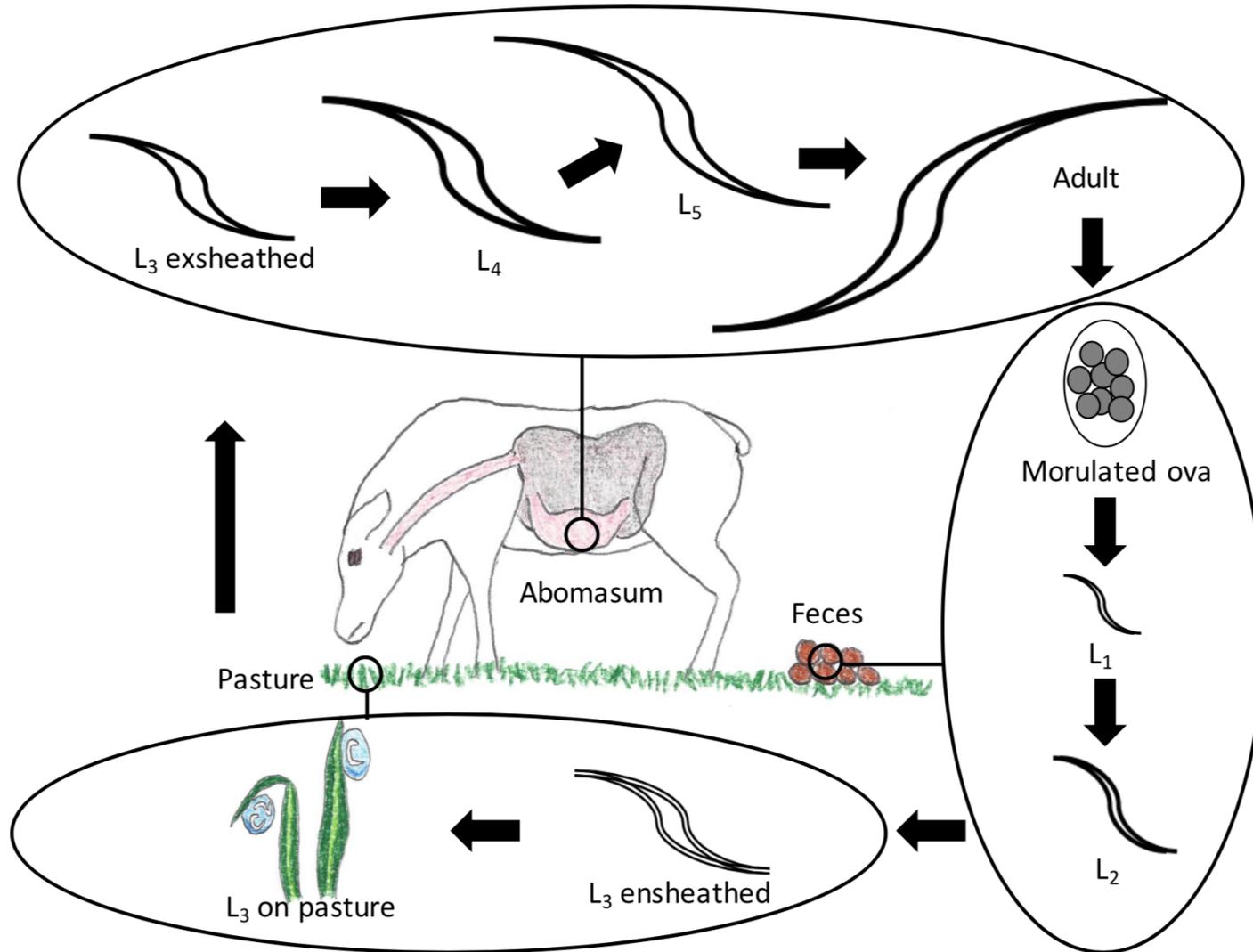


Figure 1.2. Selected mechanisms of the innate response to gastrointestinal nematodes. Red arrows indicate inhibitory signals, green arrows indicate stimulatory signals. ROS = reactive oxygen species; RNS = reactive nitrogen species; PAMPs = pathogen associated molecular patterns; DAMPs = damage associated molecular patterns; IL = interleukin.

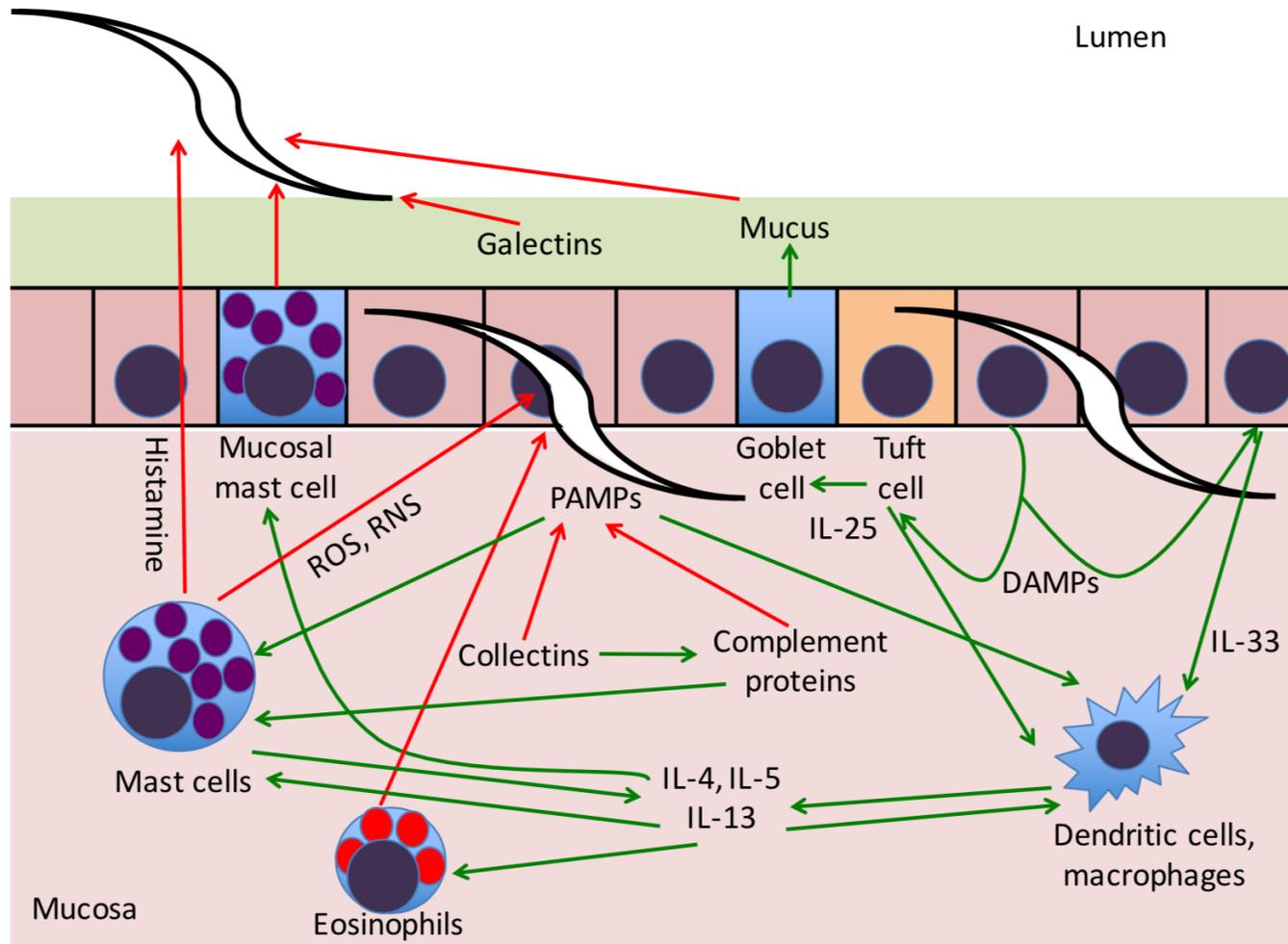


Figure 1.3. Selected mechanisms of the acquired response to gastrointestinal nematode protein antigens. Red arrows indicate inhibitory signals, green arrows indicate stimulatory signals. TGF = transforming growth factor; Treg = T regulatory; CD = cluster of differentiation; IL = interleukin; MHC = major histocompatibility complex; Th = T helper; Ig = immunoglobulin; FcεR = fraction crystallizable epsilon receptor.

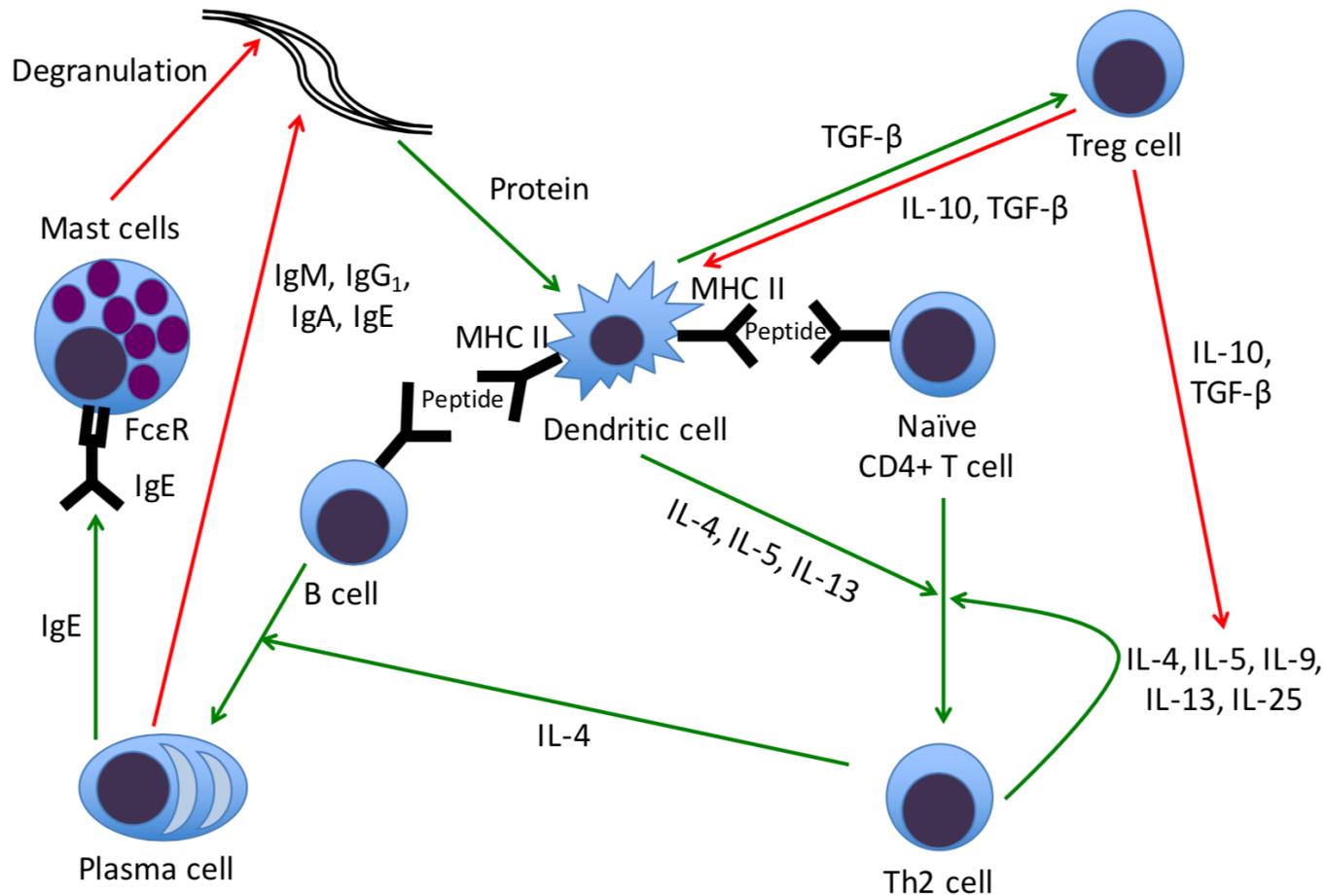
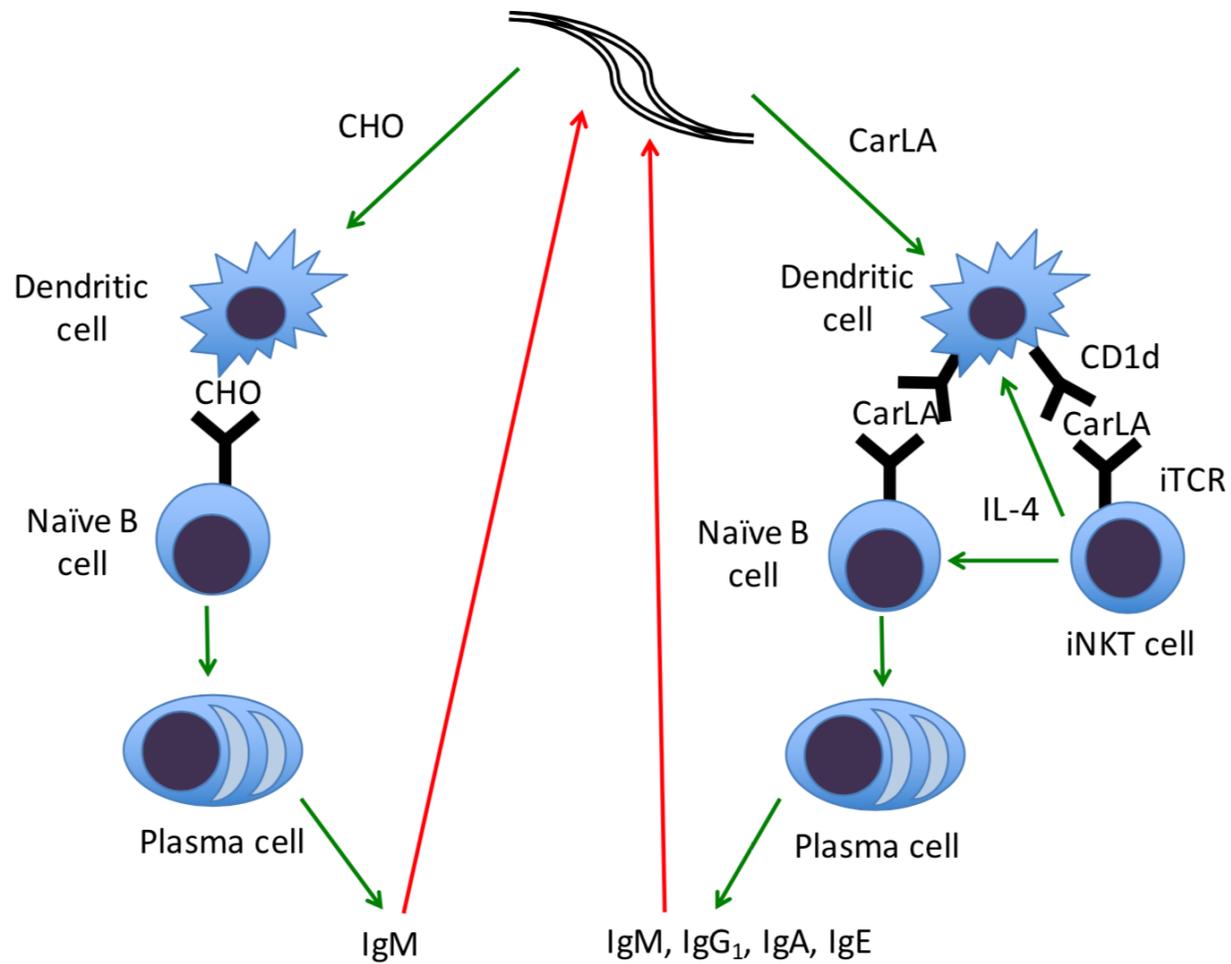


Figure 1.4. The antibody response to gastrointestinal nematode non-protein antigens. Red arrows indicate inhibitory signals, green arrows indicate stimulatory signals. CHO = carbohydrate; CarLA = carbohydrate larval antigen (prototypical glycolipid antigen); CD = cluster of differentiation; iTCR = invariant T cell receptor; IL = interleukin; iNK = invariant natural killer; Ig = immunoglobulin.



CHAPTER TWO:

**COMPARISON OF DEEP AMPLICON SEQUENCING WITH
MORPHOLOGICAL IDENTIFICATION TO QUANTIFY
GASTROINTESTINAL NEMATODE SPECIES COMPOSITION IN
SMALL RUMINANT FECES**

Based on a manuscript in preparation for submission to *Veterinary Parasitology*.

Abstract

Mixed gastrointestinal nematode (GIN) infections are a common and significant cause of financial loss for small ruminant producers. Morphologic speciation of third-stage larvae (L₃s) can be used to identify species composition in feces but is seldom used due to the requirement for specialized expertise and the extensive time, labour and cost involved. Deep amplicon sequencing of L₃s mitigates risk of misidentification and allows for higher throughput with reduced labour. However, larval attrition during culture to the third stage is typically high. Culture and sequencing of first-stage larvae (L₁s) is faster, further reduces labour, and fewer larvae may be lost between hatching and sequencing due to shorter culture duration. However, results of deep amplicon sequencing have not been previously compared with morphologic speciation in small ruminants. This study used deep amplicon sequencing of L₁s and morphologic speciation of L₃s to compare the species proportions of small ruminant gastrointestinal nematodes generated from seven pooled fecal samples. Larval recovery as a proportion of cultured ova was also compared

between L₁ and L₃ cultures. Significantly higher proportions of *Teladorsagia circumcincta* (odds ratio = 3.1, p = 0.008) and lower proportions of *Trichostrongylus* spp. (p = 0.009) were identified using deep amplicon sequencing of L₁s than morphologic speciation of L₃s, consistent with previous reports of biased survival of these species in L₃ cultures. Larval recovery rate was significantly higher from L₁ cultures than from L₃ cultures (p < 0.0001); eggs were 238.7 times more likely to develop to L₁s than to L₃s (95 % confidence interval for odds ratio 80.0-712.0). These results indicate that deep amplicon sequencing of L₁s may reduce bias introduced by differential GIN survival to L₃s in small ruminants.

Keywords

Nemabiome, sheep, goat, nematodes, *Haemonchus*, *Trichostrongylus*, *Teladorsagia*

2.1 Introduction

Parasitic gastrointestinal nematodes (GINs) are ubiquitous on sheep and goat farms, and are a significant cause of morbidity, mortality, and loss of productivity worldwide (Mederos et al., 2010; Mavrot et al., 2015). While infections generally consist of a mixture of species, fecal egg counts (FECs), the most common method of monitoring such infections, cannot differentiate among identical ova produced by multiple GIN species (Mederos et al., 2010; Zajac and Conboy, 2012; Avramenko et al., 2015; Taylor et al., 2016; Avramenko et al., 2017; Redman et al., submitted). Confirmation of the infecting GIN species offers the opportunity for selection of optimal anthelmintic treatment, the use of narrow-spectrum closantel for predominately *Haemonchus contortus*

infection for example (Taylor et al., 2016). It also allows for more detailed study of interactions among co-infecting GIN species and with the developing host immune response (Avramenko et al., 2015).

Traditionally, GIN speciation has been performed via morphologic identification of third-stage larvae (L₃s) isolated from fecal culture (Ministry of Agriculture, Fisheries and Food, 1984; Zajac and Conboy, 2012; van Wyk and Mayhew, 2013). However, this method is labour-intensive, requires expertise, and is prone to error due to the subtle morphologic differences among certain species; as a result, morphologic identification is performed infrequently (Avramenko et al., 2015). Moreover, larval mortality during development to L₃s is high (Ministry of Agriculture, Fisheries, and Food, 1984; van Wyk and Mayhew, 2013). Recently, a novel GIN speciation method using deep amplicon sequencing of the internal transcribed spacer-2 (ITS-2) rDNA locus has been validated for GIN L₃s in cattle and sheep (Avramenko et al., 2015; Avramenko et al., 2017; Redman et al., submitted). The use of deep amplicon sequencing reduces labour requirements and risk of error relative to morphologic speciation (Avramenko et al., 2015; Avramenko et al., 2017; Redman et al., submitted). However, as with morphologic speciation, sequencing of L₃s requires several days to culture eggs to L₃s. Thus, larval recovery is similarly potentially limited by high mortality during culture (Ministry of Agriculture, Fisheries, and Food, 1984; van Wyk and Mayhew, 2013). Moreover, optimal culture conditions vary between GIN species, which can lead to differential survival and bias speciation results (Dobson et al., 1992). Sequencing of first-stage larvae (L₁s) requires a shorter fecal culture duration and, unlike L₃ cultures, L₁ cultures do not require

additional heat during incubation or daily stirring and misting to ensure adequate humidity (Ministry of Agriculture, Fisheries, and Farming, 1984; Mes et al., 2007). Shorter culture duration to L₁s may also increase the proportion of surviving larvae, but this has not been assessed (Mes et al., 2007; Burgess et al., 2012; Avramenko et al., 2017; Redman et al., submitted).

The objectives of this study were to: a) compare GIN species composition generated by deep amplicon sequencing of L₁s with that generated by morphologic speciation of L₃s from small ruminant fecal samples, and b) compare larval recovery from small ruminant fecal samples following fecal culture to L₁s with culture to L₃s.

2.2 Materials and Methods

2.2.1 Preparation of pooled fecal samples

Fresh fecal samples collected per rectum were obtained from five small ruminant farms from across Ontario, Canada, with a history of GIN parasitism. Samples were submitted by referring veterinarians (farms 2 and 3) and collected directly by the researchers (farms 1, 4, and 5), and stored at room temperature (~20 °C) in airtight plastic bags. Samples from one farm (farm 3) were mistakenly refrigerated at 4 °C for an unknown length of time prior to submission. The farms included four sheep farms (farms 1-4) and one goat dairy (farm 5). All samples were received by the laboratory and processed within 48 hours of collection. Equal weights of feces from 4-10 adult (> 1 year) animals per farm were pooled and homogenized to yield a total pooled sample weight of 120 g. One pooled

sample each was generated from farms 1, 2, 3, and 5, and three pooled samples taken at two different time points were generated from farm 4 (see Table 2.1).

2.2.2 Fecal egg count

A modified McMaster fecal egg count method that yielded a minimum level of sensitivity of 8.33 eggs per gram was performed on each pooled sample to estimate the total number of GIN eggs in each culture (Zajac and Conboy, 2012). Briefly, a 4 g aliquot of feces was suspended in 26 mL of saturated sodium chloride (NaCl, specific gravity 1.2), the solution strained using a metal tea strainer (mesh size approximately 1.0 mm) to remove particulates, and egg count estimated using a three-chamber McMaster slide with a volume per chamber of 0.3 mL (Chalex Corporation, Utah, USA). Pooled sample fecal egg counts are presented in Table 2.1.

2.2.3 Fecal culture to L₃

Culture of eggs to L₃s was performed in triplicate for each pooled fecal sample. For each culture, a 30 g aliquot of feces was placed in a ceramic ramekin, covered with perforated foil, and incubated at 27 °C with relative humidity at 60-70 % for seven days (Ministry of Agriculture, Fisheries, and Food, 1984; Zajac and Conboy, 2012). Each day, incubating cultures were misted with tap water and stirred to inhibit fungal growth and maintain even moisture and oxygen exposure. Following removal from the incubator on the seventh day of incubation, L₃s were collected for speciation using a Baermann apparatus (Ministry of Agriculture, Fisheries, and Food, 1984; Zajac and Conboy, 2012). Liquid collected from the Baermann apparatus was agitated manually for 10 seconds to evenly

disperse the larvae, and speciation of the first 100 L₃s examined was performed using morphologic features described in detail by Zajac and Conboy (2012) and van Wyk and Mayhew (2013). Features used in morphologic speciation are summarized in Table 2.2. Proportions of *Chabertia* spp. and *Oesophagostomum* spp. were reported as a combined value, as intestinal cell shape and number were frequently obscured by preparation artifact.

2.2.4 Fecal culture to L₁

Eggs were isolated from the pooled fecal samples for L₁ culture using a protocol modified from Mes et al. (2007) and Redman et al. (submitted). An aliquot of 6 g of feces was suspended in 10 mL of saturated NaCl (specific gravity 1.2) and strained through a 150 µm sieve into a 50 mL Falcon tube to remove particulates. The resulting filtrate was centrifuged for 2 minutes at 715 X g. The supernatant was then poured through a 25 µm sieve to collect parasite ova in the sieve, and the ova washed with water to remove residual salt solution. Ova were flushed into a 10 cm petri dish and cultured in water for 48 hours at 20 °C. The resulting L₁s were collected and preserved in 70 % ethanol. This method was performed in triplicate using three separate 6 g aliquots of each pooled fecal sample. Culture methods (L₁ and L₃) are summarized in Figure 2.1.

2.2.5 Estimation of larval recovery

For L₃ cultures, the total number of ova cultured was determined by multiplying the pooled sample fecal egg count by the weight cultured (30 g). The L₃s/mL of liquid collected from the Baermann apparatus was determined using the following formula:

$$L_3\text{s/mL} = 100 \times (1 \text{ mL} / \text{volume required to obtain } 100 L_3\text{s})$$

Larval recovery as a proportion of cultured ova was determined using the following formula:

$$\text{Larval recovery proportion (\%)} = (L_3\text{s/mL} \times \text{total volume collected}) / \text{ova cultured} \times 100$$

For L_1 cultures, larval recovery as a proportion of cultured ova was determined by using a dissecting microscope to count the number of L_1 s and unhatched ova. Cultures were agitated briefly to evenly disperse their contents, and larvae and ova were counted in three randomly selected fields at 4X magnification (field area 0.95 cm²). The proportion of larvae recovered as a proportion of cultured ova was defined as:

$$\text{Larval recovery proportion (\%)} = L_1\text{s} / (L_1\text{s} + \text{unhatched ova}) \times 100$$

2.2.6 L_1 DNA lysate preparation

Lysates were prepared according to protocols reported by Redman et al. (submitted). Prior to DNA extraction, ethanol-fixed L_1 s were placed in 1 mL of lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM MgCl₂, 0.45 % Nonidet P-40, 0.45 % Tween 20, 0.01 % w/v gelatin) and centrifuged at 2500 X g for 5 minutes. The supernatant was removed and the process repeated twice for a total of three washings prior to suspension of the L_1 s in 100 μ L of lysis buffer. The suspended larvae were heated for 15 minutes at 95 °C, followed by freezing at -80 °C for 1 hour then 150 μ L of lysis buffer containing 120 μ g/mL proteinase K was added to the thawed samples. The larval suspension was then incubated for 2 hours at 60 °C and shaken during incubation at 750 RPM using an orbital shaker (Thomas Scientific Inc., New Jersey, USA). Proteinase K was inactivated via

incubation for 20 minutes at 95 °C. Lysates were diluted 1:10 using molecular grade water.

2.2.7 Deep amplicon sequencing of L_1 rDNA ITS-2

Sequencing was performed according to the protocol reported by Redman et al., (submitted). Eight pairs of forward primers and reverse primers, listed in Table 2.3, were used to amplify the rDNA ITS-2 locus. For sequencing, 4 μ L of 1:10 diluted DNA lysate was combined with 5 μ L KAPA HiFi HotStart Fidelity Buffer (5X) (KAPA Biosystems, Massachusetts, USA), 0.75 μ L NC1+ Adapter Primer (10 μ M), 0.75 μ L NC2+ Adapter Primer (10 μ M), 0.75 μ L dNTPs (10 mM), 0.5 μ L KAPA HiFi HotStart Polymerase (0.5 U) (KAPA Biosystems), and 13.25 μ L ddH₂O. The solution was heated at 95 °C for 3 minutes, then PCR cycled 25 times through successive temperatures of 98 °C for 20 seconds, 62 °C for 15 seconds, and 72 °C for 15 seconds. A final extension was performed at 72 °C for 2 minutes, and amplified products were purified using AMPure XP Magnetic Beads (1X) (Beckman Coulter Inc., California, USA). Limited cycle PCR amplification was used to add Illumina sequencing tags to the amplicons. For limited cycle PCR, 2 μ L of the initial amplicon was combined with 5 μ L KAPA HiFi HotStart Fidelity Buffer (5X), 1.25 μ L Forward Primer (N501-508) (10 μ M), 1.25 μ L Reverse Primer (N701-712) (10 μ M), 0.75 dNTPs (10 mM), 0.5 μ L KAPA HiFi Polymerase (0.5 U), and 14.25 μ L H₂O. The solution was heated at 98°C for 45 seconds, then PCR cycled 7 times through successive temperatures of 98 °C for 20 seconds, 63 °C for 20 seconds, and 72 °C for 2 minutes. Purification using AMPure XP Magnetic Beads (1X) was repeated and a sequencing library generated by pooling 50 ng of each product. The

KAPA qPCR Library Quantification Kit (KAPA Biosystems) was used to determine the concentration of the pooled library, which was then run on an Illumina MiSeq Desktop Sequencer (Illumina, California, USA). The sequencer used a 500-cycle-pair-end reagent kit (MiSeq Reagent Kits v2, MS-103-2003) at a concentration of 12.5 nM with the addition of 25 % PhiX Control v3 (Illumina, FC-110-3001), and generated FASTQ files without post-run analysis.

2.2.8 Bioinformatic analysis

Sequence analysis was performed according to the protocol reported by Redman et al., (submitted). Percentage species composition was calculated using the following formula:

$$\text{Species percentage} = (\text{Raw reads per species} / \text{total reads per sample}) \times 100$$

Correction factors applied to species percentages were determined previously via sequencing of an artificially generated DNA lysate containing equal numbers of L₁s from 6 species of GIN commonly identified in small ruminants (Redman et al., submitted).

2.2.9 Statistical analysis

Since the responses were proportions, species composition and larval recovery proportions were subjected to logit transformation: $\text{logit} = \log[(P + k)/(100 - P + k)]$, where k is a bias correction term that also accounts for 0 % or 100 % responses (Cox and Snell, 1989). For larval recovery proportions, k was set to 0.375 based on tests for normality, while for species composition k was set to 0.25.

The design was in the form of a paired t-test with subsampling, and was analyzed using Proc Mixed (SAS 9.4, SAS Institute Inc., Cary, North Carolina, USA), treating farm as a random block (pair) and culture method by farm as the random experimental unit (thus allowing for subsampling), while culture method was a fixed effect. Results were back-transformed into proportions (percentages) with 95% confidence intervals and odds ratios (also with 95% confidence intervals). In addition, multivariate analysis was performed on species composition using Proc GLM (SAS 9.4, SAS Institute Inc.), testing farm and culture method as effects and using farm by culture method as the experimental error term. In both the univariate and multivariate analyses, residuals were plotted against predicted and explanatory variables and tests of normality used to assess for presence of outliers, conformance to model assumptions, and unequal variance.

2.3 Results

2.3.1 Nematode species proportions

The most commonly identified genera using both morphologic speciation of L₃s and deep amplicon sequencing of L₁s were *Haemonchus contortus*, *Trichostrongylus* spp., and *Teladorsagia circumcincta*. Proportions of these genera varied among farms and are summarized in Figure 2.2. *Haemonchus contortus* was the predominant (range 54.0-99.6 %) genus on farms 1, 2, 3, and 5 but was less common (range 1.9-30.4 %) in all three pooled samples from farm 4. Upon review of information submitted with the fecal samples from referring veterinarians, it was discovered that samples submitted from farm 3 had been mistakenly exposed to refrigeration (4 °C), though the duration of exposure was not known. Since exposure to cold temperatures is known to affect egg viability,

particularly for *Haemonchus contortus* (Taylor et al., 2015), data from this farm were excluded from subsequent analyses. *Cooperia* spp. and *Chabertia/Oesophagostomum* spp. were not present in five of the seven pooled samples and comprised no more than 13.0 % of larvae isolated from L₃ culture, and no more than 5.5 % of larvae speciated using L₁ PCR. Proportions of *Cooperia* spp. and *Chabertia/Oesophagostomum* spp. were not analyzed due to the low prevalence of these species in the pooled samples.

Results of univariate species proportions analysis are presented in Table 2.4. Proportions of *Teladorsagia circumcincta* and *Trichostrongylus* spp. were significantly influenced by culture method as a simple effect ($p = 0.008$) and as an interaction term with fecal sample ($p = 0.009$), respectively. The odds of identifying *Teladorsagia circumcincta* were 3.1 times higher in L₁ cultures than L₃ cultures (95 % confidence interval 1.6-6.1), and this was offset by a decrease in proportions of *Trichostrongylus* spp. in L₁ cultures. Culture method was not significantly associated with proportions of *Haemonchus contortus*. Fecal sample was significantly associated with proportions of all three species ($p < 0.01$), due to the geographical and seasonal variation in GIN species composition as demonstrated in Figure 2.2. The associations identified in univariate analysis were supported by the results of multivariate analysis, with Wilk's lambda values of 0.039, < 0.0001 , and 0.017 for culture method, fecal sample, and culture method by fecal sample, respectively.

2.3.2 Larval recovery proportion

Larval recovery proportion from L₁ and L₃ culture of each pooled sample is summarized in Figure 2.3. The odds of recovering larvae were 238.7 times higher (95 % confidence interval for odds ratio 80.0-712.0, $p < 0.0001$) following culture to L₁s than to L₃s.

Neither fecal sample as a simple effect nor the fecal sample by culture method interaction were significantly associated with larval recovery proportion (see Table 2.5).

2.4 Discussion

Despite the high prevalence of mixed GIN infections in small ruminants, and the diversity in pathogenicity and anthelmintic susceptibility among infecting species, speciation of GINs cultured from ruminant fecal samples has traditionally been an underutilized methodology due to the high cost, increased labour, and long turnaround time involved in morphologic speciation (Avramenko et al., 2015; Taylor et al., 2016). Deep amplicon sequencing is an attractive alternative to morphologic speciation of GINs due to its reduced labour and higher throughput capacity, allowing speciation of thousands of GIN larvae per sample rather than hundreds (Avramenko et al., 2015; Avramenko et al., 2017; Redman et al., submitted). Sequencing of ova eliminates the time and effort required to culture larvae but is less practical than sequencing of larval stages as variation in rate of mitosis leads to variable numbers of cells, and therefore DNA copy numbers, among ova, and can skew calculated species proportions (Avramenko et al., 2017; Redman et al., submitted). Conversely, larvae at the same developmental stage display less within- and between-species variation in copy numbers than ova, allowing for correction of species estimates for differences in size and cell

numbers among larvae of different species (Burgess et al., 2012; Avramenko et al., 2015; Avramenko et al., 2017; Redman et al., submitted).

To date, sequencing of L₃s has been the most common approach in deep amplicon sequencing (Avramenko et al., 2017). However, as is the case for morphologic speciation, culture and collection of L₃s from fecal samples requires a minimum of 8 days (7 days incubation and Baermannization for 8-12 hours) (Ministry of Agriculture, Fisheries, and Food, 1984; Zajac and Conboy, 2012; van Wyk and Mayhew, 2013). The various species of small ruminant GINs have different optimal culture conditions, and recommended temperature and humidity conditions for culture to L₃s are based on the average of optimal conditions for multiple common species (Ministry of Agriculture, Fisheries, and Food, 1984; Dobson et al., 1992; Zajac and Conboy, 2012; Taylor et al., 2016). Consequently, as culture conditions are not optimized for any particular species, mortality of larvae during development is high in all species, and can markedly differ between species (Dobson et al., 1992; Taylor et al., 2016). In the current study, larval survival to L₃s ranged from 3.1 % to 33.9 % of ova cultured. Culture samples for L₃ sequencing must therefore contain very high egg counts and/or large volumes of feces in order to generate sufficient larvae. Culture to L₁s requires a shorter duration (48 hours), allowing for species proportions to be determined more rapidly and reducing potential culture biases (Burgess et al., 2012; Avramenko et al., 2017; Redman et al., submitted). Moreover, this study determined that larval survival is significantly improved when cultured to L₁s, with L₁ cultures achieving a range of 86.5 % to 100 % survival. As a

result, L₁ cultures require smaller fecal volumes to obtain sufficient larvae for sequencing and allow for speciation of GINs in samples with lower FECs.

Both morphologic speciation of L₃s and deep amplicon sequencing of L₁s identified *Haemonchus contortus*, *Trichostrongylus* spp., and *Teladorsagia circumcincta* as the most common GINs on Ontario small ruminant farms, consistent with previous epidemiologic data reported by Mederos et al., 2010. On farm 4, the proportions of *Teladorsagia circumcincta* decreased and *Trichostrongylus* spp. increased from summer to fall, similar to previously reported temporal patterns of GIN infection in Ontario (Mederos et al; 2010). *Chabertia/Oesophagostomum* spp. and *Cooperia* spp. were infrequent in all pooled samples, and no *Cooperia* spp. L₃s were identified morphologically, consistent with low prevalence of these species reported by Mederos et al., 2010. Identification of increased proportions of *Teladorsagia circumcincta* in L₁ versus L₃ culture, and the opposite observation in *Trichostrongylus* spp., is consistent with differential attrition of these species in L₃ culture as reported by Dobson et al. (1992). Collectively, these findings suggest that speciation at the L₁ stage should reduce species bias introduced by differential survival to L₃s. Given the relatively subtle morphologic differences between L₃ of *Teladorsagia circumcincta* and *Trichostrongylus* spp. (see Table 2.2), error in morphologic speciation was also considered a possible cause of this discrepancy. However, morphologic identification was performed by a highly trained and experienced observer; therefore, misidentification was considered unlikely.

2.5 Conclusions

The results of this study indicate that deep amplicon sequencing of L₁s cultured from trichostrongyle-type eggs in small ruminant feces may reduce the risk of species bias introduced by differential survival to L₃s and is associated with markedly improved larval survival, reduced labour, and greatly reduced costs and turnaround time. Handling of fecal samples prior to larval culture may affect the resulting species proportions; this variable merits further investigation. Nonetheless, L₁ deep amplicon sequencing appears to be an alternative with many advantages to L₃ morphologic speciation for investigation of GIN infections in small ruminants.

2.6 Acknowledgements

This research was supported by the Ontario Ministry of Agriculture, Food and Rural Affairs through a Highly Qualified Personnel Scholarship, and a University of Guelph Undergraduate Research Assistantship. The authors wish to thank all veterinarians and small ruminant farms that graciously provided samples.

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2.8 Tables

Table 2.1. Pooled fecal sample characteristics and nematode egg count.

Farm	Species	Sample ¹	Number of animals	Month collected	Fecal egg count (eggs per gram)
1	Sheep		9	June	4110
2	Sheep		4	June	3197
3	Sheep		6	June	802
4 ¹	Sheep	A	10	July	607
		B	10	July	587
		C	10	October	1468
5	Goat		7	June	3892

¹Three separate pooled samples were generated from animals on farm 4. No individual animal contributed to more than one pooled sample.

Table 2.2. Features used in morphologic speciation of third-stage gastrointestinal nematode larvae cultured from sheep feces. Derived from van Wyk and Mayhew (2013) and Zajac and Conboy (2012).

Species	Total length (µm)	Tail sheath length (µm)	Head morphology	Tubercles	Intestinal cell shape and number
<i>Haemonchus contortus</i>	650-751	65-82	Tapered	None	Triangle, 16
<i>Teladorsagia circumcincta</i>	797-910	30-44	Square	None	Triangle, 16
<i>Trichostrongylus</i> spp.	622-796	18-56	Tapered	0-2	Triangle, 16
<i>Oesophagostomum</i> spp.	771-923	122-207	Tapered	None	Triangle, 18-22
<i>Chabertia</i> spp.	710-789	101-150	Tapered	None	Rectangle, 28-32
<i>Cooperia</i> spp.	711-924	39-82	Square, refractile bodies	None	Triangle, 16

Table 2.3. Primers used to amplify the rDNA ITS-2 locus in first-stage gastrointestinal nematode larvae cultured from small ruminant feces. Derived from Redman et al. (submitted).

Species	Primers (5' → 3')
<i>Haemonchus contortus</i>	Forward: GTTACAATTTTCATAACATCACGT Reverse: TTTACAGTTTGCAGAACTTA
<i>Teladorsagia circumcincta</i>	Forward: ATACCGCATGGTGTGTACGG Reverse: CAGGAACGTTACGACGGTAAT
<i>Trichostrongylus colubriformis</i>	Forward: CCCGTTAGAGCTCTGTATA Reverse: TGCGTACTCAACCACCACTAT
<i>Trichostrongylus vitrinus</i>	Forward: AGGAACATTAATGTCGTTACA Reverse: CTGTTTGTCTGAATGGTTATTA
<i>Trichostrongylus axei</i>	Forward: AGGGATATTAATGTCGTTCA Reverse: TGATAATTCCCATTTTAGTTT
<i>Cooperia curticei</i>	Forward: TATACTACAGTGTGGCTAGCG Reverse: TCATACCATTCAGAAATGTTC
<i>Chabertia ovina</i>	Forward: CATGTGTGATCCTCGTACTAGATAAGA Reverse: ATGAACCGTACACCGTTGTCA
<i>Oesophagostomum venulosum</i>	Forward: TGTTTACTACAGTGTGGCTTG Reverse: CGGTTGTCTCATTTCACAGGC

Table 2.4. Univariate analysis of gastrointestinal nematode species proportions isolated from small ruminant feces. Direction of association is shown for significant effects only.

Species	Predictor variable	p-value	Direction of association	Degrees of freedom	F-value
<i>Haemonchus contortus</i>	Fecal sample	< 0.001	Variable ¹	5	157.41
	Culture method	0.824	NA	1	0.05
	Fecal sample*culture method	0.649	NA	5	0.67
<i>Teladorsagia circumcincta</i>	Fecal sample	< 0.001	Variable ¹	5	147.27
	Culture method	0.008	See note ²	1	25.03
	Fecal sample*culture method	0.640	NA	5	0.68
<i>Trichostrongylus</i> spp.	Fecal sample	0.014	Variable ¹	5	9.51
	Culture method	0.227	NA	1	1.90
	Fecal sample*culture method	0.009	See note ³	5	4.00

¹ Direction of association varies at different levels of a categorical variable.

² Proportions of *Teladorsagia circumcincta* were higher in L₁ cultures than L₃ cultures.

³ Proportions of *Trichostrongylus* spp. were typically higher in L₃ cultures than L₁ cultures, dependent on pooled fecal sample.

Table 2.5. Larval recovery (percentage larvae recovered as a proportion of ova cultured) from first-stage (L₁) and third-stage (L₃) larval culture. Direction of association is given for significant effects only.

Predictor variable	p-value	Direction of association	Degrees of freedom	F-value
Fecal sample	0.174	NA	5	2.45
Culture method	< 0.001	See note ¹	1	165.83
Fecal sample*culture method	0.057	NA	5	2.52

¹ Larval recovery proportions were higher for L₁ culture than L₃ culture.

2.9 Figures

Figure 2.1. Schematic representation of third-stage (L_3) and first-stage (L_1) larval culture methods. NaCl = sodium chloride, SG = specific gravity.

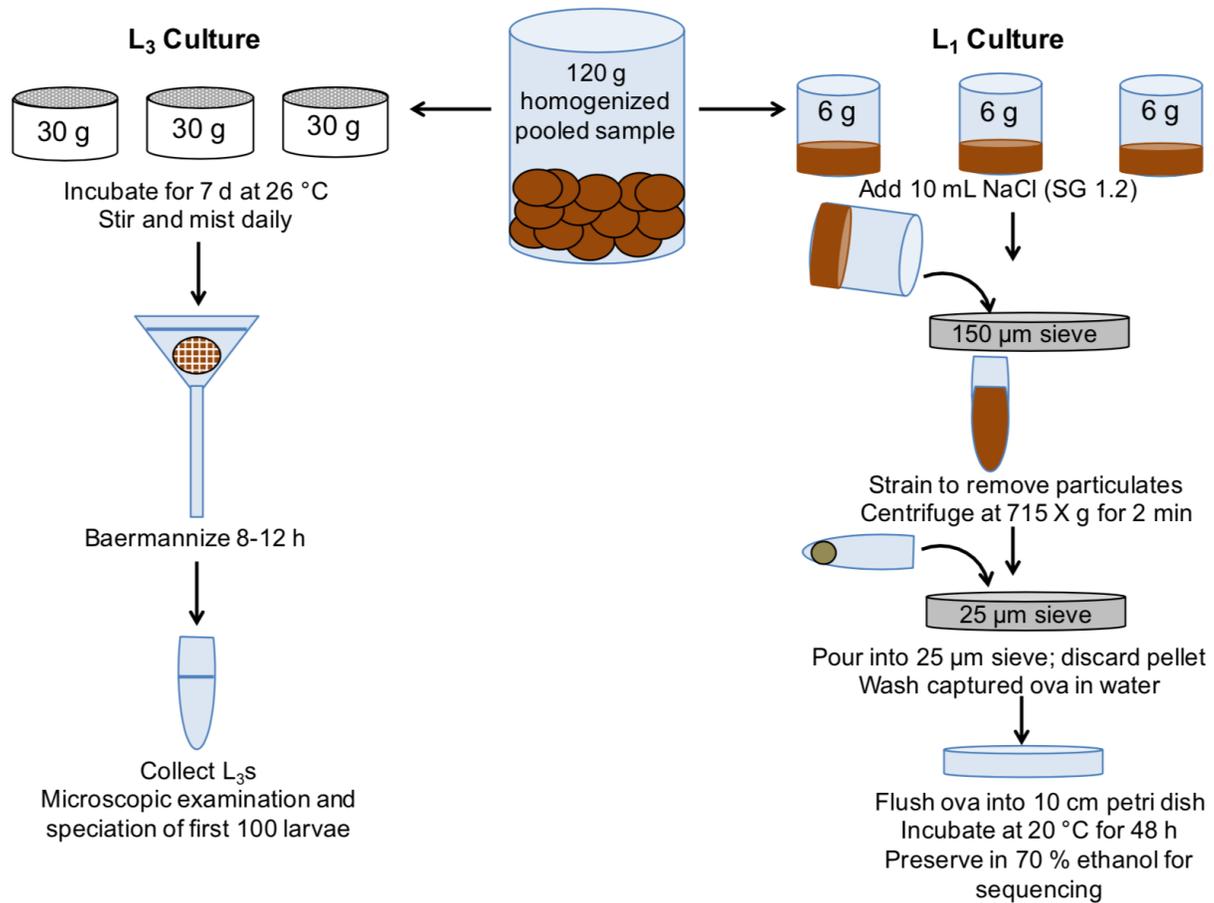


Figure 2.2. Proportions of *Haemonchus contortus*, *Trichostrongylus* spp., and *Teladorsagia circumcincta* identified by deep amplicon sequencing of first-stage larvae (L₁s) and morphologic speciation of third-stage larvae (L₃s) cultured from the same pooled fecal sample. Error bars indicate species proportion range from triplicate cultures. Samples from Farm 3 were refrigerated for an unknown duration and were excluded from subsequent analyses.

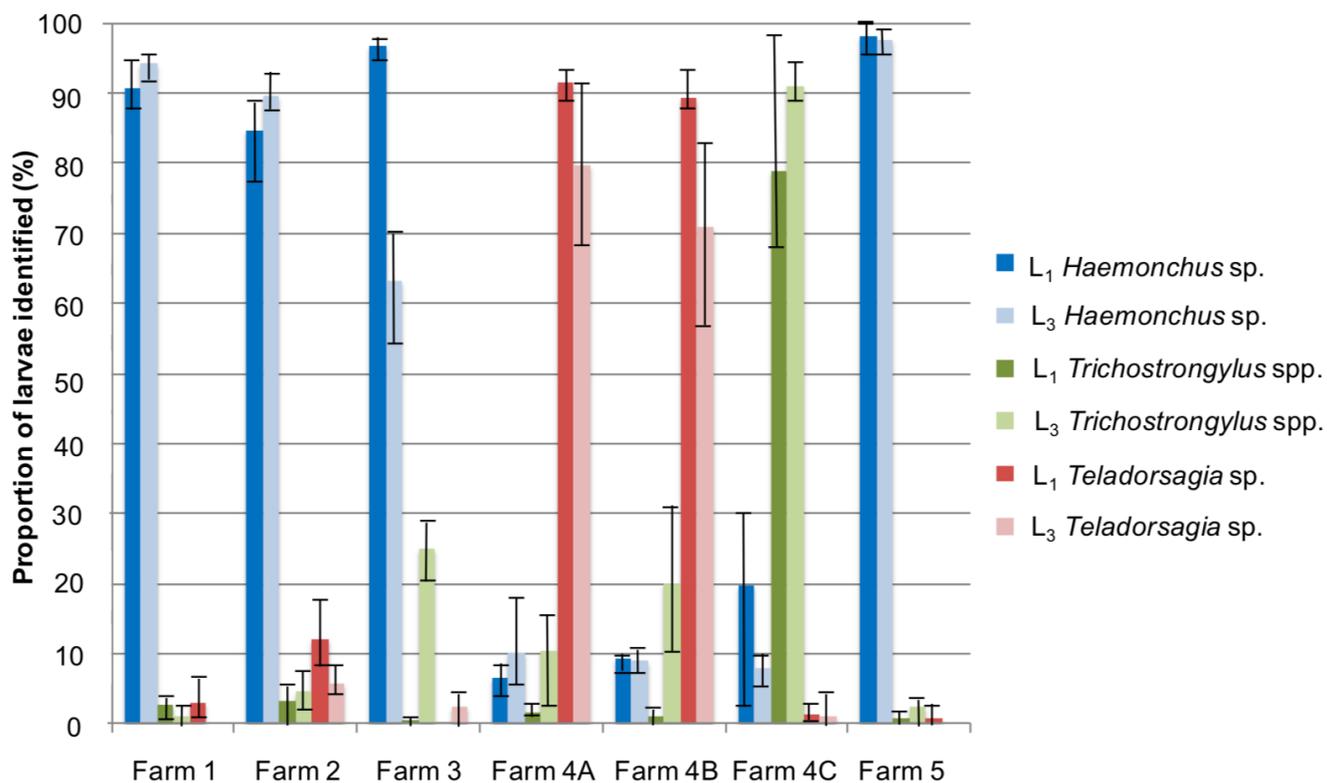
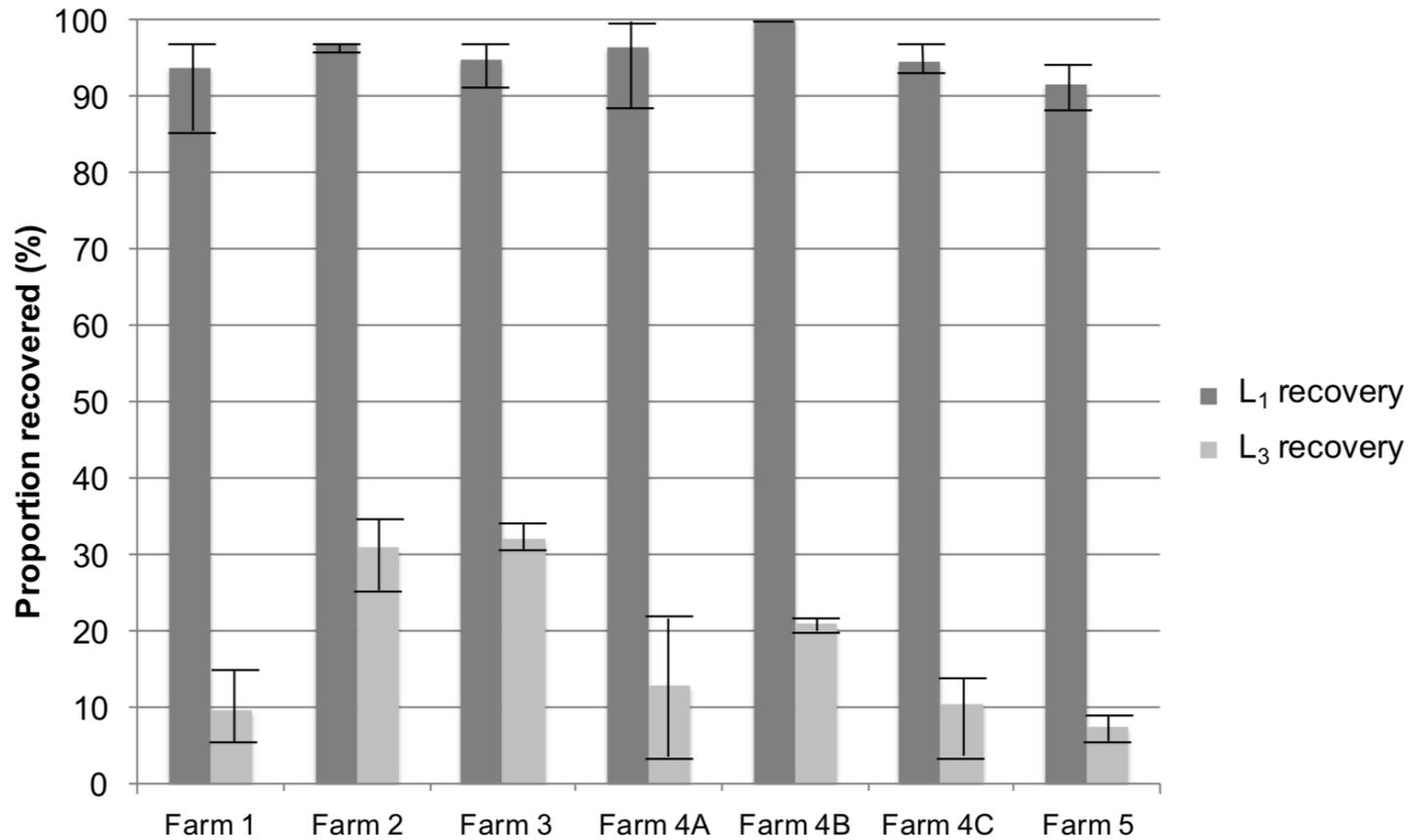


Figure 2.3. Mean larval recovery proportion from first-stage (L1) and third-stage (L3) larval culture of the same fecal sample. Error bars indicate larval recovery range from triplicate cultures. Samples from Farm 3 were refrigerated for an unknown duration and were excluded from subsequent analyses.



CHAPTER THREE:

**CORRELATION OF GASTROINTESTINAL NEMATODE
PARASITISM WITH GROWTH AND REPRODUCTIVE
PERFORMANCE IN EWE LAMBS IN ONTARIO**

Based on a manuscript in preparation for submission to *Preventive Veterinary Medicine*.

Abstract

Infection with gastrointestinal nematode parasites (GINs) is an important cause of productivity loss on sheep farms in Ontario and worldwide. However, efforts to quantify the effect of GIN infection on growth have demonstrated mixed results. Furthermore, there has been limited investigation of their effect on reproductive performance. This study evaluated the effect of GIN parasitism on growth and reproductive performance of ewe lambs under Ontario grazing conditions. Rideau-Dorset cross ewe lambs (n = 140) born in spring 2016 on a farm in central Ontario were followed for two years from before weaning through to November 2017, including their first lambing and lactation event. These animals grazed from May to November of each year, and were sampled every 6-8 weeks during both grazing seasons and once at mid-gestation in March 2017. At each sampling the ewe lambs were weighed, body condition scores assigned, fecal egg counts (FECs) performed, and pasture samples collected to assess number of infective GIN larvae. Fecal samples were cultured to determine infecting GIN species, and climate data were obtained from a weather station 26 km away from the farm. Precipitation levels and

numbers of infective larvae on pasture were low during the first grazing season, but were more typical of Ontario conditions in the second grazing season. The three most common GIN species were *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus* spp. General linear mixed models were generated for weight change over time, litter size at lambing, and weaning weights of offspring. Gastrointestinal nematode FEC was not a significant predictor of weight change in the study ewe lambs during their first two grazing seasons. However, higher FECs at lambing were associated with larger litter sizes ($p=0.05$), likely reflecting increased periparturient egg rise in ewes with larger litters. Significant positive quadratic and negative linear associations were identified between late lactation FECs and offspring weaning weights; the association between increased FECs and weaning weights changed from negative to positive at a FEC of 361 eggs per gram. Thus, subclinical GIN infection appears to have minimal impact on growth and reproductive performance in Ontario sheep.

Keywords

sheep, nematode, *Haemonchus*, *Trichostrongylus*, *Teladorsagia*, productivity

3.1 Introduction

Parasitic gastrointestinal nematodes (GINs) are common pathogens on sheep farms around the world (Mederos et al., 2010; Mavrot et al., 2015). The mechanism and degree of pathology varies with the infecting GIN species and burdens (Bowman, 2014; Mavrot et al., 2015). Infection with mixed species is typical, and on sheep farms in Ontario, Canada, *Haemonchus contortus*, *Teladorsagia circumcincta*, and *Trichostrongylus* spp.

are most commonly identified (Mederos et al., 2010). Mortality and morbidity are most commonly observed in animals with high burdens of *H. contortus*, secondary to severe anemia and protein loss (Bowman, 2014; Taylor et al., 2016). However, most GINs are present in a relatively small proportion of the flock. Thus, the majority of animals are usually subclinically infected (Miller and Horohov, 2006; Kemper et al., 2009).

Nevertheless, subclinical GIN infection impacts productivity via protein and blood loss, can impair digestion, and, especially in the case of *T. circumcincta*, may reduce appetite (Greer, 2008; Bowman, 2014; Mavrot et al., 2015; Taylor et al., 2016).

Although subclinical GIN infections are known to cause financial losses due to reduced productivity, efforts to quantify this effect have demonstrated mixed results. In a recent meta-analysis, Mavrot et al. (2015) evaluated the results of 88 studies that compared wool and milk production and growth in GIN-infected and control sheep. The studies were distributed across multiple geographic regions, though more originated from Europe (38) and Oceania (21) than from the Americas (14), Africa (10), or Asia (5), and only 5 trials were conducted in North America (Mavrot et al., 2015). The majority (88 %) of these studies identified reduced productivity in parasitized sheep, with overall reductions of 22 %, 15 % and 10 % in milk production, growth and wool production, respectively (Mavrot et al., 2015). However, 57 % of the trials did not reach statistical significance, and many of the trials evaluated performance of sheep with single-species infections, which is not representative of typical conditions on most commercial sheep farms (Mavrot et al., 2015).

There has been limited investigation of the effect of GIN parasitism on reproductive performance in sheep. One study of Barbarine rams artificially infected with *H. contortus* in Tunisia demonstrated higher proportions of nonviable sperm relative to controls via eosin-nigrosin staining (Rouatbi et al., 2016). In central Greece under natural challenge with mixed GIN infection, Lacaune dairy ewe lambs treated with long-acting moxidectin at the beginning of the breeding season attained puberty earlier and delivered more liveborn lambs compared with untreated controls (Mavrogianni et al., 2011). A study of Katahdin ewes in the United States found that GIN fecal egg counts (FECs) in ewes at parturition and at 30 days post-parturition were weakly positively correlated with litter size ($r < 0.2$); correlations between ewe FECs and lamb birth and weaning weights were also weak ($|r| < 0.1$) and both positive and negative correlations were reported (Notter et al., 2018). However, as this study evaluated flocks located south of the 41st parallel, it remains unclear whether GIN infection would have similar effects on reproductive performance in sheep raised under the shorter grazing seasons and colder winters observed farther north, or in different breeds. The objective of this study was to evaluate the effect of GIN parasitism on growth and reproductive performance of ewe lambs under Ontario grazing conditions, with the goal of improving understanding of the relationship between GIN infection, growth and reproduction. In turn, this will facilitate better informed decisions regarding the management of GINs in replacement breeding stock.

3.2 Materials and Methods

3.2.1 Farm and animal enrollment

The study was conducted on a commercial sheep farm located in central Ontario, Canada. It met the following criteria: a) the farm retained at least 100 ewe lambs each year as replacement animals for the breeding flock, b) ewes and lambs were grazed on pasture beginning no later than early May and remained on pasture until at least mid-November of each year, c) breed and management practices were representative of Ontario sheep farms (Kennedy, 2012), d) the farm had a history of clinical gastrointestinal nematode parasitism, e) records of individual animal growth and reproductive performance were maintained as part of participation in the Canadian sheep genetic improvement program GenOvis (La Pocatière, Québec, Canada), and f) the producer agreed to repeated sampling of the flock by the research team for the two-year study period. In addition, the flock was of high health status and known to be free of scrapie, Maedi-Visna, and *Coxiella burnetii*. A sample size of 100 was selected in order to detect correlation coefficients of at least 0.30 using a single group of animals, with type I error set at 0.05 and type II error set at 0.20 (University of California, San Francisco, 2018). In order to allow for loss of animals due to mortality and normal culling practices including failure to conceive or lamb during the project, 140 Rideau-Dorset cross ewe lambs, randomly selected from the participating farm, were enrolled May 10-11, 2016. Lambs were eligible for enrollment if they were a) female, b) healthy at the time of enrollment, c) born between March 28 and April 24 2016, and d) born in a litter of three or more lambs. This latter criterion was required by the flock owners, who elected to only retain ewe

lambs born as triplets. Lambs that were not reared to weaning by a ewe, i.e. were raised artificially on milk replacer, were excluded from the study. Where possible, reason for loss to follow-up was recorded for all animals that did not complete the study. Animal use approval was obtained from the University of Guelph Animal Care Committee (Animal Use Protocol Number 3380).

3.2.2 Animal management

The ewe lambs enrolled in this study were managed as a single flock from birth until mid-gestation, and from lambing until completion of the study. Mean age on the day of enrollment (May 10 or 11, 2016) was 30 days (range 16-43 days), and the study ewe lambs were weaned at a mean age of 60 days. In 2016, the flock grazed on pasture from May 1 (mean age 20 days) until December 1 (mean age 229 days). A ration of dry distiller's grain, shelled corn, minerals, and a commercial lamb protein supplement was offered to the lambs from birth until weaning. Following weaning, a ration of shelled corn mixed with trace mineral salt was fed at the rate of 0.23 kg per head per day while the lambs were grazing pasture until housing on December 1, 2016. Access to water was provided ad libitum at all times.

A total mixed ration of corn silage, 0.68 kg per head per day wet distiller's grain, and mineral consumed at 15 g per head per day was provided when the study ewe lambs were housed indoors from December 1, 2016 to January 15, 2017. The study ewe lambs were exposed to rams for breeding from December 15, 2016 until January 15, 2017 (245-276 days of age). On January 15, 2017, they were housed outdoors on a dry lot with access to

shelter and were fed 1.81 kg per head per day each of ensiled alfalfa and mixed fruit waste in addition to the total mixed ration described above. Fetal numbers were counted via trans-abdominal real-time ultrasound on March 15, 2017 (mean age 342 days). Study ewe lambs bearing twin or single lambs returned to the dry lot on the same diet until April 1, 2017, when the ration of distiller's grain was increased to 1.36 kg per head per day. Those bearing litters of three or more lambs were brought indoors, shorn, and maintained on the total mixed ration until April 1, 2017, when half the corn silage offered was substituted for ensiled second cut hay.

Lambing occurred between May 3 and June 6 2017, when the study ewe lambs were 380-410 days of age. One or two offspring were removed from triplet-bearing animals for cross-fostering or artificial rearing. Single and twin-bearing study ewe lambs were brought indoors, housed, and fed with the triplet-bearing study ewe lambs from approximately one week prior to lambing (April 24, 2017) until the flock was turned out on pasture after the majority of the ewes had lambed (May 15, 2017). Study ewe lambs that had not lambed by May 15, 2017 were turned out with the flock within 48 hours of lambing. Each study ewe lamb's lambing date, litter size including stillborn and mummified offspring, and sex and weaning weights of offspring were recorded. Following turnout on May 15, the study ewe lambs, now > 1 year of age, were provided 15 g mineral per head per day but other supplemental feeding was discontinued. They were shorn and their offspring weaned on August 9, 2017, at a mean age at weaning of 86 days (range 64-100 days). In mid-late autumn, the study ewe lambs were offered 0.45 kg

per head per day of shelled corn while grazing pasture from October 16, 2017 (550 days of age) until completion of the study on November 10, 2017 (570 days of age).

3.2.3 Sampling of sheep and pasture

The study ewe lambs were sampled every 6-8 weeks between May and November in 2016 and 2017, and at mid-gestation in March 2017 for a total of 11 time points (see Table 3.1). Samples were collected at mean ages of 30 (nursing), 90 (weaned), 130 (pre-breeding), 190 (pre-breeding), 220 (pre-breeding), 350 (mid-gestation), 400 (lambing), 450 (mid-lactation), 490 (late lactation), 550 (dry) and 570 (dry) days of age. At each visit, the study ewe lambs were weighed, assigned body condition scores by a single observer (J.A.) based on a five-point scale (Russel et al., 1969), and fecal samples were obtained per rectum from each animal. Weights of single and twin-bearing study ewe lambs obtained at mid-gestation, lambing, and mid-lactation were reduced by 4 kg (average fleece weight on farm as reported by the flock owner) to account for the delay in shearing of single and twin-bearing study ewe lambs relative to triplet-bearing study ewe lambs. Herbage samples were also collected from pasture that had been grazed by the study ewe lambs for at least 14 consecutive days, immediately prior to each visit in order to evaluate numbers of third-stage larvae (L_{3s}). A maximum of 500 g of herbage was obtained by walking two “W” paths across the field, stopping every 20 paces to cut herbage as close to the soil as possible while avoiding contamination by soil or fecal material (Ministry of Agriculture, Fisheries, and Food, 1984). Pasture samples were not collected in March 2017, as the herbage was buried under snow and the study ewe lambs were housed indoors at that time.

3.2.4 Climate data

Climate data for May 2016 to November 2017 were obtained from the nearest weather station to the farm (26 kilometers distance). Hourly measurements were used to determine monthly minimum, mean and maximum temperature. Total monthly precipitation was calculated from daily precipitation amounts.

3.2.5 Laboratory methods

Gastrointestinal nematode FECs for each study ewe lamb were determined using a modified McMaster method that yielded a minimum level of sensitivity of 8.33 eggs per gram (epg) (Zajac and Conboy, 2012). Any study ewe lamb with FEC exceeding 500 epg was treated with either levamisole at a dose of 7.5 mg/kg per os (Huvepharma, Missouri, USA) in 2016, or albendazole at a dose of 5 mg/kg per os (Zoetis Canada Inc., Quebec, Canada) in 2017 to prevent morbidity and mortality due to GIN parasitism during the study. Treatment was administered at least 4 weeks prior to the next sampling to allow reinfection and completion of a full prepatent period (Taylor et al., 2016). Pasture herbage wet weight was recorded and L₃s were collected using a method that has previously been described (Ministry of Agriculture, Fisheries, and Food, 1984). In brief, the herbage was washed using 1 mL of soap in 5 L of water prior to filtration, and a total of three wash and sediment filtration steps were performed. Gastrointestinal nematode L₃s in the sediment were counted and speciated using morphologic features described in detail by van Wyk and Mayhew (2013).

At each time point, the study ewe lambs were stratified into groups with high (over 1000 epg), medium (500-1000 epg) and low (200-499 epg) FECs, and up to five randomly selected fecal samples from each group were cultured separately to extract first-stage larvae (L₁s) according to a protocol modified from Redman et al. (submitted). Eggs extracted from 6 g of feces were cultured for 48 hours at 20 °C; a minimum threshold for culture of 200 GIN eggs per gram was used to ensure sufficient L₁s were collected for speciation. Less than 15 animals in total exceeded the minimum threshold for culture in November 2016 and August 2017. Thus, fecal samples from all animals exceeding 200 epg were cultured in these months (n = 14 and n = 7, respectively). Infecting GIN species were determined using deep amplicon sequencing of a minimum of 100 ethanol-fixed L₁s, performed according to protocols described by Redman et al. (submitted). Arithmetic mean proportions of *H. contortus*, *T. circumcincta* and *Trichostrongylus* spp. were calculated at each sampling time point.

3.2.6 Statistical analysis

Analyses were performed using Proc Mixed and Proc Glimmix (SAS version 9.4, SAS Institute Inc., Cary, North Carolina, USA). General linear mixed models were generated for change in weight of the study ewe lambs between consecutive time points and individual weaning weights of offspring of the study ewe lambs, and a generalized linear mixed model for Poisson distributed data was generated for litter size at lambing. Fixed effects evaluated in the models are listed and defined in Table 3.2. All quadratics as well as two- and three-way interactions between fixed effects were evaluated for significance. Effects that were not significant ($p > 0.05$) were removed via backward elimination prior

to reintroduction to confirm lack of significance and assess for multicollinearity.

Nonsignificant effects involved in a significant interaction term were retained to preserve model hierarchy. Unique animal identification number was included in the models as a random effect.

For outcome variables with repeated measures, an error structure was fitted and examined for best fit using the Akaike information criterion (Dohoo et al., 2014). Random effects, autoregressive, heterogenous autoregressive, Toeplitz, heterogenous Toeplitz, and unstructured error structures were evaluated. In all models, residuals were plotted against the model's predicted and explanatory variables to determine conformance to model assumptions, assess normality, and to identify outliers and unequal variance. Normality was assessed using the Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises and Anderson-Darling tests (Ghasemi and Zahediasl, 2012). Outliers were removed to reassess model fit, and maintained in the model if not found to be due to data error.

3.3 Results

3.3.1 Climate, pasture contamination, and infecting GIN species

Monthly minimum, mean and maximum temperatures and total precipitation are shown in Figure 3.1. The 2016 grazing season was characterized by low precipitation, with a total of 319.5 mm of rainfall between May and November. In contrast, there was abundant rainfall during the 2017 grazing season, amounting to a total of 726.7 mm between May and November. Temperatures were similar in both years, with slightly lower peak temperatures occurring during the 2017 grazing season.

Pasture L₃ contamination and species-specific FECs of GIN species obtained from L₁ fecal cultures are summarized in Figure 3.2. Insufficient larvae (less than 100) were obtained from pasture samples to yield an accurate estimate of species proportions on pasture at any sampling point. Pasture contamination was low (less than 50 L₃s/kg dry matter) between July and October 2016, corresponding to dry conditions and high temperatures, which would have impaired survival of L₃s on pasture. The cause of the increase in pasture contamination in November 2016 (148 L₃s/kg dry matter) is unclear, but may reflect improved survival of larvae due to cool daytime temperatures at the beginning of the month prior to onset of overnight ground frost. Numbers of L₃s remained low from May to July 2017, peaked in August (264 L₃s/kg dry matter), and declined in October and November.

No GIN species proportions could be determined for May 2016, as no study ewe lambs exceeded the minimum threshold of 200 eggs per gram for fecal culture. The most commonly identified GINs at all other sampling points were *H. contortus*, *T. circumcincta* and *Trichostrongylus* spp., consistent with results reported for Ontario by Mederos et al. (2010). *Nematodirus spathiger* and *Cooperia* spp. were identified sporadically at low levels (less than 5 %) in individual animals. Proportions of *H. contortus*, *T. circumcincta* and *Trichostrongylus* spp. varied widely between individual study ewe lambs at each sampling point. However, mean proportions of each GIN species within the high, middle and low fecal egg count groups were similar, therefore the groups were combined to calculate overall flock mean GIN species proportions at each sampling

point. Species-specific FECs for the three most common GIN species were calculated using mean species proportions and flock mean FECs at each time point (see Figure 3.2). Species-specific FECs varied over the two-year interval, though *H. contortus*, *T. circumcincta* and *Trichostrongylus* spp. followed a similar seasonal pattern in both years. Fecal egg counts of all three species gradually increased in spring, peaked mid-summer and sharply declined in fall. Peak FECs occurred slightly earlier in 2017 (July) than in 2016 (August). The differences between peak and trough levels were greatest for *H. contortus*, and FECs of *Trichostrongylus* spp. showed the least variation, remaining relatively constant from October 2016 until May 2017.

3.3.2 Losses to follow-up and anthelmintic treatment

Of the 140 ewe lambs enrolled at the start of the study in May 2016, 33 were lost to follow-up by the end of the study in November 2017. Reasons included mortality (n = 22), failure to conceive (n = 5), illness (n = 3), and escape from the study group into other management groups on the farm (n = 3). Detailed information on animals lost to follow-up is presented in Table 3.3. The timing and flock distribution of anthelmintic treatment in the study ewe lambs is summarized in Table 3.1. All study ewe lambs were treated with levamisole per os at 7.5 mg/kg following weaning at 90 days of age, according to routine farm management practices.

3.3.3 Effect of parasitism on weight change in study ewe lambs

Fecal egg counts were subjected to natural logarithmic transformation prior to modeling to normalize distribution; zero counts were corrected to half the minimum detection limit

of the McMaster test (4.16 epg), which was associated with the best conformance to normality (Schisterman et al., 2006; Westers et al., 2016). Predicted weight change was modeled using a general linear mixed model for repeated measures with a heterogeneous Toeplitz error structure. Study ewe lambs lost to follow-up before 130 days of age ($n = 12$) were excluded from the analyses; study ewe lambs lost to follow-up after 130 days of age ($n = 21$) were included in analyses up to the last sampling point before loss to follow-up. Predictors retained in the final model are listed in Table 3.4. Body condition scores of 1-4 were observed during the study; however, a body condition score of 1 was assigned only once during the two-year observation period. Therefore, this observation was excluded from analysis of the effect of body condition score on weight change. Fecal egg count was not significantly associated with weight change ($p > 0.80$), but was included in the model as a variable of interest.

Mean changes in weight for study ewe lambs with body condition score 3 that were raised as triplets, stratified by litter size, are presented in Figure 3.3. Study ewe lambs that did not deliver offspring included those open at pregnancy check ($n = 5$), or confirmed pregnant via ultrasound but aborted sufficiently early in gestation so that no fetuses were identified and lactation did not occur ($n = 5$). Study ewe lambs that were open at pregnancy check were removed from the study before 350 days of age and were excluded from analysis beyond 220 days of age. Weight changes between 90-190 (post-weaning and pre-breeding) and 450-550 days of age (post-lambing) were not significantly different between study ewe lambs bearing singles, twins, triplets and those that failed to lamb ($p > 0.05$ for all comparisons). Single-bearing study ewe lambs gained

significantly less weight between 190-350 days of age (pre-breeding to mid-gestation) than those bearing twins or triplets ($p < 0.05$ for all comparisons). Triplet-bearing study ewe lambs gained significantly more weight than any other litter size between 220-350 days of age, but also lost the most weight between 350-400 days of age ($p < 0.05$ for all comparisons), corresponding with losses likely associated with lambing. Study ewe lambs that were confirmed pregnant but failed to lamb also lost weight between 350-400 days of age, but unlike those that delivered lambs and entered lactation, they did not continue losing weight between 400-490 days of age.

Rates of weight change varied depending on the age of the study ewe lambs. Predicted weight change of a typical commercial ewe lamb (raised as a triplet, body condition score 3, FEC fixed at two-year group average of 662 epg, gave birth to twin lambs) over different age intervals is presented in Figures 3.4 and 3.5. For example, a ewe lamb weighing 55 kg at 220 days of age would be predicted to gain approximately 20.5 kg by 350 days of age (see Figure 3.4). If the same ewe lamb weighed 75 kg at 350 days of age, a loss of approximately 15.6 kg would be predicted by the time of lambing at 400 days of age (see Figure 3.5). Weight gains were predicted in all intervals prior to lambing, corresponding with expected growth from weaning to puberty. The model predicted the greatest weight loss between mid-gestation (350 days of age) and lambing (400 days of age), milder weight loss during lactation (400-490 days of age), and weight gain from 490 days of age onwards, after the study ewe lambs' offspring were weaned. Study ewe lambs with higher initial weight were predicted to experience greater changes in weight

at all intervals. At all ages, study ewe lambs in lower body condition gained more and lost less weight than others of comparable weight in higher body condition.

3.3.4 Effect of parasitism on litter size in replacement ewe lambs

Overall reproductive performance of the study ewe lambs is presented in Table 3.5.

Stillborn and mummified offspring delivered at term were included in evaluation of litter size. Ewe lambs that did not survive to breeding age ($n = 13$), that died while pregnant ($n = 7$), or for which litter size was not recorded ($n = 2$), were excluded from analyses.

Study ewe lambs that did not deliver lambs included those that were open at pregnancy check ($n = 5$) or were confirmed pregnant but aborted early in gestation such that no fetuses were identified ($n = 5$). Two variables were identified as significant ($p < 0.05$) predictors of litter size: weight of the study ewe lamb at 350 days old, corresponding with mid-gestation, and fecal egg count of the study ewe lamb at 400 days old, corresponding with lambing. Effect of parasitism shortly prior to breeding (220 days of age) and during gestation were considered variables of interest, therefore FECs of the study ewe lambs at 220 and 350 days of age were retained in the model despite lack of significance ($p > 0.40$, see Table 3.6).

Fecal egg counts at 220 (pre-breeding), 350 (mid-gestation) and 400 days of age (lambing) were fixed at flock average FECs at each age (287, 320 and 749 epg, respectively) to determine predicted litter size in study ewe lambs with varying weights at 350 days of age, presented in Figure 3.6. Greater weight at 350 days of age was associated with increased litter size, with study ewe lambs weighing 50 kg at 350 days

predicted to deliver 1.5 offspring on average, and those weighing 65 kg or more at the same age predicted to deliver two or more offspring. Change in litter size per 200 epg increase in FEC at 220, 350 or 400 days of age was greatest at low FECs and reached a plateau in study ewe lambs with higher FECs (see Figure 3.7). For example, the model predicted 1.5 offspring would be born on average to study ewe lambs weighing 58 kg (flock average weight) at 350 days of age with a FEC of 400 epg at 400 days of age. Similar study ewe lambs with higher FEC at 400 days of age (lambing) were predicted to give birth to larger litters. Fecal egg counts at 220 (pre-breeding) and 350 days of age (mid-gestation) were not significant predictors of total litter size ($p > 0.40$).

3.3.5 Effect of maternal parasitism on lamb weaning weight

The outcome of interest was each study ewe lamb's ability to wean offspring rather than individual offspring performance. Therefore, for each study ewe lamb, the total weaning weight of all offspring weaned was divided by the number of offspring weaned to yield average weaning weight per offspring for analysis. Triplet offspring were cross-fostered or artificially reared such that no study ewe lamb nursed more than two offspring. Sex was also classified across offspring weaned, with three possible categories: all female, all male, or mixed sex. Study ewe lambs were excluded from analysis if they did not lamb ($n = 30$), failed to deliver live offspring and also did not raise a foster lamb ($n = 2$), rejected their offspring at birth ($n = 1$), died after lambing without weaning any offspring ($n = 4$), or had incomplete weaning records ($n = 7$). Those that delivered live offspring which died prior to weaning of undetermined cause ($n = 16$) were included in analyses as weaning zero offspring. One study ewe lamb that did not deliver live offspring, but raised

a foster lamb to weaning, was included in the analyses. Predictors retained in the final model are presented in Table 3.7. Unequal variance in weaning weights between offspring from all-female, all-male and mixed sex litters was accommodated in the model by including a repeated statement for sex (SAS Institute Inc., 2018); all-female litters had the greatest variance, and weaning weights of mixed sex litters were the least variable.

Study ewe lambs that were heavier at 400 days of age, concurrent with lambing, weaned heavier offspring. Predicted weaning weights of twin offspring weaned at the flock average weaning age (86 days), born to a study ewe lamb with FEC of 2800 epg at 450 days of age (flock average), are presented in Figure 3.8. Twin male offspring born to a study ewe lamb weighing 60 kg at lambing were predicted to weigh approximately 21.3 kg at weaning. Offspring from mixed sex litters were significantly lighter than those from male- or female-only litters ($p < 0.01$ for both comparisons). Although offspring from male-only litters had the highest predicted weaning weights, weaning weights were not significantly different between offspring from male-only and female-only litters ($p = 0.48$).

Natural logarithm-transformed fecal egg counts in study ewe lambs at 450 days of age, corresponding with late lactation, had significant negative linear and positive quadratic associations with weaning weights of their offspring. The FEC at which the positive quadratic effect exceeded the negative linear effect of FEC was calculated using the following formula:

Reflection = $e^{[(-1) \times \text{linear FEC 450 estimate}] / [2 \times \text{quadratic FEC estimate}]}$

Weaning weights were predicted to decrease with increasing dam FEC from 0 to 361 epg, and increase above 361 epg. Predicted weaning weights of twin male offspring weaned at 86 days of age that were born to study ewe lambs weighing the flock average (51 kg) at 400 days of age are shown in Figure 3.9. For example, a ewe lamb with FEC of 6500 epg at 450 days old was predicted to wean twin male offspring weighing approximately 25.5 kg each, 5.0 kg heavier than a similar ewe with a FEC of 3000 epg. Single offspring had significantly higher weaning weights than those born as either twins or triplets ($p < 0.001$ for both comparisons), but there was no significant difference between those born as twins or triplets ($p = 0.19$), regardless of study ewe FEC.

3.4 Discussion

Although the 2016 grazing season was characterized by low precipitation, the infecting GIN species identified and seasonal fluctuations in their relative proportions and FECs were consistent with the epidemiology of GINs in Ontario reported by Mederos et al. (2010). This indicates that the parasite challenge conditions during the study were likely representative of those under typical Ontario grazing conditions. The study ewe lambs had comparable average birth weight (3.3 kg), 50 day weight (20.1 kg) and 100 day weight (36.1 kg) to provincial (3.7 kg, 21.4 kg and 36.7 kg, respectively) and national (3.8 kg, 23.4 kg and 39.5 kg, respectively) averages for the Rideau breed in 2016 (GenOvis, 2018). The average number of offspring born per study ewe lamb in 2017 (1.85) was lower than the provincial (2.46) or national (2.58) averages of ewes of all ages (GenOvis, 2018). Inclusion of mature ewes likely accounts for the observed difference in

average litter size, as primiparous ewe lambs typically have smaller litters than mature ewes (Koycegiz et al., 2009; Notter et al., 2018). Given the similarity between flock productivity measures and national and provincial averages, performance in the study flock appeared representative of Rideau and Rideau-crossbred sheep both in Ontario and throughout Canada.

Of the three performance indicators modeled, predicting weight change proved the most complex but followed a predictable pattern that correlated with reproductive activity. Study ewe lambs grew steadily prior to breeding at 245 days of age, with more rapid weight gain by mid-gestation at 350 days of age and abrupt loss of weight at parturition (400 days of age). Study ewe lambs that delivered lambs, but not those that aborted early in gestation, continued to lose weight from 400-490 days of age; this corresponds with lactation when energy demands are very high. All of the study ewe lambs resumed gaining weight from 490-570 days of age, between the end of their first lactation and their second breeding.

Weight loss at parturition (400 days of age) in triplet-bearing study ewe lambs was approximately three times that of single-bearing study ewe lambs (see Figure 3.3), suggesting that the majority of periparturient weight loss was directly due to offspring birth weight. However, study ewe lambs that were confirmed pregnant but failed to deliver offspring lost a similar amount of weight between 350-400 days of age as single-bearing study ewe lambs. Weight loss in this group is not likely to be due solely to loss of fetuses, as the aborted fetuses were too small to be identified. Nutritional stress is also

considered unlikely, as high rainfall around lambing in May 2017 led to lush pasture growth and nutritional supplementation was provided to all study ewe lambs throughout gestation. Furthermore, late gestation nutritional management was similar for all of the study ewe lambs. All five study ewe lambs that aborted early in gestation survived to the termination of the study, but the cause of abortion was not determined. It is possible that abortion in these ewe lambs was the result of an underlying disease process, which could have contributed to their observed weight loss. Only study ewe lambs that aborted gained weight during the lactation period and, as GIN FEC was not found to be a significant predictor of weight change, this suggests that weight loss in lactating ewe lambs was primarily due to the nutritional demands of lactation. The association of higher body condition scores with less weight gain or more weight loss, depending on the time interval, likely reflects increased capacity to lose weight but less potential for weight gain relative to lean animals of similar weight (Tolkamp et al., 2006).

It is unclear whether weight at 350 days of age (mid-gestation) influenced litter size or vice versa. The heaviest study ewe lambs at mid-gestation may have grown faster and achieved a greater proportion of their adult weight. If this was the case, they would be expected to face lower energy demands for continued growth, allowing more energy to be directed to fetal development than in lighter ewe lambs. However, weight of fetuses would be expected to contribute more to the weight of a mid-gestation ewe lamb bearing a larger litter than a ewe lamb bearing a single fetus, therefore mid-gestation weight may simply be a direct reflection of fetal numbers. Similarly, FEC at 400 days of age (lambing) is more likely a consequence of the physiological stresses associated with litter

size than causal of litter size. Ewes are known to experience a periparturient relaxation of GIN immunity, leading to an increase in fecal egg output referred to as the periparturient egg rise (PPER) (Falzon et al., 2013; Notter et al., 2018). This PPER is believed to be related to partitioning of energy to lactation and fetal development in late gestation; thus, the increased metabolic demand of higher numbers of fetuses would be predicted to result in a higher PPER (Kerr et al., 2017).

Although increasing FEC in the study ewe lambs prior to breeding at 220 days of age and at mid-gestation at 350 days of age had slight negative associations with litter size, these associations were not significant. As the study ewe lambs were not exposed to infective L₃s on pasture between 220 and 350 days of age, FECs at 350 days of age reflect infective burdens acquired up to sampling in November 2016 at 220 days of age. Compared with summer and early fall 2016, pasture L₃ contamination had increased in November 2016 prior to breeding, but the lack of a corresponding increase in flock FECs indicates that the majority of ingested L₃s likely became hypobiotic (see Figure 3.2). As burdens of adult GINs prior to breeding and at mid-gestation were low, resulting gastrointestinal damage and/or protein loss appeared insufficient to cause a significant reduction in litter size (Greer, 2008; Mavrot et al., 2015). Administration of an anthelmintic either prior to or after breeding was not a significant predictor of litter size, in contrast to the findings reported by Mavrogianni et al. (2011). However, pre-breeding arithmetic mean FEC in the untreated ewe lambs reported here (164 epg) was much lower than the 767 epg reported by Mavrogianni et al. (2011).

Several significant associations were identified between offspring-related factors and their weaning weights. Predictably, younger offspring and those born in litters of two or more were lighter at weaning than older or single lambs. Weaning weights of offspring born as twins or triplets did not differ significantly. However, since at least one lamb was artificially reared or cross-fostered from study ewe lambs that delivered triplets, offspring born as triplets were reared as twins, or, less commonly, as a single lamb. Milk available per nursing lamb would therefore be expected to be comparable between lambs born as triplets or twins. The lighter weights of offspring from mixed sex litters compared with single sex litters is likely due at least in part to the fact that lambs weaned from mixed sex litters by definition included only those reared as twins, while all-female and all-male litters included both twins and single lambs.

Two dam-related factors were also significant predictors of weaning weights. Heavier study ewe lambs at 400 days of age, corresponding with lambing, weaned heavier offspring. This finding is in agreement with results reported in North American Targhee sheep by Borg et al. (2009), and multiple explanations for this relationship are possible. As postulated previously, ewe lambs that were heavier at lambing may have attained a greater proportion of mature weight, requiring less energy for continued growth and leaving more available for lactation. Heavy ewe lambs may also have had greater adipose reserves for mobilization during lactation, though body condition score reflects frame size and adipose reserves more accurately than weight (Borg et al., 2009) and dam body condition score was not significantly associated with offspring weaning weight. Lighter ewe lambs may have delivered twins or triplets and partitioned more of their energy

reserves into fetal growth in late gestation while leaving less for lactation, but this was considered less likely given that the interaction between litter size and dam weight was not significant. Given the heritability of growth potential, it is also possible that a genetic effect contributed to the positive association between dam weight at lambing and offspring weaning weight (Borg et al., 2009).

The presence of opposing linear and quadratic effects of ewe lamb FEC at 450 days of age (late lactation) on offspring weaning weights reflects the complex relationship between GIN parasitism and metabolism in ewes and lambs. Nematode infection is expected to exert increased metabolic demand in ewes, diverting energy from lactation (Greer, 2008; Mavrot et al., 2015). However, the effect of GINs on milk production in small ruminants is partly dependent on infecting GIN species. In dairy goats, does with higher milk yields typically have lower FEC if *Trichostrongylus* sp. and/or *T. circumcincta* are the primary infecting species, but higher FEC if *H. contortus* is most common (Heckendorn et al., 2017). Evidence for these associations in sheep is less conclusive, as studies investigating the relationship between FEC and milk yield typically involve comparison of treated and untreated ewes (Cringoli et al., 2009; Suarez et al., 2009; Cruz-Rojo et al., 2012). However, recently Kordalis et al. (2019) reported increased prevalence of subclinical mastitis in ewes with increased GIN burdens, predominately of *Teladorsagia* spp. This may also explain the negative association between *T. circumcincta*-specific FEC and milk yield in goats; however, the reason for the positive association between *H. contortus*-specific FEC and milk yield remains unclear. As *H. contortus* accounted for approximately 50 % of the fecal egg output in late

lactation (July 2017, see Figure 3.2), it is possible that a similar relationship existed in the study flock and contributed to the increased predicted weaning weights when FEC was greater than 361 epg. However, given the pathogenicity of *H. contortus*, this positive association would not be expected to continue indefinitely. A low number of study ewe lambs (n = 17) had late lactation FECs between 5000 and 12,500 epg; consequently, the 95 % confidence interval for predicted weaning weights increased progressively as FEC increased (see Figure 3.9). Likewise, the 95 % confidence interval for predicted weaning weight at late lactation FECs between 0 and 361 epg was wide due to relatively few observations within this FEC range (n=19), and the lower limit indicated the possibility that predicted weaning weights increased with FEC across the entire observed range of late lactation FECs (see Figure 3.9). Therefore, the precise association between offspring weaning weights, milk yield and GIN FEC in late lactation, particularly at FECs below 361 epg and exceeding 5000 epg, merits further investigation in sheep.

3.5 Conclusions

Throughout the study, *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* accounted for greater than 95 % of fecal egg shedding, with *T. circumcincta* predominant in spring, *H. contortus* in summer, and *T. colubriformis* in fall and winter. Despite the prevalence of these pathogenic species, subclinical gastrointestinal nematode infection, as measured by FEC, was not found to be a significant predictor of growth in Ontario ewe lambs during their first two grazing seasons. However, higher FEC in primiparous ewe lambs at lambing was associated with

larger litter sizes. This association likely reflects increased PPER in ewes delivering larger litters. Pre-breeding and mid-gestation FECs were not significantly associated with litter size. Both significant positive quadratic and negative linear effects were identified between late lactation FEC and offspring weaning weights, though the positive quadratic effect predominated above FEC of 361 epg. However, very few ewes exceeded FECs of 5,000 epg in late lactation. Therefore, further investigation of the relationship between ewe FEC and offspring weaning weight at ewe FECs above 5,000 epg is warranted.

3.6 Acknowledgements

The authors wish to thank Rebecca Chant, Stéphanie Bourgon, Ziwei Li, Leah Lourenco, Samantha Dixon, Mike Alcorn, Tracy To, Courtney Lun, Alaina Macdonald, Tori Brown and Peyton Tam for providing laboratory and field assistance. Thanks also to Dennis Inglis and Neil Moore for providing access to climate data. The authors give special thanks to the sheep producer who participated in this study.

This work was supported by the Canadian Agricultural Adaptation Program, the Ontario Agri-Food Innovation Alliance, Ontario Sheep Farmers, and a University of Guelph Undergraduate Research Assistantship.

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3.8 Tables

Table 3.1. Timing of anthelmintic treatment and proportion of Rideau-Dorset cross ewe lambs treated, 2016-2017, in a commercial flock in central Ontario.

Date (month/year)	Mean age (days)	Production stage	Study group size	Number treated	Percent treated
05/2016	30	Nursing	140	0	0.0
07/2016	90	Weaned	133	133	100.0
08/2016	130	Pre-breeding	128	115	89.8
10/2016	190	Pre-breeding	127	15	11.8
11/2016	220	Breeding	127	20	15.7
03/2017	350	Mid-gestation	119	27	22.7
05/2017	400	Lambing	110	74	67.3
07/2017	450	Mid-lactation	108	88	81.5
08/2017	490	Late lactation	107	1	0.9
10/2017	550	Dry	107	30	28.0
11/2017	570	Dry	107	6	5.6

Study ewe lambs were treated with 7.5 mg/kg levamisole per os in 2016, and 5 mg/kg albendazole per os in 2017. All study ewe lambs were treated when weaned in 07/2016 according to routine farm management practices. At all other time points treatment was administered to individual ewe lambs with fecal egg count equal to or greater than 500 eggs per gram.

Table 3.2. Parameters evaluated as fixed effects in models of growth, litter size, and individual offspring weaning weight of Rideau-Dorset cross ewe lambs, 2016-2017, in a commercial flock in central Ontario. Observed ranges are given in parentheses for categorical variables.

Variable	Definition	Model(s)
Ewe born as	Birth litter size (range 3-5)	Growth, litter size, weaning weight
Ewe raised as	Offspring raised to weaning by dam or foster dam of ewe lamb (range 1-3)	Growth, litter size, weaning weight
Ewe age	Age of ewe lamb in days	Growth, litter size, weaning weight
Litter size	Total number of offspring born to ewe lamb (range 0-3)	Growth, weaning weight
Previous weight	Weight of ewe lamb at start of 6-8 week interval	Growth
Weight day X	Weight of ewe lamb on day X	Litter size, weaning weight
Ln FEC (day X)	Natural logarithmic transformation of ewe lamb GIN fecal egg count on day X	Growth, litter size, weaning weight
Treatment (day X)	Whether ewe lamb was treated with anthelmintic on day X (yes/no)	Growth, litter size, weaning weight
BCS	Ewe lamb's body condition score (2-4)	Growth, litter size, weaning weight
Day	Days since study began (May 10, 2016)	Growth
Weaned	Number of offspring weaned by ewe lamb (0-2)	Weaning weight
Age at weaning	Age of ewe lamb's offspring at weaning	Weaning weight
Sex(es)	Sex of ewe lamb's offspring (all male, all female, or mixed sex pair)	Weaning weight

Ln = natural logarithm; FEC = fecal egg count; GIN = gastrointestinal nematode; BCS = body condition score.

Table 3.3. Causes of loss to follow-up in Rideau-Dorset cross ewe lambs (n = 33), 2016-2017, in a commercial flock in central Ontario.

	Cause	Number of sheep
2016	Mortality	
	Misadventure	3
	Coccidiosis	2
	Pneumonia	2
	Predation	2
	Unknown cause	3
	Removal	
	Illness (unknown cause)	1
2017	Mortality	
	Listeriosis	3
	Lambing complications	3
	Mastitis	1
	Metritis	1
	Unknown cause	2
	Removal	
Failure to conceive	5	
Escape	3	
	Illness (vaginal prolapse)	2
Total		33

Table 3.4. Predictors in a general linear mixed model of weight change over 6-8 week intervals of Rideau-Dorset cross ewe lambs (n = 140), 2016-2017, in a commercial flock in central Ontario. Direction of association is given for significant effects only.

Predictor variable	p-value	Direction of association	NDF	DDF	F-value	Proportion of variance explained (%)¹
Day	0.003	Variable ²	8	773	3.74	3.73
BCS	0.010	Negative	2	116	4.84	7.70
Ewe raised as	0.360	NA	2	108	1.03	1.87
Litter size	0.365	NA	3	108	1.07	2.89
Previous weight	0.688	NA	1	773	0.16	0.02
Ln GIN FEC	0.820	NA	1	773	0.05	0.006
Ewe raised as*Litter size*Day	< 0.001	Variable ²	31	773	3.82	13.28
Ewe raised as*Day	< 0.001	Variable ²	16	773	3.05	5.94
Litter size*Day	< 0.001	Variable ²	24	773	5.36	14.27
Previous weight*Day	< 0.001	Variable ²	8	773	5.93	5.78
Ewe raised as*Litter size	0.829	Variable ²	4	108	0.37	1.35

NDF = numerator degrees of freedom; DDF = denominator degrees of freedom; BCS =

body condition score; NA = not applicable; Ln = natural logarithm; GIN = gastrointestinal nematode; FEC = fecal egg count.

¹ Estimated using formulas described by Dohoo et al. (2014), page 330.

² Direction of association varies at different levels of a categorical variable.

Table 3.5. Reproductive performance of Rideau-Dorset cross ewe lambs (n = 108), May-August 2017, in a commercial flock in central Ontario.

	Measure	Number of lambs
Offspring born May-June 2017	Birth status	
	Liveborn	182
	Stillborn	14
	Mummified	4
	Litter size	
	Single	28
	Twin	136
	Triplet	36
	Total	200
Offspring weaned August 2017	Offspring sex	
	Male	55
	Female	59
	Offspring weaned as single	
	Male	26
	Female	26
	Offspring weaned as twin	
	Male	14
	Female	18
	Mixed	30
		Total

Table 3.6. Predictors in a generalized linear mixed model of litter size of Rideau-Dorset cross ewe lambs (n = 118), May-June 2017, in a commercial flock in central Ontario.

Direction of association is given for significant effects only.

Predictor variable	p-value	Direction of association	NDF	DDF	F-value	Proportion of variance explained (%)¹
Ewe weight at 350 days of age (mid-gestation)	0.025	Positive	1	101	5.16	4.86
Ewe Ln GIN FEC at 400 days of age (lambing)	0.050	Positive	1	101	3.94	3.75
Ewe Ln GIN FEC at 220 days of age (pre-breeding)	0.610	NA	1	101	0.26	0.26
Ewe Ln GIN FEC at 350 days of age (mid-gestation)	0.452	NA	1	101	0.57	0.56

NDF = numerator degrees of freedom; DDF = denominator degrees of freedom; Ln =

natural logarithm; GIN = gastrointestinal nematode; FEC = fecal egg count.

¹ Estimated using formulas described by Dohoo et al. (2014), page 330.

Table 3.7. Predictors in a general linear mixed model of individual weaning weights of offspring raised by Rideau-Dorset cross ewe lambs (n = 96), August 2017, in a commercial flock in central Ontario. Direction of association is given for significant effects only.

Predictor variable	p-value	Direction of association	NDF	DDF	F-value	Proportion of variance explained (%)¹
Litter size	< 0.001	Negative	2	70	14.48	29.26
Age of lambs at weaning	0.002	Positive	1	70	10.32	12.85
Ewe weight at 400 days of age (lambing)	0.007	Positive	1	70	7.74	9.56
Lamb sex(es)	< 0.001	See note ²	2	70	10.06	22.33
Ewe Ln GIN FEC at 450 days of age (mid-lactation)	0.044	Negative	1	70	4.22	5.69
Ewe Ln GIN FEC at 450 days of age X Ewe Ln GIN FEC at 450 days of age	0.029	Positive	1	70	4.97	6.63

NDF = numerator degrees of freedom; DDF = denominator degrees of freedom; Ln = natural logarithm; GIN = gastrointestinal nematode; FEC = fecal egg count.

¹ Estimated using formulas described by Dohoo et al. (2014), page 330.

² Offspring raised as single-sex litters predicted to be heavier than those raised as mixed-sex litters. No significant difference between offspring raised as male-only and female-only litters.

3.9 Figures

Figure 3.1. Monthly maximum, minimum and mean temperatures and total monthly precipitation in central Ontario from May 2016- November 2017. Error bars indicate 95 % confidence interval for mean monthly temperature. Weather data were obtained from a station 26 km away from the enrolled farm.

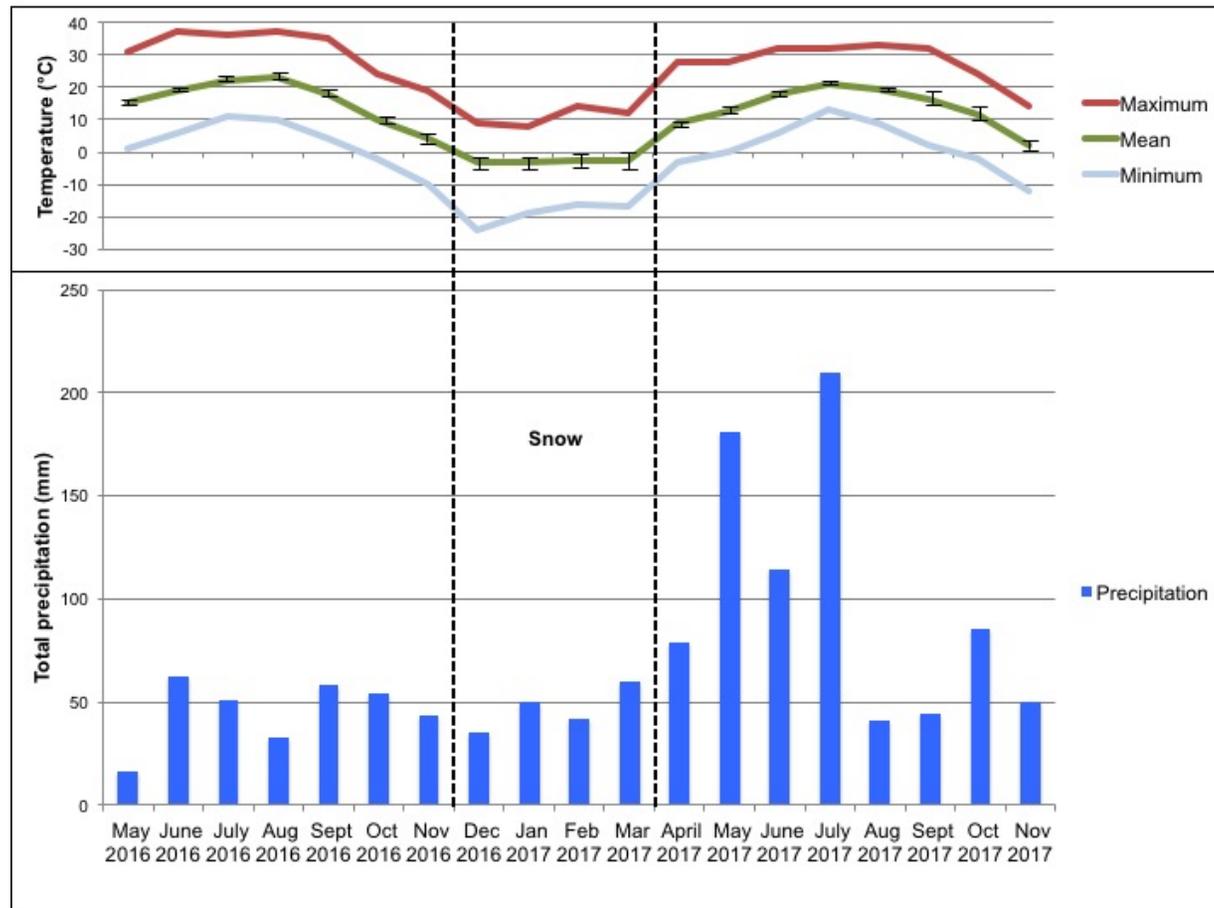


Figure 3.2. Total infective nematode third-stage larvae (L₃s) per kilogram dry matter (DM) on pasture and mean gastrointestinal nematode (GIN) species-specific fecal egg counts (FEC), May 2016-November 2017, on a commercial sheep farm in central Ontario. Error bars indicate 95 % confidence interval for GIN species-specific FEC. Arithmetic mean GIN species proportions were calculated from 15 individual fecal cultures at each time point except November 2016 and August 2017, when only 14 and 7 samples, respectively, exceeded the minimum FEC for culture of 200 epg. Species-specific FEC was determined for each GIN species using the following formula:

$$\text{Species-specific FEC} = \text{Arithmetic mean species proportion} \times \text{Flock mean FEC}$$

*No animals exceeded the minimum egg count for culture in May 2016; hence species proportions could not be determined.

**Pasture forage was snow-covered in March 2017 and was not collected for enumeration of L₃s per kg dry weight.

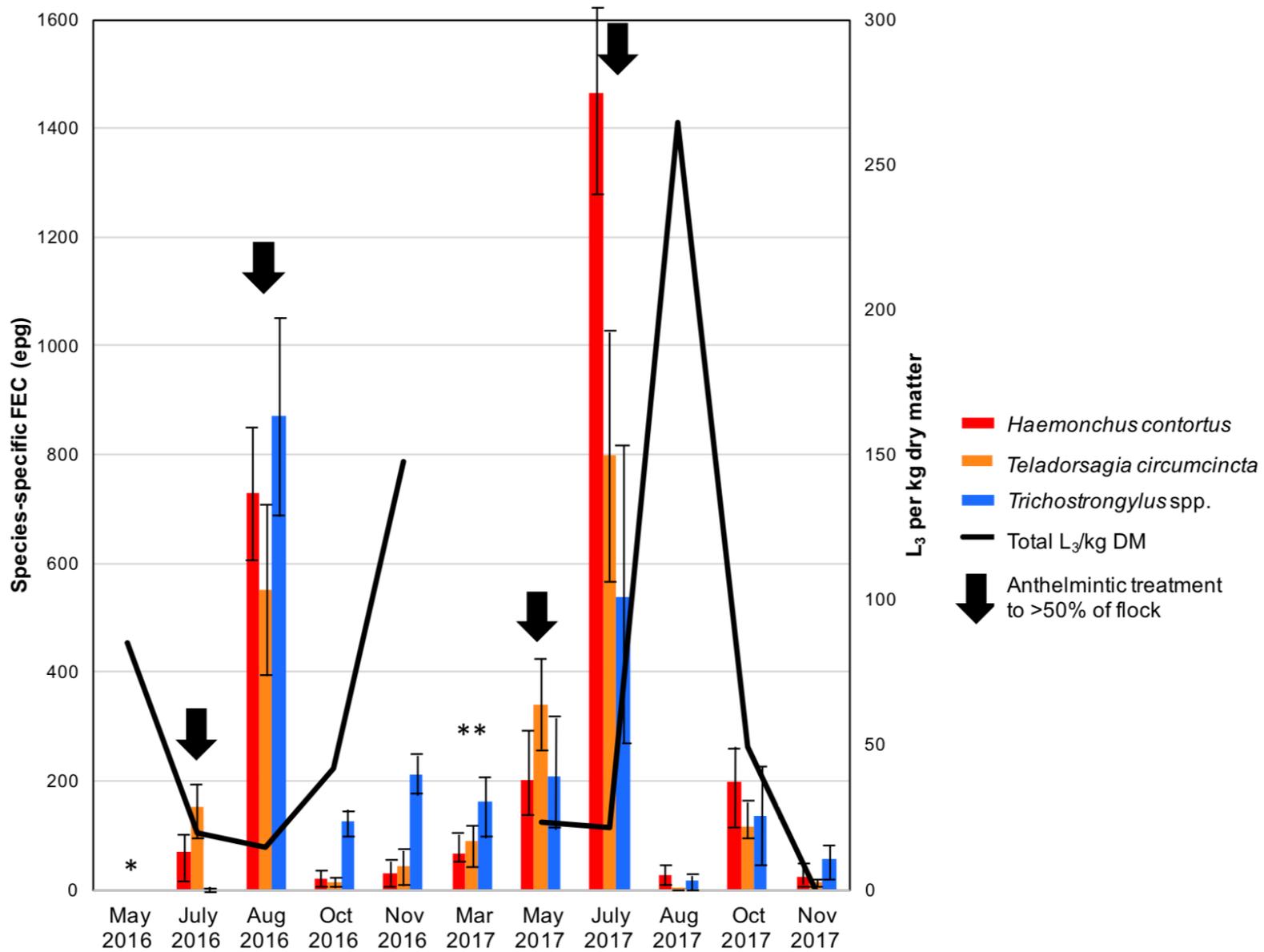


Figure 3.3. Average change in weight of Rideau-Dorset cross ewe lambs raised as triplets and with body condition score 3 over the indicated age interval, stratified by number of offspring delivered at lambing, 2016-2017, on a commercial sheep farm in central Ontario. Error bars indicate 95 % confidence intervals for the means.

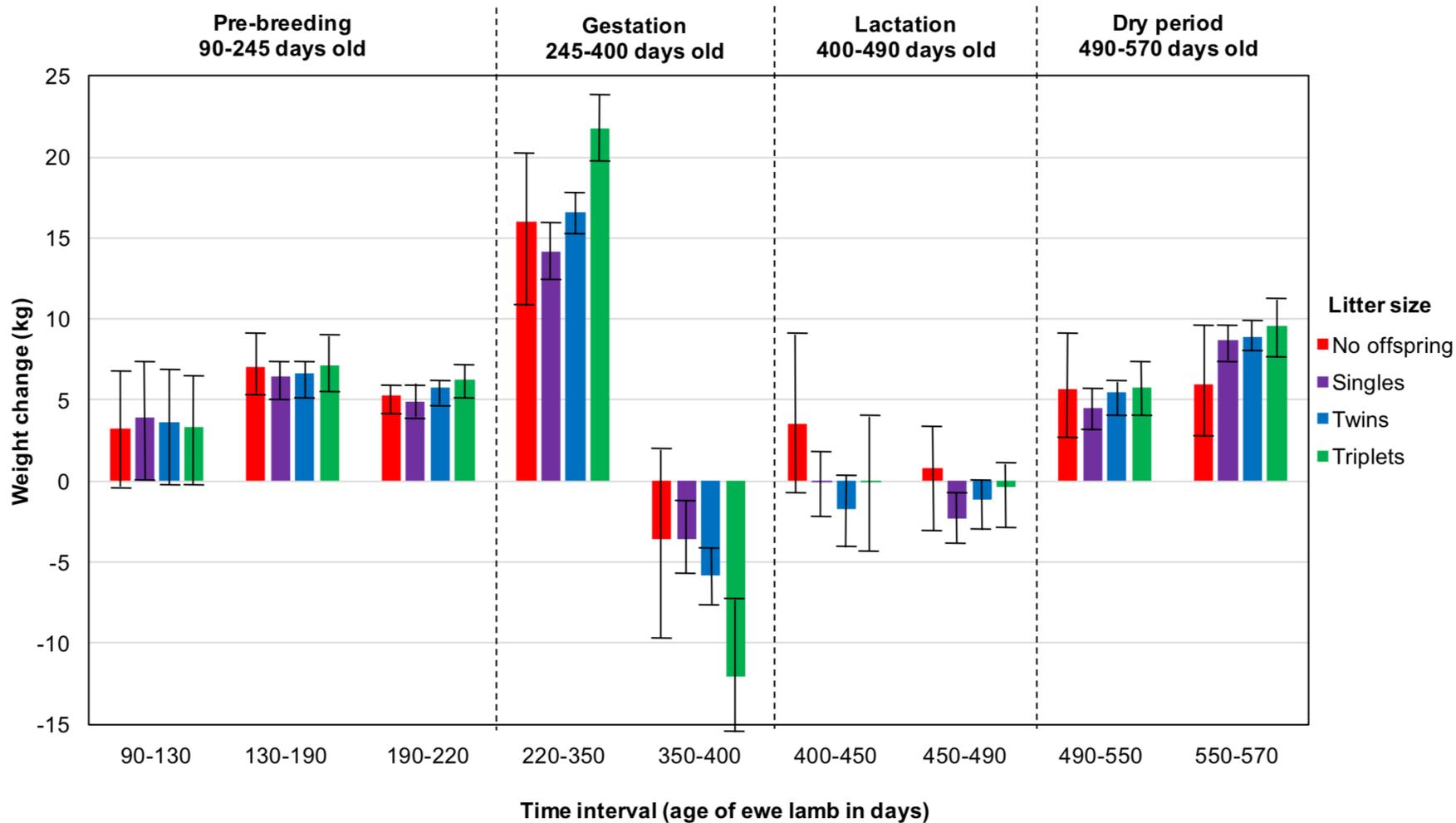


Figure 3.4. Predicted weight change of Rideau-Dorset cross ewe lambs prior to their first lambing, July 2016-March 2017, on a commercial sheep farm in central Ontario. Predictions are for study ewe lambs raised as triplets, with body condition score 3, two-year flock average gastrointestinal nematode fecal egg count (662 eggs per gram), and that gave birth to twin offspring at 400 days of age. Line length indicates the observed range of weights at the start of each time interval.

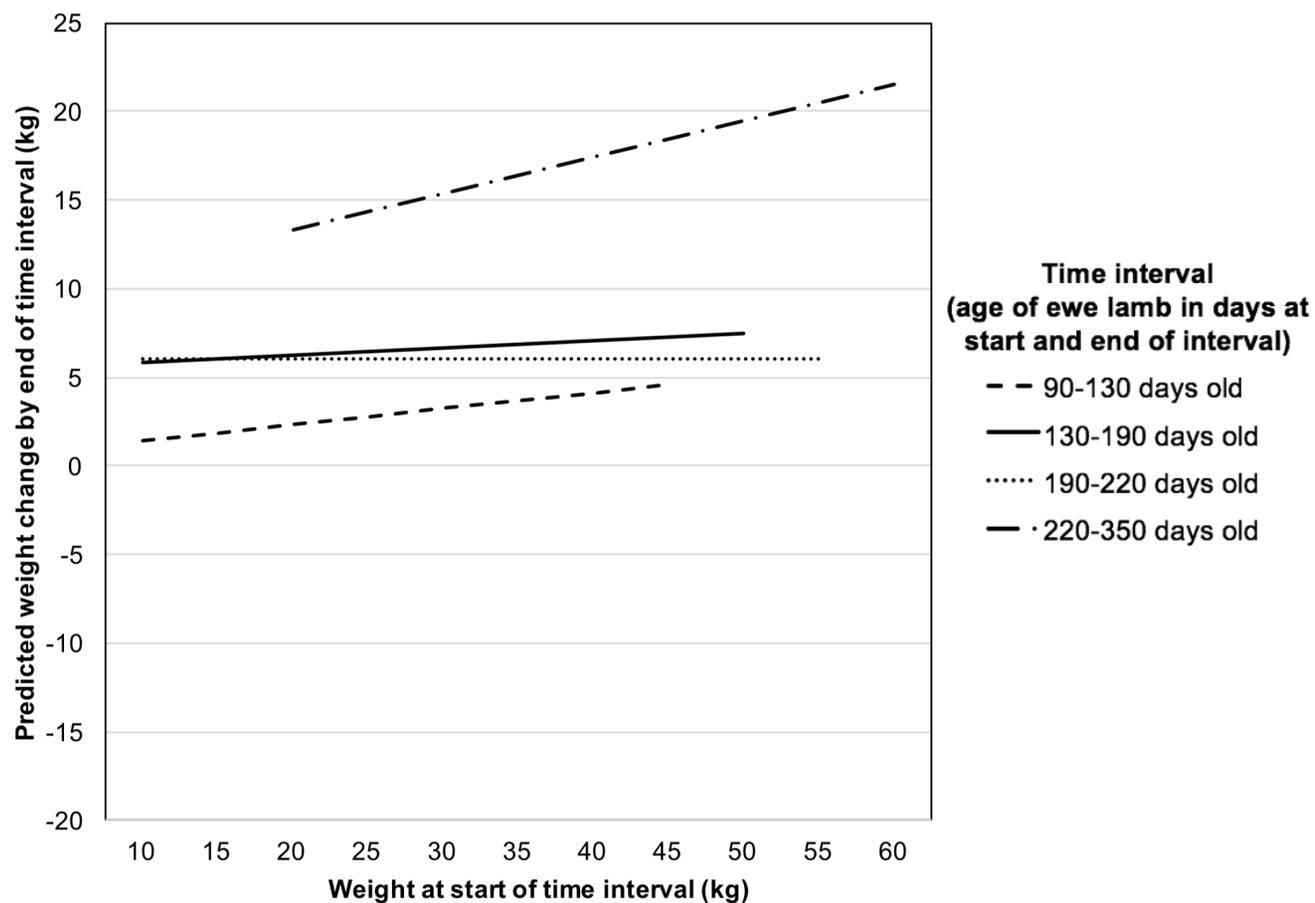


Figure 3.5. Predicted weight change of Rideau-Dorset cross ewe lambs following lambing at 400 days of age, March-November 2017, on a commercial sheep farm in central Ontario. Predictions are for study ewe lambs raised as triplets, with body condition score 3, two-year flock average gastrointestinal nematode fecal egg count (662 eggs per gram), and that gave birth to twin offspring at 400 days of age. Line length indicates the observed range of weights at the start of each time interval.

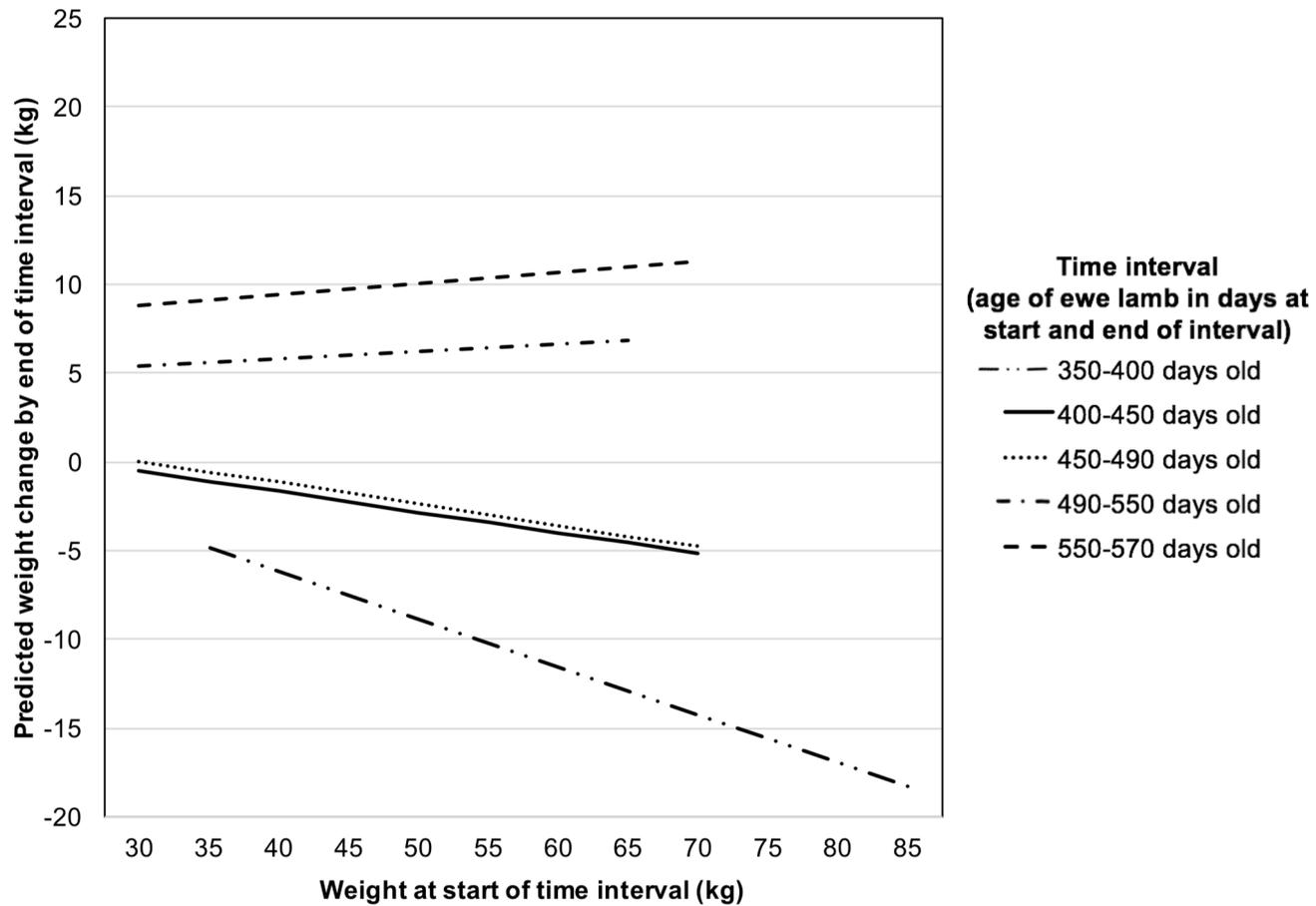


Figure 3.6. Predicted litter size, including stillborn and mummified lambs, of Rideau-Dorset cross ewe lambs with various weight at 350 days of age (mid-gestation), on a commercial sheep farm in central Ontario. Predictions are for ewe lambs with flock average gastrointestinal nematode fecal egg counts at 220, 350 and 400 days of age (287, 320, and 749 eggs per gram, respectively).

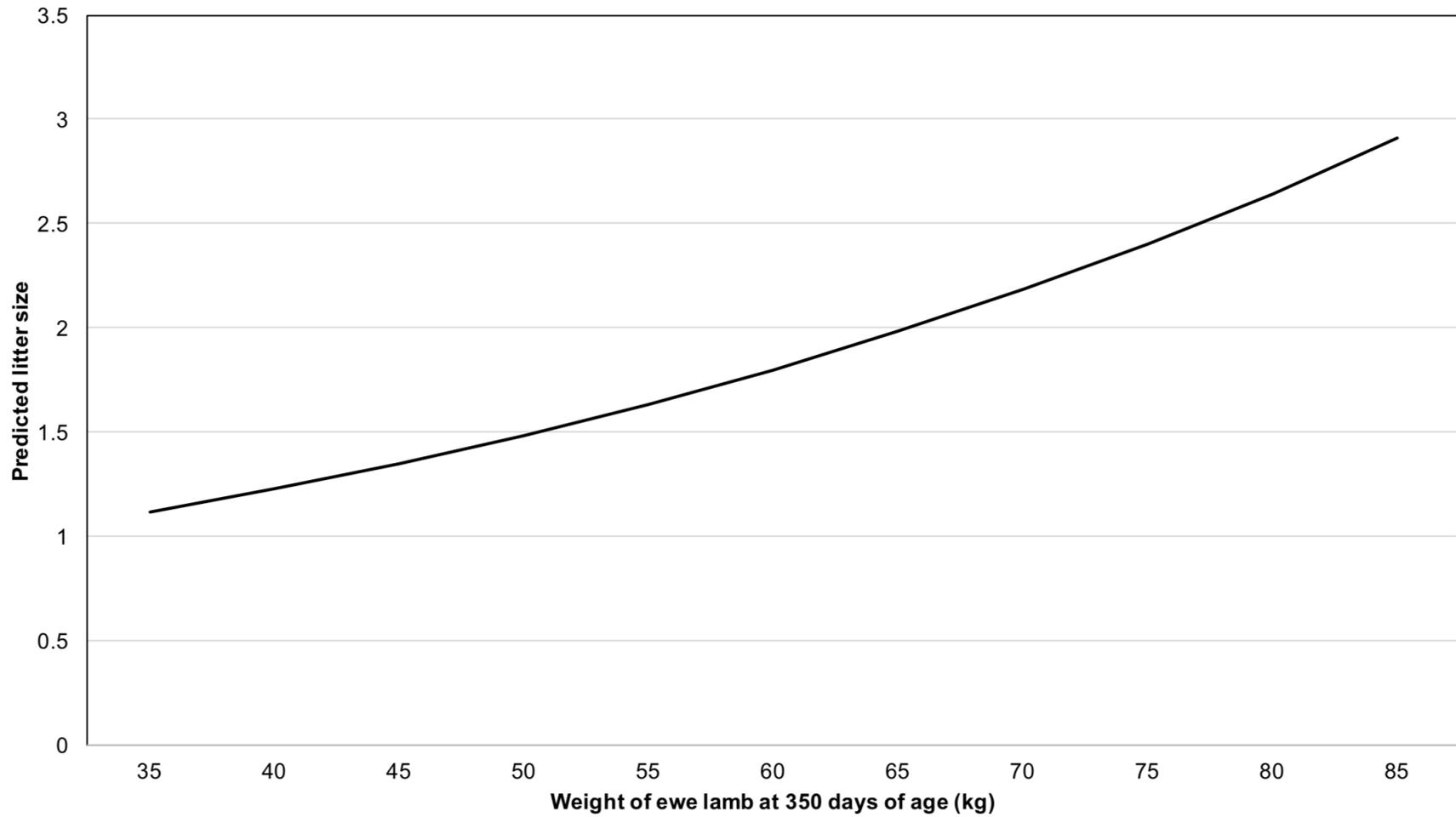


Figure 3.7. Predicted litter size, including stillborn and mummified offspring, of Rideau-Dorset cross ewe lambs with various gastrointestinal nematode fecal egg count (FEC) shortly prior to breeding (220 days old), at mid-gestation (350 days old), and at lambing (400 days old), on a commercial sheep farm in central Ontario. Predictions are for ewe lambs that weighed 58 kg at 350 days of age (flock average).

*Ewe lamb FECs at 350 and 220 days of age were not significantly associated with predicted litter size ($p > 0.40$).

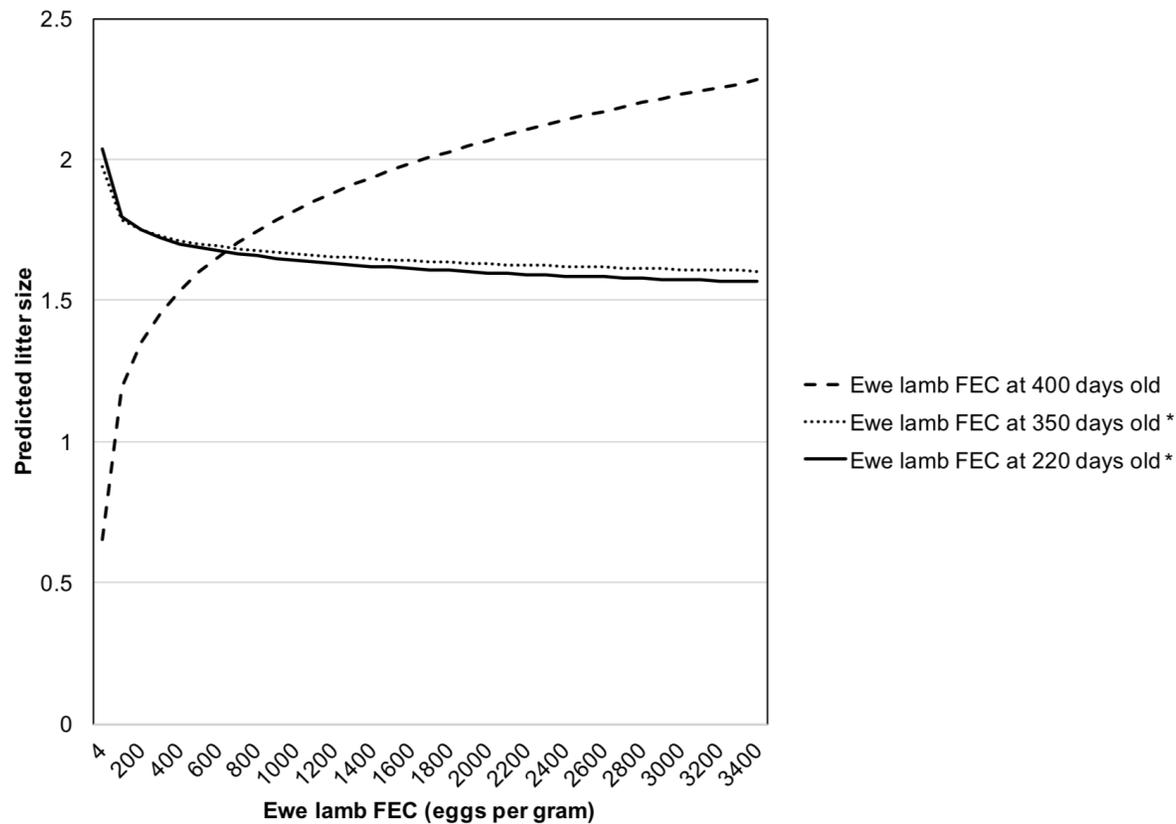


Figure 3.8. Predicted weaning weights of 86-day-old twin offspring raised by Rideau-Dorset cross ewe lambs on a commercial sheep farm in central Ontario. Predictions are based on dam weight at lambing (400 days of age). Predictions are for ewe lambs with flock average gastrointestinal nematode fecal egg count at 450 days of age (2800 eggs per gram).

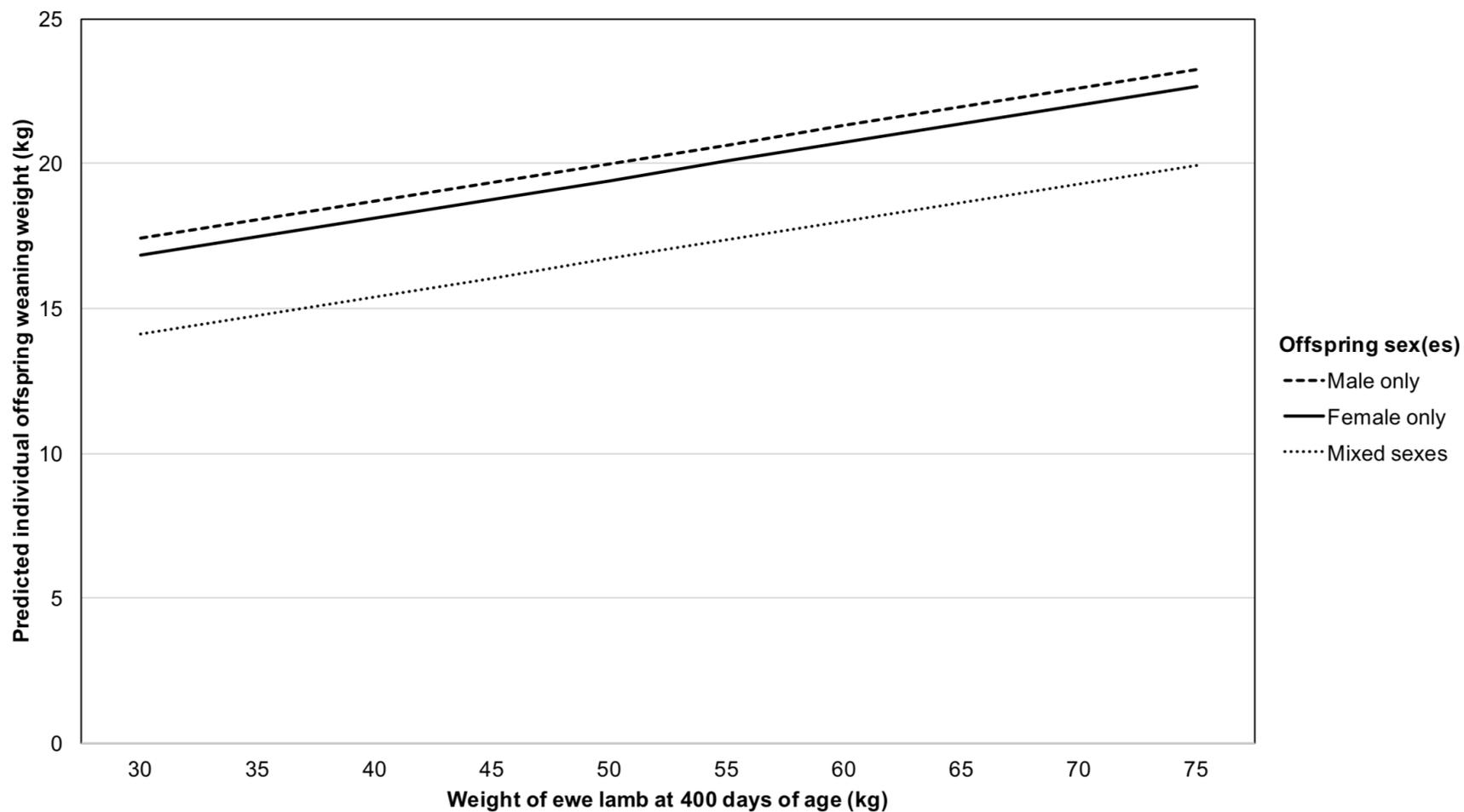
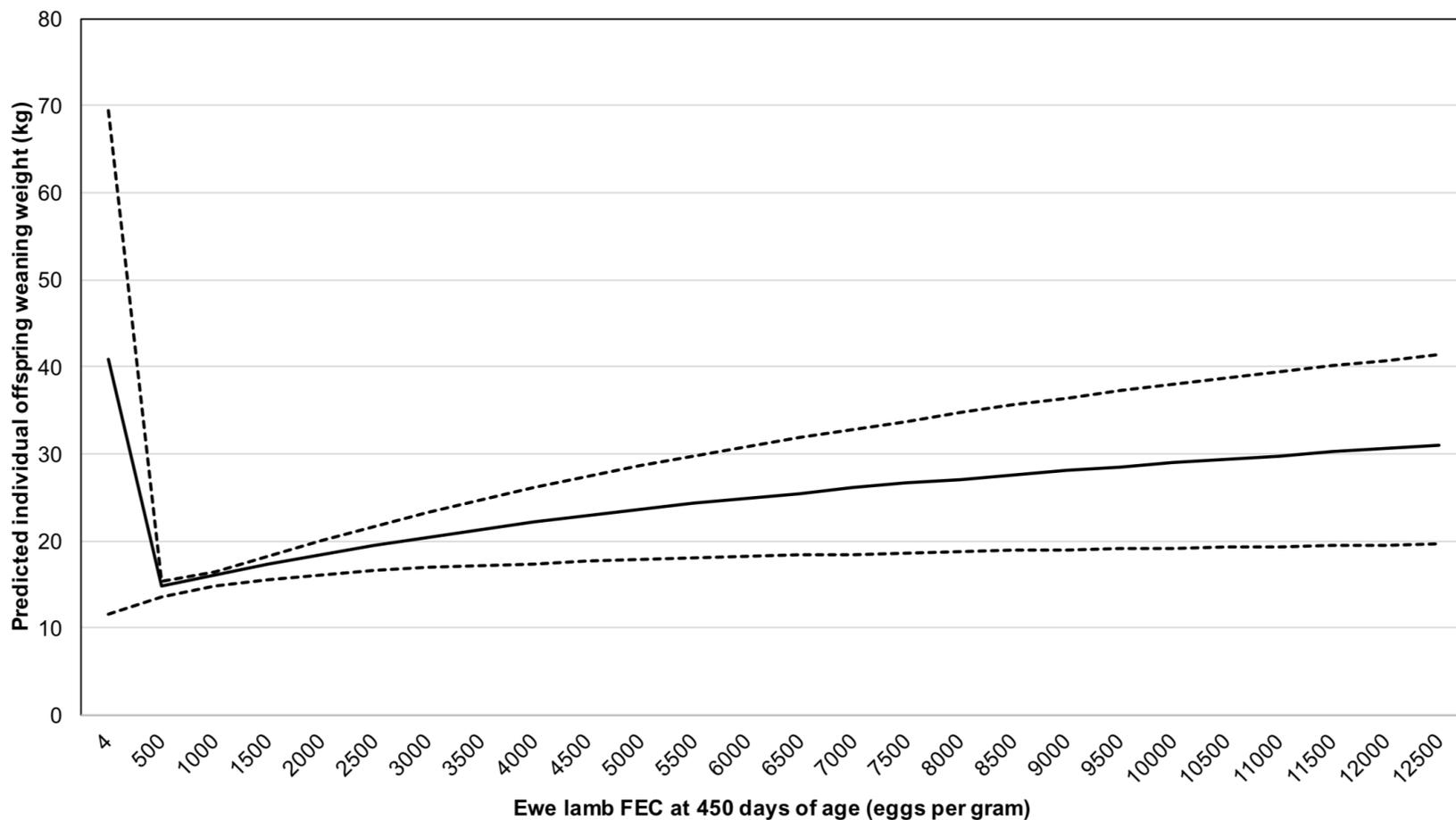


Figure 3.9. Predicted weaning weight per lamb of 86-day-old twin male lambs raised by Rideau-Dorset cross ewe lambs in central Ontario, based on dam gastrointestinal nematode fecal egg count (FEC) in late lactation (450 days of age). Dashed lines indicate 95 % confidence interval for predicted weaning weight. Predictions are for ewes that weighed 51 kg at 400 days of age (flock average weight).



CHAPTER FOUR:

**CORRELATION OF SALIVARY ANTIBODY TO CARBOHYDRATE
LARVAL ANTIGEN (CARLA) WITH HEALTH AND
GASTROINTESTINAL NEMATODE PARASITISM IN SHEEP UNDER
ONTARIO GRAZING CONDITIONS**

Based on a manuscript in preparation for submission to *Veterinary Parasitology*.

Abstract

Leveraging mucosal immunity is a promising method for controlling gastrointestinal nematode (GIN) parasitism in sheep. Salivary antibody to carbohydrate larval antigen (sCarLA), a heritable measure of immunity to third-stage GIN larvae (L₃S), has been successfully applied to genetic improvement programs in New Zealand. However, sCarLA levels wane in the absence of ongoing GIN exposure. New Zealand's temperate climate permits year-round exposure to L₃S, but cold winters in boreal regions such as Ontario, Canada interrupt exposure for five months or more. This study investigated associations between sCarLA levels, GIN parasitism and indicators of overall health in sheep grazing under Ontario conditions. A commercial flock of 140 Rideau-Dorset cross ewe lambs was followed from approximately 30 days of age in May 2016 until November 2017, including lambing and lactation in the spring of 2017. Every 6-8 weeks during the grazing season and at mid-gestation in March 2017, fecal egg counts were performed, blood collected to assess serum albumin, globulin and hematocrit, and pasture samples obtained to confirm exposure to infective larvae. Measurements of sCarLA level were

performed at the beginning, middle and end of each grazing season, and at mid-gestation. Spearman's rank correlation coefficients were calculated to compare sCarLA levels over time, and general linear mixed models created to evaluate associations between sCarLA levels, GIN fecal egg count, hematocrit, serum albumin and globulin. Levels of sCarLA followed a similar seasonal pattern to GIN fecal egg counts with a 6-8 week delay; much higher sCarLA levels were observed in the second grazing season. The proportion of the flock with detectable sCarLA was 68.3 % by the end of the first grazing season, declined over winter, and approached 100 % by the middle of the second grazing season. Correlations between sCarLA levels over time were consistently positive, of weak to moderate strength, and significant ($p < 0.05$). Higher sCarLA level was significantly ($p < 0.0001$) associated with reduced fecal egg counts at all time points. The flock displayed minimal variability in hematocrit, serum albumin, and serum globulin; none of which were significantly associated with sCarLA levels. These results suggest that sCarLA can be maintained over winter and may be a suitable measure of immunity to GINs in sheep under Ontario grazing conditions.

Keywords

Sheep, nematode, carbohydrate larval antigen, *Haemonchus*, *Trichostrongylus*, *Teladorsagia*

4.1 Introduction

Parasitism of sheep by gastrointestinal nematodes (GINs) is a significant cause of morbidity, mortality and financial losses on sheep farms across the globe (Mederos et al., 2010; Mavrot et al., 2015). However, the success of anthelmintic control of GINs is increasingly being limited by widespread anthelmintic resistance (Kaplan and Vidyashankar, 2012; Falzon et al., 2013). In

Ontario, Canada, resistance to ivermectin and fenbendazole was identified in 97 % and 95 %, respectively, of sheep farms reporting anthelmintic failure (Falzon et al., 2013). In light of the rising prevalence of anthelmintic resistance, strategies to leverage the host immune response to GINs are being developed to complement and reduce reliance on anthelmintics (Shaw et al., 2012; Shaw et al., 2013). One such strategy is production of GIN vaccines; a vaccine for *Haemonchus contortus* has been approved and is commercially available in the United Kingdom, Australia and South Africa (Barbervax®, Department of Agriculture and Food, Australia) (Bassetto and Amarante, 2015; WormBoss, 2019), and a recombinant antigen vaccine for *Teladorsagia circumcincta* has shown promise in early trials in the United Kingdom (Nisbet et al., 2013; Nisbet et al., 2016). However, both vaccines require repeated administration to achieve and maintain protective immunity, and neither are licensed in North America (Nisbet et al., 2013; Bassetto and Amarante, 2015; Emery et al., 2016; Nisbet et al., 2016; WormBoss, 2019).

Variation in immunity to GINs was recognized in sheep long before vaccine development began (Albers et al., 1987), and genetic selection for superior immune response can be combined with anthelmintics in the short term, and vaccination in the longer term, to control GINs. Several indirect indicators of GIN burden including fecal egg counts (FECs), dag scores (numerical score of fecal breech soiling), and Faffa Malan Chart (FAMACHA©) anemia scores have been used as tools in genetic selection for GIN immunity (Williams, 2011; Saddiqi et al., 2012; Pickering et al., 2015). However, each has significant limitations. For dag and FAMACHA© scoring, clinically apparent diarrhea and anemia are not specific to GIN infection. Thus, resistant animals may appear susceptible if harbouring an unrelated disease (Saddiqi et al., 2012; Pickering et al., 2015). Although FEC is specific to GIN infection, anthelmintic treatment suppresses FEC and

must be delayed or withheld for FEC to reflect the animal's immune competence, placing health and productivity at risk (Douch et al., 1996). Moreover, the considerable temporal variation in FEC often necessitates repeated measurement to accurately reflect GIN immunity, reducing its practicality in many flocks (Douch et al., 1996; Saddiqi et al., 2012; Shaw et al., 2012).

Levels of GIN-specific immunoglobulins (Igs) are dependent on exposure to the inciting GIN antigens, and are therefore a direct and highly specific measure of the immune response to GIN infection (Douch et al., 1996; Shaw et al., 2012). Immunoglobulin production is generally unaffected by conventional anthelmintic treatment and assessment of Ig levels does not require treatment to be withheld (Shaw et al., 2013). Further, it is possible to identify a single optimal time to measure Ig levels for a given climate or region based on predictable patterns in GIN epidemiology and exposure (Douch et al., 1996; Shaw et al., 2013). Multiple Ig isotypes have been evaluated as indicators of GIN immunity, including circulating IgE, IgG1 and IgA, and mucosal IgA; all are moderately heritable and negatively associated with GIN burdens and FEC (Douch et al., 1996; Shaw et al., 2012). However, elevated circulating IgE and IgG1 have been associated with reduced growth rates and increased dag scores, limiting their utility in genetic selection (Douch et al., 1996). Circulating IgA does not appear as strongly associated with reduced productivity or with fecal consistency, but the proportion of animals with high serum IgA is too low to be a practical basis for genetic selection (Strain et al., 2002; Shaw et al., 2012).

Levels of GIN-specific mucosal IgA at the gastrointestinal mucosal surface are higher than those of the circulating isoform, and elevated mucosal GIN-specific IgA has been associated with increased growth in lambs (Strain et al., 2002; Shaw et al., 2012; Shaw et al., 2013). Although

GIN-specific IgA is typically highest at the site of infection, IgA levels in saliva are reflective of abomasal and small intestinal IgA (Shaw et al., 2012; Shaw et al., 2013). Moreover, saliva can be collected easily and noninvasively from live sheep (Shaw et al., 2012; Shaw et al., 2013). In New Zealand, assessment of salivary IgA specific to a carbohydrate larval antigen (CarLA) is available as a commercial test for GIN immunity (The CarLA Saliva Test®, AgResearch Inc., New Zealand) (Shaw et al., 2012; Shaw et al., 2013). CarLA is present on the surface of all infective third-stage GIN larvae (L₃s) tested to date; thus, CarLA-specific IgA is protective across multiple GIN species (Shaw et al., 2012; Shaw et al., 2013). However, CarLA is not a protein antigen and development of immunologic memory is weak, so mucosal IgA declines quickly if exposure is not continuous (Harrison et al., 2008; Shaw et al., 2012; Jaurigue and Seeberger, 2017). In lambs under New Zealand grazing conditions, Shaw et al. (2013) demonstrated the optimal time to perform selection based on levels of salivary antibody to CarLA (sCarLA) was within two months after weaning (between 90 and 150 days of age). Selection for GIN immunity based on salivary IgA directed at antigens, likely including CarLA, from homogenized *Teladorsagia circumcincta* L₃s has also proven promising in Lleyn sheep raised in the United Kingdom (Fairlie-Clarke et al., 2019). However, the climates of New Zealand and the United Kingdom are classified as humid warm temperate with cool to warm summers, allowing year-round grazing and relatively continuous L₃ exposure (World Maps of Köppen-Geiger Climate Classification, 2019). In contrast, the climate in Ontario, Canada is humid boreal with snowy sub-zero winters and warm summers; sheep are typically housed off pasture and not exposed to L₃ challenge for five or more months during the winter (Falzon et al., 2013; World Maps of Köppen-Geiger Climate Classification, 2019). Development and maintenance of salivary antibody to CarLA or other L₃ antigens has not been characterized in

sheep raised under these climate and management conditions. Thus, the utility of the CarLA Saliva Test® under Ontario grazing conditions is not known. The objectives of this study were therefore to (i) describe the salivary antibody response to CarLA in ewe lambs managed under Ontario grazing conditions, (ii) evaluate the association between salivary antibody to CarLA and GIN parasitism and (iii) investigate the relationship between the salivary antibody response to CarLA and overall health. The resultant data should yield insight into the potential utility of salivary CarLA-specific IgA antibody as a phenotype for genetic selection of sheep raised in Ontario and similar climates.

4.2 Materials and Methods

4.2.1 Farm and animal enrollment

The study ewe lambs were raised on a commercial sheep farm in central Ontario, Canada, that met previously reported selection criteria (Chapter 3, Section 3.2.1). Criteria included a history of GIN parasitism on the farm, management practices representative of Ontario flocks (Kennedy, 2012), and pasturing of the flock from at least early May until late November each year. The flock was of high health status, and known to be free from Maedi-Visna virus, scrapie, and *Coxiella burnetii* infection. A target sample size of 100 animals was chosen to allow identification of correlation coefficients of 0.30 or greater with type I error of 0.05 and type II error of 0.20 (University of California, San Francisco, 2018). Loss of animals due to mortality or management practices, such as culling open ewes following pregnancy diagnosis, was accounted for by enrolling 140 Rideau-Dorset cross ewe lambs at the start of the study in May 2016. All ewe lambs enrolled were born between March 28 and April 24, 2016, raised by their dam or a foster ewe, and born as a litter of 3-5 lambs; the farm retained lambs born in litters of three or

more as breeding stock. Reason for removal or loss to follow-up was recorded for all study ewe lambs that did not complete the study. Animal use approval was obtained from the University of Guelph Animal Care Committee (Animal Use Protocol Number 3380).

4.2.2 Animal management

The mean age of the study ewe lambs at enrollment on May 10 or 11, 2016 was 30 days (range 16-43 days), and mean age at weaning was 60 days (range 46-73 days). The study flock was kept on pasture from May 1 to December 1, 2016, and housed indoors from December 1, 2016 until January 15, 2017. The study ewe lambs were housed with rams from December 15, 2016 (mean age 245 days) until January 15, 2017 (mean age 276 days) for breeding, and turned out on a snow-covered dry lot from January 15 to March 15, 2017. Fetal numbers were determined using trans-abdominal real-time ultrasound on March 15, 2017 (mean age 342 days).

The study ewe lambs were fed and managed as one group from birth until completion of the study, apart from a 40-day interval in late gestation (March 15 to April 24, 2017). During this interval, triplet-bearing study ewe lambs were shorn, housed indoors, and fed a total mixed ration of 0.68 kg per head per day distiller's grain, 15 g per head per day mineral, and corn silage, with half the corn silage replaced by ensiled hay after April 1, 2017. The single- and twin-bearing study ewe lambs were not shorn and remained outdoors on the dry lot where they were fed mixed fruit waste, 1.81 kg per head per day ensiled alfalfa, and the same total mixed ration as triplet-bearing ewes until April 1, 2017, when the distiller's grain offered was increased to 1.36 kg per head per day. Single- and twin-bearing study ewe lambs were housed indoors with the triplet-bearing study ewe lambs from April 24, 2017 until lambing (see below). All study ewe

lambs were offered the same diet throughout the remainder of the study; detailed nutritional management of the flock during the study was described in Chapter 3, Section 3.2.2. Water was available *ad libitum* at all times.

The study ewe lambs lambed between May 3 and June 6, 2017, at 380-410 days of age. Lambing date, number of offspring including stillborn and mummified lambs, and sex of liveborn offspring were recorded. Study ewe lambs that gave birth to three or more live lambs had at least one offspring removed for cross-fostering or artificial rearing, so that no study ewe lamb reared more than two offspring. The study group returned to pasture on May 15, 2017, when the majority had lambed; any that had not lambed remained indoors until lambing, and then were turned out with the group within 48 hours of lambing. Offspring were weaned on August 9, 2017, at a mean age of 86 days (range 64-100 days). All study ewe lambs were shorn on the same day their offspring were weaned, and returned to pasture until the end of the study on November 10, 2017.

4.2.3 Sampling of animals

Samples were collected from the study ewe lambs at 11 different time points, corresponding with nursing (mean age 30 days), pre-breeding (mean ages of 90, 130, 190, and 220 days), mid-gestation (mean age 350 days), lambing (mean age 400 days), mid-lactation (mean age 450 days), late lactation (mean age 490 days) and the dry pre-breeding period (mean ages 550 and 570 days). At each sampling, each animal was weighed and assigned a body condition score according to the five-point scale reported by Russel et al. (1969). The difference in fleece weight between triplet-bearing study ewe lambs shorn on March 15, 2017, and single- or twin-bearing

study ewe lambs that were not shorn until August 9, 2017, was accounted for by reducing mid-gestation, lambing, and mid-lactation weights of single- and twin-bearing animals by 4 kg (average fleece weight based on farm records). All body condition scores were assigned by a single observer (J.A.) to ensure consistency in scoring. Fecal samples were collected directly from the rectum, and venous blood samples were obtained from the jugular vein for assessment of hematocrit, total protein, globulin and albumin levels, complete erythrocyte and leukocyte counts and differential leukocyte counts. Saliva samples were collected according to a protocol reported by Shaw et al. (2012). Briefly, a cotton dental swab (Richmond Dental & Medical Inc., USA) clamped in hemostatic forceps was introduced into the buccal space and moved back and forth for 10 seconds. The swab was then placed in a conical 5 mL screw-top tube (Fisher Inc., USA) and frozen at -80°C until measurement of CarLA-specific IgA.

4.2.4 Environmental monitoring

Weather data collected between May 2016 and November 2017 was obtained from a monitoring station 26 kilometers away from the farm. In order to determine numbers of L₃s on pasture, at each sampling time point up to 500 g of herbage was collected from pasture the study ewe lambs had been grazing. Pasture samples were collected according to the protocol reported by the Ministry of Agriculture, Fisheries, and Food (1984). Pasture samples were not collected in March 2017 as the herbage was still snow-covered and the study ewe lambs were housed on a dry lot at that time.

4.2.5 Laboratory methods

A modified McMaster method yielding a sensitivity of 8.33 eggs per gram of feces was used to determine gastrointestinal FEC (Zajac and Conboy, 2012). Animals exceeding 500 eggs per gram (epg) were treated with an anthelmintic at least 4 weeks prior to the next sampling, in order to prevent GIN-related morbidity while allowing reinfection and completion of at least one full prepatent period between treatment and sampling (Taylor et al., 2016). In 2016, animals requiring treatment were administered levamisole per os at 7.5 mg/kg (Huvepharma, Missouri, USA). In 2017, albendazole (Zoetis Canada Inc., Quebec, Canada) was administered per os at 5 mg/kg. Species of GIN ova shed in feces were determined at each time point by separately culturing up to 15 individual fecal samples and extracting first-stage (L₁s) larvae for deep amplicon sequencing (Redman et al., submitted). At least 100 L₁s preserved in ethanol were required for speciation; therefore, 200 epg was used as the minimum threshold for culture and collection of sufficient larvae from 6 g fecal samples. Larvae in pasture herbage samples were collected using the method described by the Ministry of Agriculture, Fisheries, and Food (1984). Morphologic speciation was performed using criteria described by van Wyk and Mayhew (2013).

Salivary CarLA-specific IgA was assessed in samples collected at the beginning (May), middle (August), and near the end (October) of each grazing season (2016 and 2017), and at mid-gestation in March 2017 when the study ewe lambs had been housed indoors and not exposed to L₃s for 3.5 months. This yielded results from a total of seven time points, corresponding with mean ages of 30, 130, 190, 350, 400, 490, and 550 days (see Figure 4.1). Saliva was extracted from the swabs and sCarLA level measured using an enzyme-linked immunosorbent assay (The

CarLA Saliva Test®, AgResearch Inc., New Zealand), according to the protocol reported in detail by Shaw et al. (2012). The minimum detection limit of the assay was 0.3 units/mL (Shaw et al., 2013).

4.2.6 Statistical analysis

Analysis of correlation between sCarLA levels over the seven time points was performed using RStudio Version 0.99.484 (RStudio Inc., USA). Spearman's rank correlation coefficient was calculated for each pairwise comparison between sCarLA levels measured at the seven time points. General linear mixed models for repeated measures were generated for fecal egg count, albumin and globulin levels, and hematocrit of the study ewe lambs using Proc Mixed (SAS version 9.4, SAS Institute Inc., Cary, North Carolina, USA). All fixed effects tested in the models are outlined in Table 4.1; all possible two- and three-way interactions and quadratics of the tested fixed effects were also evaluated. Nonsignificant effects ($p > 0.05$) were removed in stepwise fashion in order of decreasing p-value, then reintroduced to rule out multicollinearity and confirm lack of significance. Where nonsignificant effects were involved in a significant interaction term, model hierarchy was maintained by retaining the component effects. All models included animal identification number as a random effect. Fit of random effects, autoregressive, heterogenous autoregressive, Toeplitz, heterogenous Toeplitz, and unstructured error structures were evaluated using the Akaike information criterion (Dohoo et al., 2014), and the error structure with best fit was applied. Unequal variance, normality, and conformance to model assumptions were tested by plotting residuals against predicted and explanatory variables and applying the Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises and Anderson-Darling

tests of normality (Ghasemi and Zahediasl, 2012). Model fit was re-evaluated with outliers removed, and outliers examined for data errors or confounding.

4.3 Results

4.3.1 Climate and GIN epidemiology

Mean temperatures during the grazing season were similar in 2016 and 2017, gradually increasing from March to June and peaking June to August, though monthly maximum temperatures were approximately 5 °C higher between June and September in 2016 than in 2017. Mean temperature steadily declined from August to December, remaining relatively constant at -5 °C from December 2016 until March 2017. Total precipitation between May and November in 2017 was approximately double the amount during the same interval in 2016. The moderate to severe drought conditions in 2016 (Agriculture and Agri-Food Canada, 2018) corresponded with low numbers of L₃s in pasture herbage through most of the grazing season (less than 50 L₃s/kg dry matter from July to October 2016), with slightly higher levels in May and November. The majority of the precipitation in 2017 occurred between May and July, with a sharp decline from 210.0 mm in July to 40.7 mm in August. Numbers of L₃s had been low (less than 25 L₃s/kg dry matter) from May to July 2017, and rapidly increased to greater than 250 L₃s/kg dry matter in August 2017 before returning to low levels (less than 50 L₃s/kg dry matter) in October and November.

Changes in the average FEC of the study flock followed a similar pattern during both grazing seasons. Fecal egg counts rapidly increased from spring to mid-summer, peaked mid to late summer, and sharply declined to low levels in the fall, remaining low during the winter. Peak

FECs occurred slightly later in 2016 (August) than in 2017 (July). Gastrointestinal nematode eggs were identified in feces of only two study ewe lambs at the first sampling in May 2016. Neither lamb exceeded the minimum FEC threshold of 200 epg; thus, fecal culture was not performed and infecting species could not be determined at this time point. At all other time points at least seven animals met the minimum threshold for fecal culture. Infecting species proportions were averaged across all individually cultured fecal samples to estimate GIN species proportions within the overall flock. *Haemonchus contortus*, *Teladorsagia circumcincta*, and *Trichostrongylus* spp. accounted for the majority of larvae cultured from fecal samples at all time points during the study. Low numbers of *Nematodirus spathiger* and *Cooperia* spp. larvae were isolated from individual fecal cultures, never exceeding 5 % of larvae identified in an individual sample.

4.3.2 Losses to follow-up

During the study, 33 of the 140 study ewe lambs initially enrolled in the study were lost to follow-up. Of these, 13 were lost during the first grazing season (May to November 2016), and the remaining 20 were lost during the second year (March to November 2017). Mortality was the most common reason for loss to follow-up (n = 22). Cause of death was not determined in five animals; in the remaining 17, mortality was not related to nematode parasitism.

Of the five animals with an unknown cause of death, three died in the first grazing season (June to September 2016) and two died early in the second grazing season (May and June 2017). One study ewe lamb that died in 2016 died prior to peak GIN FECs in July; the other two were unusually small at enrollment and consistently gained less weight than the rest of the flock,

despite having low FECs. The two study ewe lambs that died in 2017 also both died prior to peak GIN infection levels that year. Thus, death due to GIN parasitism is considered unlikely in all five of these lambs.

Other reasons for removal from the study included culling (sold to slaughter) due to failure to conceive (n = 5) or vaginal prolapse (n = 2), escape into other management groups (n = 3), and illness of undetermined cause (n = 1). The three animals that escaped into other management groups could not be located until after termination of the study, and were therefore unavailable for sampling. The study ewe lamb removed due to illness had low FECs and did not respond to anthelmintic treatment; therefore, GIN parasitism was not likely the cause of disease.

4.3.3 Kinetics of salivary CarLA-specific IgA

Saliva samples collected from study ewe lambs that did not survive to the end of the first year (n = 13), and samples collected in 2017 from animals that did not complete the study (n = 20) were not analyzed. One study ewe lamb could not be located for saliva collection at 190 days of age (August 2016). Variation in mean sCarLA levels and FECs is summarized in Figure 4.2, and the proportion of the study group with sCarLA exceeding the minimum detection limit (0.3 units/mL) is shown in Table 4.2.

Higher peak sCarLA levels were observed during the second grazing season than in the first, but changes in mean sCarLA levels otherwise followed a similar pattern to seasonal variation in FEC with a 6- to 8-week delay (see Figure 4.2). No study ewe lambs had sCarLA exceeding the minimum detection limit (0.3 units/mL) when first sampled in May 2016 at 30 days of age (see

Table 4.2). The proportion of study ewe lambs with detectable sCarLA and flock mean sCarLA level increased slightly by 130 days of age (August 2016). During the first year of life, the highest proportion of the flock with detectable sCarLA (68.3 %) and the highest mean levels (1.2 units/mL) occurred at 190 days of age (October 2016). This proportion and the mean sCarLA level declined to 50.5 % and 0.7 units/mL, respectively, by mid-gestation at 350 days of age in March 2017, and declined further to 43.9 % and 0.5 units/mL by lambing at 400 days of age. After lambing, when the study ewe lambs were turned out on pasture for their second grazing season, nearly the entire flock (99.1 %) demonstrated detectable sCarLA by 490 days of age (August 2017), concurrent with the maximum mean sCarLA level (13.9 units/mL). The high prevalence of detectable sCarLA was maintained until the final measurement at 550 days of age in October 2017, but mean levels decreased slightly to 10.9 units/mL.

4.3.4 Correlation of salivary CarLA-specific IgA levels between repeated measurements in Ontario study ewe lambs

The salivary CarLA-specific IgA data were not normally distributed. Therefore, IgA levels were subjected to natural logarithmic transformation prior to analyses (Shaw et al., 2012). Samples below the detection limit of the assay (0.3 units/mL) were assumed to be normally distributed between 0 and 0.29 units/mL, and a correction factor of 0.15 units/mL (half the detection limit) was applied to these values prior to transformation (Schisterman et al., 2006).

Spearman's rank correlation coefficients between paired sCarLA levels are presented in Table 4.3. Correlation between the first sCarLA measurement at 30 days of age and subsequent samplings was not possible, as no study ewe lambs had detectable sCarLA at the first

measurement. All other pairwise correlations were positive and of weak to moderate strength (rho 0.17-0.59). Correlations were significant ($p = 2.1 \times 10^{-11}$ to 0.047) for all comparisons except between sCarLA measured during the first grazing season (130 and 190 days of age) and the final sCarLA measurement at 550 days of age (p values 0.081 and 0.060, respectively). The strongest correlations were between sCarLA measured at 350 days of age (mid-gestation) and subsequent sCarLA levels (rho = 0.40 to 0.52, all comparisons $p < 0.0001$), and between the final two sCarLA measurements at 490 and 550 days of age (rho = 0.59, $p = 2.1 \times 10^{-11}$) (see Figure 4.3).

4.3.5 Association between salivary CarLA-specific IgA and parasitism in study ewe lambs

In order to improve conformation to Gaussian distribution prior to modeling, a correction factor of half the minimum detection limit of the modified McMaster test (4.16 epg) was applied to fecal samples with a FEC of zero, and all FEC were subjected to natural logarithmic transformation (Schisterman et al., 2006). Salivary CarLA-specific IgA levels were also logarithmically transformed prior to modeling as described in Section 4.3.4. A general linear mixed model for repeated measures with a heterogeneous autoregressive error structure was used to model predicted FECs. Predictors retained in the final model are listed in Table 4.4. The initial sampling point at 30 days of age was excluded from analysis as all study ewe lambs had zero FECs at that time.

Higher sCarLA level was significantly ($p < 0.0001$) associated with reduced FECs at all time points assessed, independent of other significant effects including anthelmintic treatment. Predicted FECs based on sCarLA level in representative untreated study ewe lambs (raised as triplets, with flock average leukocyte count and serum globulins, and that gave birth to twin

offspring in 2017) are presented in Figures 4.4 and 4.5. At all time points, the sharpest decline in predicted FEC relative to increases in sCarLA level occurred at sCarLA levels below 1.0 units/mL, which is considered the minimum level indicative of protective GIN immunity in New Zealand (Shaw et al., 2013), and reached a plateau at higher sCarLA levels. Effect on predicted FEC was greatest per unit change in sCarLA during the first grazing season. The decline in predicted FEC with increased sCarLA level was greatest at 130 days of age (pre-breeding), coinciding with the highest flock mean FECs observed in 2016 (2152 epg, see Figure 4.2). Predicted change in FECs decreased until 350 days of age (mid-gestation), when the study ewe lambs had been on a dry lot and not exposed to L₃s for 3.5 months and mean flock FECs were low (320 epg). During the second grazing season (400, 490, and 590 days of age), predicted FEC did not decrease as rapidly with increased sCarLA level as in the first grazing season; however, mean FECs at all three sCarLA measurement timepoints in the second grazing season were low (see Figure 4.2). The lowest rate of change in predicted FEC with increases in sCarLA level were observed at 490 days of age (late lactation), corresponding with the timing of the lowest flock mean FECs observed during the study (44 epg).

Administration of an anthelmintic at the previous sampling (6-8 weeks prior) was significantly associated with predicted FEC ($p = 0.0021$), but was affected by an interaction with day of sampling ($p < 0.0001$). As a result, predicted FEC in untreated study ewe lambs was not always higher than that of treated animals (see Figure 4.6). Predicted FEC was significantly ($p < 0.001$) higher in untreated animals at 190 (pre-breeding) and 490 days of age (late lactation). At 400 days of age (lambing), the difference in predicted FEC between treated and untreated animals approached significance ($p = 0.051$), but FEC was predicted to be lower in untreated animals; p-

values at all other time points ranged between 0.333 and 0.964. Litter size ($p = 0.0027$), leukocyte count ($p = 0.0295$) and their interaction ($p = 0.0023$) were all significantly associated with FEC, with increased litter size and decreased leukocyte count generally associated with higher predicted FEC. However, litter size was not significantly associated with FEC in animals with leukocyte counts between the 25th ($6.7 \times 10^9/L$) and 75th percentile ($9.6 \times 10^9/L$). Like anthelmintic treatment, the number of siblings raised with the study ewe lamb was significantly associated with FEC ($p = 0.005$), but affected by an interaction with serum globulin level ($p = 0.002$). Increased number of siblings co-raised with the study ewe significantly ($p < 0.01$) increased predicted FEC above serum globulin levels of the 50th (36 g/L) percentile, but did not significantly affect predicted FEC below the 25th percentile (32 g/L).

4.3.6 Association of salivary CarLA-specific IgA and health indicators in study ewe lambs

General linear mixed models for repeated measures were generated to predict hematocrit, serum albumin, and serum globulin in the study ewe lambs. An unstructured error structure was fitted to the models of hematocrit and serum globulin, and a heterogeneous Toeplitz error structure applied to the model predicting serum albumin. Serum albumin levels within the study flock were unusually high at 90 days of age (post-weaning), and multiple outliers were identified with serum albumin greater than three standard deviations above the two-year flock average (33 g/L). The date of sampling at 90 days of age (July 12-13, 2016) corresponded with unusually high environmental temperatures for this region, with ambient temperature during sample collection ranging between 30-32°C. Measurements collected at 90 days of age from all study animals were excluded from analysis of albumin levels due to the high likelihood of bias due to heat stress and dehydration. A single outlier with serum globulin greater than 7 standard deviations above the

study flock mean (36 g/L) was identified at 190 days of age (pre-breeding). Upon review of the data, the animal also had a leukocyte count nearly double the two-year study flock average ($16.3 \times 10^9/L$, flock average $8.2 \times 10^9/L$), but a low FEC (17 epg). As a result, the elevations in serum globulin and leukocyte count in this animal were presumed to be due to an underlying disease process unrelated to GIN parasitism, and it was excluded from analysis of serum globulin at 190 days of age. Predictors retained in the final models of hematocrit, serum albumin, and serum globulin are presented in Tables 4.5, 4.6 and 4.7, respectively.

The study flock displayed limited variability in hematocrit, serum albumin and serum globulin throughout the study (see Figure 4.7), and flock means consistently remained within reference intervals for healthy sheep (Animal Health Laboratory, 2019). Salivary CarLA was not a significant predictor of hematocrit, serum albumin, or serum globulin ($p > 0.05$). A three-way interaction involving litter size at birth (range 3-5), litter size at lambing (range 0-3) and number of siblings co-raised with the study ewe lamb (range 1-3) was identified as a significant predictor of serum globulin ($p < 0.0001$, see Table 4.7). However, the study ewe lambs were distributed across only 16 of the 36 possible levels of this interaction; thus, comparison of serum globulin at all levels of the interaction's three component effects could not be performed to resolve the entanglement. Similar entanglement was identified between litter size at lambing (range 0-3), body condition score (range 1-5), and day (11 sampling points) in the model predicting hematocrit (see Table 4.5). This entanglement was also impossible to resolve due to insufficient data distribution across the 220 possible levels of this interaction.

Weight was significantly associated with all three health indicators ($p < 0.0001$), with heavier study ewe lambs predicted to have higher hematocrit and serum albumin but lower serum globulin. Increased FEC was significantly associated with reduced hematocrit ($p < 0.0001$), but was not significantly associated with serum globulin ($p > 0.05$). Serum albumin was predicted to increase with increasing FEC at 350 (mid-gestation) and 370 days of age (lambing), and decrease at the eight other sampling time points ($p = 0.002$). Total leukocyte count was significantly and positively associated with hematocrit ($p = 0.002$), negatively associated with serum albumin ($p < 0.0001$), and was not significantly associated with serum globulin ($p > 0.05$). Hematocrit was a significant predictor of both albumin ($p < 0.0001$) and globulin in an interaction with day ($p = 0.002$). However, while increased hematocrit was consistently associated with increased serum albumin, the association between hematocrit and serum globulin varied depending on day of sampling. Hematocrit and serum globulin were negatively associated at 30 (nursing), 130 (pre-breeding), 400 (lambing), 490 (late lactation), and 550 days of age (dry), and positively associated at 90 (pre-breeding), 190 (pre-breeding), 220 (pre-breeding), 350 (mid-gestation), 450 (mid-lactation), and 590 days of age (dry). However, the clinical relevance of these associations is likely minimal, given the lack of variation in the study flock (see Figure 4.7).

4.4 Discussion

The work described was carried out to evaluate salivary antibody response to carbohydrate larval antigen and its association with GIN parasitism in sheep grazing under boreal climate conditions in Ontario. The three predominant infecting GIN species and patterns of change in study flock mean FECs were consistent with the epidemiology of GINs in Ontario as previously reported by Mederos et al. (2010). The observation of peak FECs earlier in the second grazing season (during

lactation) than in the first grazing season (as naïve lambs) is also expected in sheep on farms in Ontario (Mederos et al., 2010). Climate patterns in 2016 and 2017 were also similar to those observed in 2006-2007 by Mederos et al. (2010), with comparably low total precipitation in 2007 and 2016. Collectively, these findings indicate that climate conditions and GIN challenge during the study were representative of those typical in Ontario flocks. Despite the low numbers of L₃s in pasture samples during the dry 2016 grazing season, the peak study flock mean FEC (2152 epg) in July 2016 and high prevalence of detectable sCarLA in October 2016, at 190 days of age, indicate that larval challenge in 2016 was sufficient to stimulate an immune response. Moreover, exposure to GINs during the study was confirmed in all of the study ewe lambs, as every animal that survived to the end of the study had a FEC exceeding the minimum detection limit of 8.33 epg at five or more of the 11 sampling time points. Bias due to preferential loss to follow-up of heavily parasitized animals is considered unlikely as the cause of loss to follow-up was confirmed to be unrelated to GIN parasitism in all but six of the 33 animals that did not complete the study.

Two components likely contributed to the 6-8 week delay observed between changes in mean FEC and changes in sCarLA levels. The first is the time required for ova shed in feces to develop to L₃s on pasture. Under ideal environmental conditions, development from ovum to L₃ may take as little as 5-14 days; however, cooler temperatures or low humidity may prolong this interval for several weeks (O'Connor et al., 2006). Humidity also affects migration of L₃s from fecal matter into pasture herbage, with low rainfall trapping *H. contortus* L₃s within fecal samples and delaying exposure to grazing sheep (Wang et al., 2014). Following ingestion of L₃s, the second component of the delay involves the time required to mount an immune response to CarLA and

generate mucosal IgA. In previously exposed sheep, there was a 9-day interval between GIN challenge and peak IgA levels within gastric lymph (Halliday et al., 2007). The involvement of invariant natural killer T cells in immunity to glycolipid antigens such as CarLA suggests that immunologic memory is possible (Avci et al., 2013). The 9-day delay between GIN exposure and peak IgA in previously exposed sheep would therefore be expected to reflect development of a secondary (anamnestic) immune response; consequently, this delay is likely longer in naïve animals mounting a primary antibody response to GINs. However, all but three study ewe lambs had detectable GIN ova in feces by the second sampling at 90 days of age, indicating exposure to L₃ challenge for at least the parasites' prepatent period (i.e. approximately 21 days). The second sCarLA measurement was performed 40 days later at 130 days of age, when the study ewe lambs had likely been exposed to L₃ challenge for at least 61 days. With L₃ exposure of this duration, sCarLA at 130 days would be more consistent with a secondary than primary antibody response. Thus, a delay of approximately 6 weeks between peak FEC and corresponding peak sCarLA levels appears biologically plausible, though repeated measurement of both FEC and sCarLA level at shorter intervals would be necessary to confirm this hypothesis.

The decline in both flock mean sCarLA level and the proportion of animals with detectable sCarLA in the winter is consistent with previously described waning of the mucosal IgA response in the absence of L₃ exposure (Harrison et al., 2008; Shaw et al., 2012). However, the ability of numerous study ewe lambs to maintain some degree of sCarLA for several months through winter, and the markedly higher sCarLA levels observed during the second grazing season, suggest that immunologic memory to this antigen was retained between years, even under conditions where L₃ challenge is interrupted for a prolonged period of time. Moreover, as

in New Zealand (Shaw et al., 2012; Shaw et al., 2013), levels of sCarLA over time were significantly correlated, and higher sCarLA levels were consistently associated with reduced FEC under Ontario grazing conditions. Measuring sCarLA level, therefore, appears to be a promising approach for selection of sheep with superior GIN immunity under Ontario grazing conditions, and possibly in other regions with a similar boreal climate. Although sCarLA measured at 350 days of age (mid-gestation) was most strongly correlated with subsequent sCarLA measurements, evaluation of sCarLA level would ideally be performed prior to selection of breeding stock. In the study flock, the end of the first grazing season (190 days of age) appeared to be the ideal time to perform selection based on sCarLA levels. Levels of sCarLA at this age were significantly correlated with sCarLA levels at all subsequent measurements except the final measurement at 550 days of age, by which time the entire study flock had likely been exposed to sufficient L₃ challenge to mount a strong sCarLA response. Moreover, the proportion of the flock with detectable sCarLA at 190 days of age (68.3 %) indicates that all animals had sufficient exposure to L₃s to develop a response, and that individuals with no detectable sCarLA likely had a weak or delayed immune response. Therefore, the end of a lamb's first grazing season appears to be a suitable time to perform selection for GIN immunity based on sCarLA level under Ontario climate conditions, though evaluation of sCarLA in other flocks is needed to confirm this timing. Moreover, factors influencing CarLA levels in replacement ewe lambs at the end of their first grazing season, such as lamb weight and body condition score, merit investigation to further validate use of CarLA as a selection parameter.

The lack of significant associations between sCarLA level and hematocrit, serum albumin and serum globulin suggest that selection for elevated sCarLA would not negatively impact the

general health of sheep. This is consistent with the current understanding of how mucosal anti-CarLA IgA confers protection to GINs. Gastrointestinal inflammation leads to increased loss of protein and blood into the gastrointestinal tract, and has been implicated as the underlying cause of increased dag scores in sheep selected for low FEC (Douch et al., 1996; Williams, 2011; Shaw et al., 2013). IgA within the gastrointestinal tract binds CarLA on the surface of L₃s and impairs establishment of infection, growth and egg production while causing less mucosal inflammation than other mechanisms of GIN immunity (Stear et al., 1999; Harrison et al., 2008; Shaw et al., 2012; Shaw et al., 2013). However, the study flock displayed limited variation in hematocrit, serum albumin and serum globulin, likely due to administration of anthelmintic treatment to animals with FEC exceeding 500 epg. As a result, although statistically significant associations were observed between weight, FEC, leukocyte count, hematocrit, serum albumin and serum globulin, the predicted changes in health indicators were too small to be biologically relevant. It is unclear whether these associations would remain significant across wider ranges of hematocrit, serum albumin and serum globulin. Thus, further investigation of the association between sCarLA levels and overall health of sheep is needed. Specifically, assessment of the relationship between sCarLA level, hematocrit, serum albumin and serum globulin in sheep with clinically apparent GIN parasitism is warranted.

4.5 Conclusions

The results of this study indicate that sheep grazing under boreal climate conditions in Ontario mount a detectable salivary antibody response to GINs, and that there is individual variation in this response. Changes in salivary CarLA-specific IgA follow a similar pattern to fluctuation in GIN FEC under Ontario grazing conditions, with an approximately 6-8 week delay. Levels of

sCarLA are weakly to moderately correlated over time, and, although levels and prevalence of detectable antibody decline during the winter, Ontario sheep are capable of maintaining detectable sCarLA without being exposed to infective larvae for more than five months. Elevated sCarLA level is significantly associated with reduced fecal egg counts throughout the first two grazing seasons in Ontario sheep. Although limited variation in hematocrit, serum albumin and serum globulin was observed in the study flock, sCarLA level was not significantly associated with any of these indicators and therefore appears unlikely to negatively impact health. Since most of the naïve lambs developed detectable sCarLA before the end of their first grazing season, assessment of sCarLA levels at the end of the first grazing season appears to be a promising tool for identification of sheep with superior GIN immunity in Ontario.

4.6 Acknowledgements

This research was supported by the Canadian Agricultural Adaptation Program, Ontario Sheep Farmers, and the Ontario Agri-Food Innovation Alliance. The authors wish to thank Ziwei Li, Rebecca Chant, Stéphanie Bourgon, Leah Lourenco, Samantha Dixon and Mike Alcorn for their laboratory and field assistance. Climate data were graciously provided by Dennis Inglis and Neil Moore, and salivary CarLA testing was coordinated by Richard Shaw of AgResearch Inc., New Zealand. Special thanks to the sheep producer who participated in this study.

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4.8 Tables

Table 4.1. Parameters evaluated as fixed effects in models of gastrointestinal nematode fecal egg count, serum albumin and globulin, and hematocrit in ewe lambs in a commercial flock in central Ontario, 2016-2017. Observed ranges are given in parentheses for categorical variables.

Variable	Definition	Model(s)
Ewe born as	Birth litter size (range 3-5)	FEC, SA, HCT, SG
Ewe raised as	Offspring raised to weaning by dam or foster dam of ewe lamb (range 1-3)	FEC, SA, HCT, SG
Litter size	Total number of offspring born to ewe lamb (range 0-3)	FEC, SA, HCT, SG
Ln CarLA	Natural logarithmic transformation of ewe lamb CarLA-specific salivary immunoglobulin A on day of sampling ¹	FEC, SA, HCT, SG
Lagged Ln CarLA	Natural logarithmic transformation of CarLA-specific salivary immunoglobulin A 6-8 weeks prior to sampling	FEC, SA, HCT, SG
Weight	Weight of ewe lamb on day of sampling	FEC, SA, HCT, SG
Log FEC	Natural logarithmic transformation of ewe lamb GIN fecal egg count on day of sampling	SA, HCT, SG
Lagged treatment	Whether ewe lamb was treated with anthelmintic 6-8 weeks prior to sampling (yes/no)	FEC, SA, HCT, SG
BCS	Ewe lamb's body condition score (2-4)	FEC, SA, HCT, SG
Day	Number of days since study began (May 10, 2016)	FEC, SA, SG
Total protein	Total serum protein of ewe lamb on day of sampling	FEC, HCT
SG	Serum globulin of ewe lamb on day of sampling	FEC, HCT
SA	Serum albumin of ewe lamb on day of sampling	FEC, HCT
WBC	Total leukocyte count of ewe lamb on day of sampling	FEC, SA, HCT, SG
HCT	Hematocrit of ewe lamb on day of sampling	SA, SG

FEC = fecal egg count; SA = serum albumin; HCT = hematocrit; SG = serum globulin; Ln =

natural logarithm; CarLA = carbohydrate larval antigen; GIN = gastrointestinal nematode; BCS = body condition score; WBC = white blood cell count.

¹ Day of sampling refers to the date on which FEC, HCT, SA and SG were determined.

Table 4.2. Proportion of Rideau-Dorset cross ewe lambs with salivary carbohydrate larval antigen-specific immunoglobulin A levels exceeding assay minimum detection limit (0.3 units/mL), 2016-2017, in a commercial flock in central Ontario.

Date (month/year)	Mean age (days)	Production stage	Study group size	Number with detectable anti- CarLA IgA	Percent with detectable anti- CarLA IgA
05/2016	30	Nursing	127 ¹	0	0.0
08/2016	130	Pre-breeding	127	13	10.2
10/2016	190	Pre-breeding	126 ²	86	68.3
03/2017	350	Mid-gestation	107 ³	54	50.5
05/2017	400	Lambing	107	47	43.9
08/2017	490	Late lactation	107	106	99.1
10/2017	550	Dry	107	106	99.1

CarLA = carbohydrate larval antigen; IgA = immunoglobulin A.

¹ 13 of the 140 study ewe lambs recruited initially did not survive to the end of 2016 and were not analyzed.

² One study ewe lamb could not be located for saliva sampling.

³ Saliva samples collected in 2017 from the 20 study ewe lambs that survived to the end of 2016 but did not complete the study were not analyzed.

Table 4.3. Spearman’s rank correlation coefficients (rho) for logarithmic transformation of salivary carbohydrate larval antigen-specific immunoglobulin A measured at six different time points in Rideau-Dorset cross ewe lambs (n = 140), 2016-2017, in a commercial flock in central Ontario. Cells with duplicated comparisons are left blank.

	sCarLA 130 days of age	sCarLA 190 days of age	sCarLA 350 days of age	sCarLA 400 days of age	sCarLA 490 days of age
sCarLA 190 days of age	rho = 0.25 p = 4.5 e ⁻³	NA			
sCarLA 350 days of age	rho = 0.35 p = 2.1 e ⁻⁴	rho = 0.30 p = 1.8 e ⁻³	NA		
sCarLA 400 days of age	rho = 0.20 p = 0.036	rho = 0.19 p = 0.047	rho = 0.52 p = 1.2 e ⁻⁸	NA	
sCarLA 490 days of age	rho = 0.28 p = 3.8 e ⁻³	rho = 0.28 p = 3.5 e ⁻³	rho = 0.40 p = 2.3 e ⁻⁵	rho = 0.33 p = 4.5 e ⁻⁴	NA
sCarLA 550 days of age	rho = 0.17 p = 0.081	rho = 0.18 p = 0.060	rho = 0.45 p = 1.2 e ⁻⁶	rho = 0.45 p = 1.1 e ⁻⁶	rho = 0.59 p = 2.1 e ⁻¹¹

sCarLA = salivary carbohydrate larval antigen-specific immunoglobulin A; NA = not applicable.

Table 4.4. Predictors in a general linear mixed model of gastrointestinal nematode (GIN) fecal egg count (FEC) measured over 6-8 week intervals in Rideau-Dorset cross ewe lambs (n = 140), 2016-2017, in a commercial flock in central Ontario. Direction of association is given for significant effects only.

Predictor variable	p-value	Direction of association	NDF	DDF	F-value	Proportion of variance explained (%)¹
Ln sCarLA	< 0.001	Negative	1	396	18.98	4.57
Litter size	0.003	Positive	3	112	5.01	11.83
Lagged treatment	0.002	Variable ²	1	108	9.91	8.40
Day	< 0.001	Variable ²	5	396	17.59	18.17
WBC	0.003	Negative	1	396	4.78	1.19
SG	0.099	NA	1	396	2.74	0.69
Ewe raised as	0.005	Positive ³	2	112	5.56	9.03
WBC*Litter size	0.002	Variable ²	3	396	4.92	3.59
Lagged treatment*day	< 0.001	Variable ²	5	396	17.41	18.02
SG*Ewe raised as	0.002	Variable ²	2	396	6.60	3.23

NDF = numerator degrees of freedom; DDF = denominator degrees of freedom; Ln = natural logarithm; sCarLA = salivary carbohydrate larval antigen-specific immunoglobulin A; WBC = white blood cell count; SG = serum globulins; NA = not applicable.

¹ Estimated using formulas described by Dohoo et al. (2014), page 330.

² Direction of association varies at different levels of a categorical variable.

³ Significant positive association only at serum globulin level \geq 50th percentile (36 g/L).

Table 4.5. Predictors in a general linear mixed model of hematocrit measured over 6-8 week intervals in Rideau-Dorset cross ewe lambs (n = 140), 2016-2017, in a commercial flock in central Ontario. Direction of association is given for significant effects only.

Predictor variable	p-value	Direction of association	NDF	DDF	F-value	Proportion of variance explained (%)¹
Litter size	0.990	NA	3	114	0.04	0.11
BCS	0.125	NA	2	114	2.12	3.59
Day	< 0.001	Variable ²	10	114	3.88	25.39
Weight	< 0.001	Positive	1	114	33.81	22.87
WBC	0.002	Positive	1	114	10.26	8.26
Ln FEC	< 0.001	Negative	1	114	17.05	13.01
Litter size*day	0.031	Entangled ³	30	114	1.66	30.40
BCS*day	0.008	Entangled ³	14	114	2.30	22.02
Litter size*BCS	0.484	Entangled ³	6	114	0.92	4.62
Litter size*BCS*day	0.372	Entangled ³	22	114	1.09	17.38
Weight*day	< 0.001	Positive	10	114	4.05	26.21

NDF = numerator degree of freedom; DDF = denominator degree of freedom; NA = not

applicable; BCS = body condition score; WBC = white blood cell count; Ln = natural logarithm;

FEC = fecal egg count.

¹ Estimated using formulas described by Dohoo et al. (2014), page 330.

² Direction of association varies at different levels of a categorical variable.

³ Insufficient distribution of data across all possible levels of the three-way interaction precluded resolution of entanglement; thus, direction of association could not be determined.

Table 4.6. Predictors in a general linear mixed model of serum albumin measured over 6-8 week intervals in Rideau-Dorset cross ewe lambs (n = 140), 2016-2017, in a commercial flock in central Ontario. Direction of association is given for significant effects only.

Predictor variable	p-value	Direction of association	NDF	DDF	F-value	Proportion of variance explained (%)¹
Litter size	0.002	Negative	3	114	5.28	12.20
BCS	0.005	Positive	2	130	5.54	7.85
Day	< 0.001	Variable ²	9	923	5.97	5.50
Weight	< 0.001	Positive	1	923	33.28	3.48
WBC	< 0.001	Negative	1	923	15.07	1.61
Ln FEC	0.109	NA	1	923	2.58	0.28
HCT	< 0.001	Positive	1	923	117.44	11.29
HCT*litter size	0.002	Positive	3	923	5.04	1.61
Weight*day	< 0.001	Positive	9	923	3.22	3.04
Weight*weight	< 0.001	Negative	1	923	21.41	2.27
Ln FEC*day	0.002	Variable ²	9	923	3.01	2.85

NDF = numerator degrees of freedom; DDF = denominator degrees of freedom; BCS = body

condition score; WBC = white blood cell count; Ln = natural logarithm; FEC = fecal egg count;

NA = not applicable; HCT = hematocrit.

¹ Estimated using formulas described by Dohoo et al. (2014), page 330.

² Direction of association varies at different levels of a categorical variable.

Table 4.7. Predictors in a general linear mixed model of serum globulin measured over 6-8 week intervals in Rideau-Dorset cross ewe lambs (n = 140), 2016-2017, in a commercial flock in central Ontario. Direction of association is given for significant effects only.

Predictor variable	p-value	Direction of association	NDF	DDF	F-value	Proportion of variance explained (%)¹
Day	< 0.001	Variable ²	10	102	5.03	33.03
Weight	< 0.001	Negative	1	102	14.96	12.79
HCT	0.996	NA	1	102	0	0.00
Litter size*ewe raised as*ewe born as	< 0.001	Entangled ³	15	102	4.14	37.84
HCT*day	0.002	Variable ²	10	102	2.99	22.67

NDF = numerator degrees of freedom; DDF = denominator degrees of freedom; HCT =

hematocrit.

¹ Estimated using formulas described by Dohoo et al. (2014), page 330.

² Direction of association varies at different levels of a categorical variable.

³ Insufficient distribution of data across all possible levels of the three-way interaction precluded resolution of entanglement; thus, direction of association could not be determined.

4.9 Figures

Figure 4.1. Experimental timeline. Mean age of the study ewe lambs in days is given in parentheses for each sampling time point. FEC = fecal egg count; HCT = hematocrit; SA = serum albumin; SG = serum globulin; sCarLA = salivary carbohydrate larval antigen-specific immunoglobulin A.

¹ March 15, 2017. Fetal numbers determined via trans-abdominal ultrasound, and triplet-bearing study ewe lambs shorn.

² August 9, 2017. Offspring weaned and all study ewe lambs shorn.

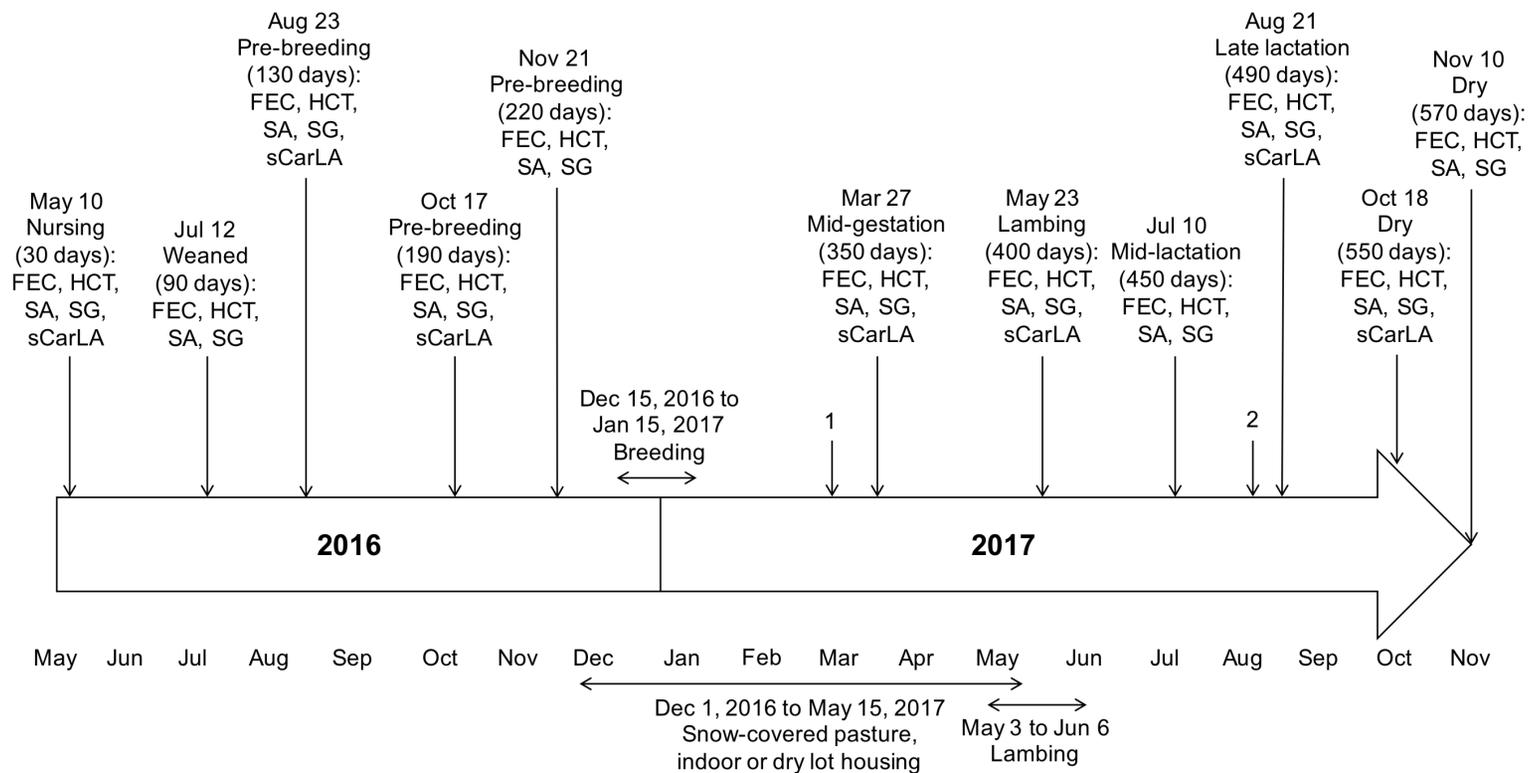


Figure 4.2. Mean gastrointestinal nematode fecal egg counts and mean salivary carbohydrate larval antigen (CarLA)-specific immunoglobulin A (IgA) levels of Rideau-Dorset cross ewe lambs (n = 140) in a commercial flock in central Ontario, 2016-2017. Error bars indicate 95 % confidence intervals for the means. Asterisks indicate sampling points at which salivary CarLA-specific IgA was not measured.

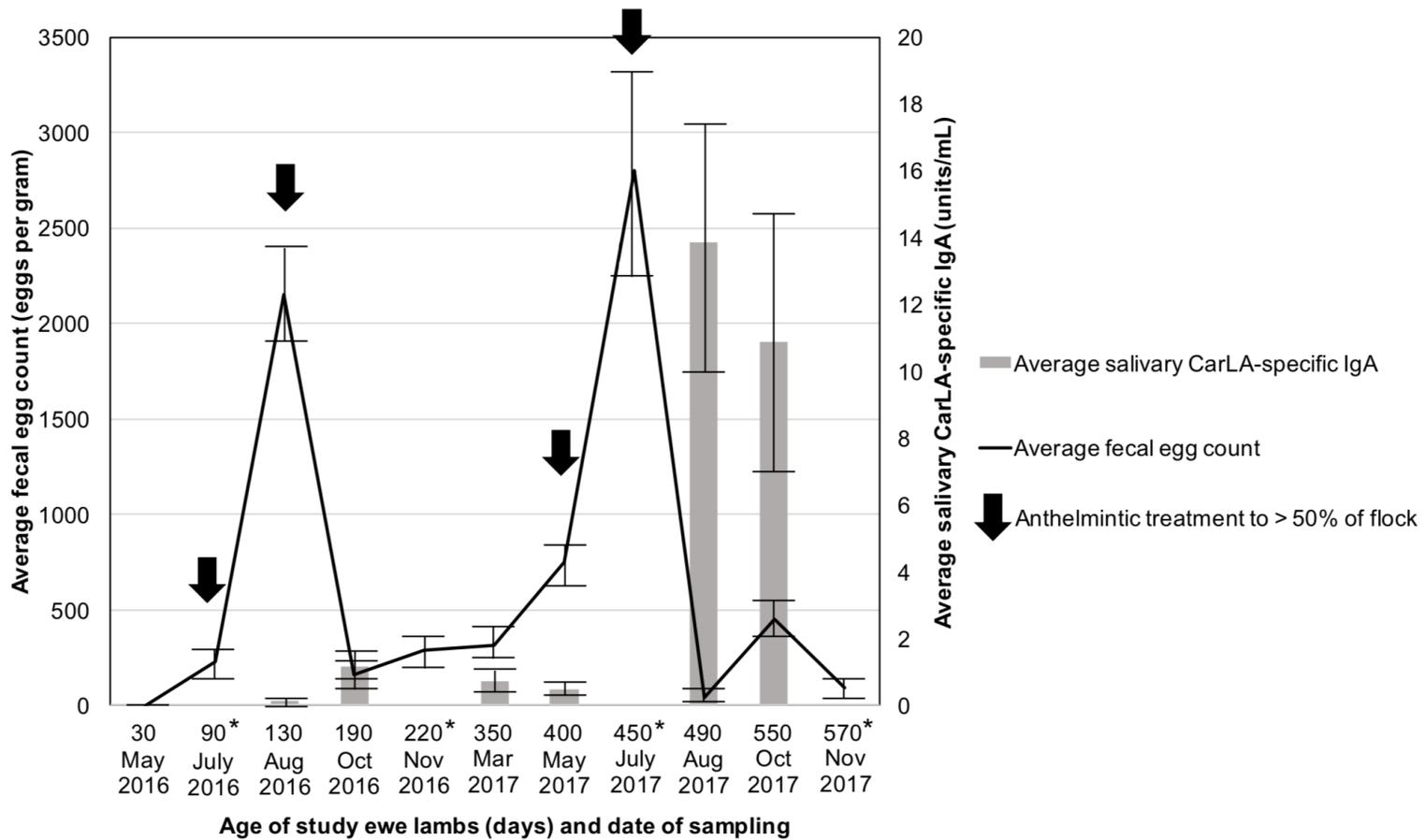


Figure 4.3. Comparison of natural logarithmically transformed salivary carbohydrate larval antigen-specific immunoglobulin A levels measured at different ages in Rideau-Dorset cross ewe lambs (n = 140), 2016-2017, in a commercial flock in central Ontario. Open circles represent individual animals; red lines represent line of best fit. Ln = natural logarithm; sCarLA = salivary carbohydrate larval antigen-specific immunoglobulin A.

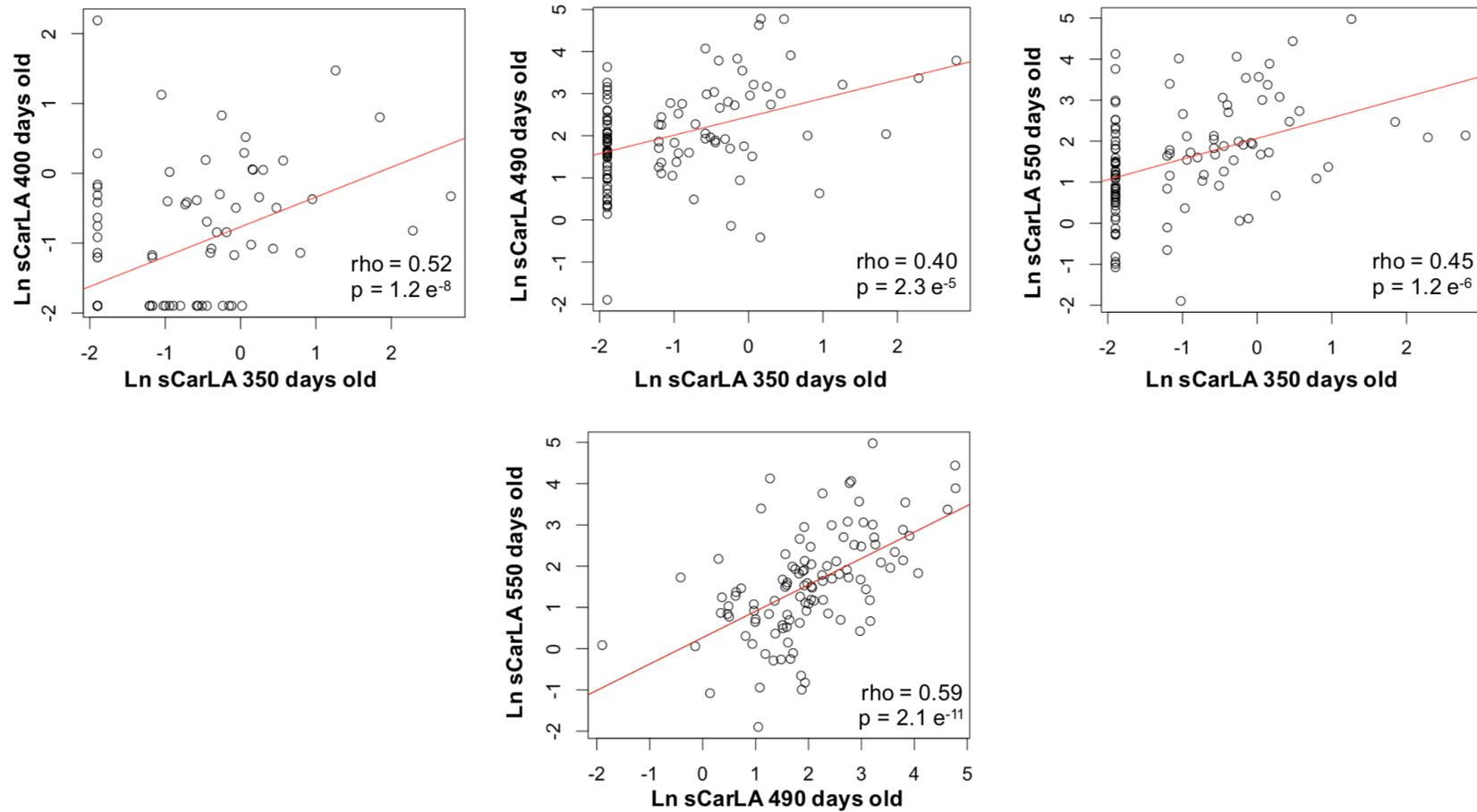


Figure 4.4. Predicted gastrointestinal nematode fecal egg count of Rideau-Dorset cross ewe lambs (n = 140) based on salivary carbohydrate larval antigen (CarLA)-specific immunoglobulin A (IgA) levels, August 2016-May 2017, in a commercial flock in central Ontario. Predictions are for study ewe lambs raised as triplets, with two-year flock average leukocyte count ($8.2 \times 10^9/L$) and serum globulins (36 g/L), that had not been treated with anthelmintics within the previous 6-8 weeks, and that gave birth to twin offspring at 400 days of age. Line length indicates the observed range of salivary CarLA-specific IgA at each age. The green line indicates salivary CarLA-specific IgA associated with strong GIN immunity (1.0 units/mL) per Shaw et al. (2013).

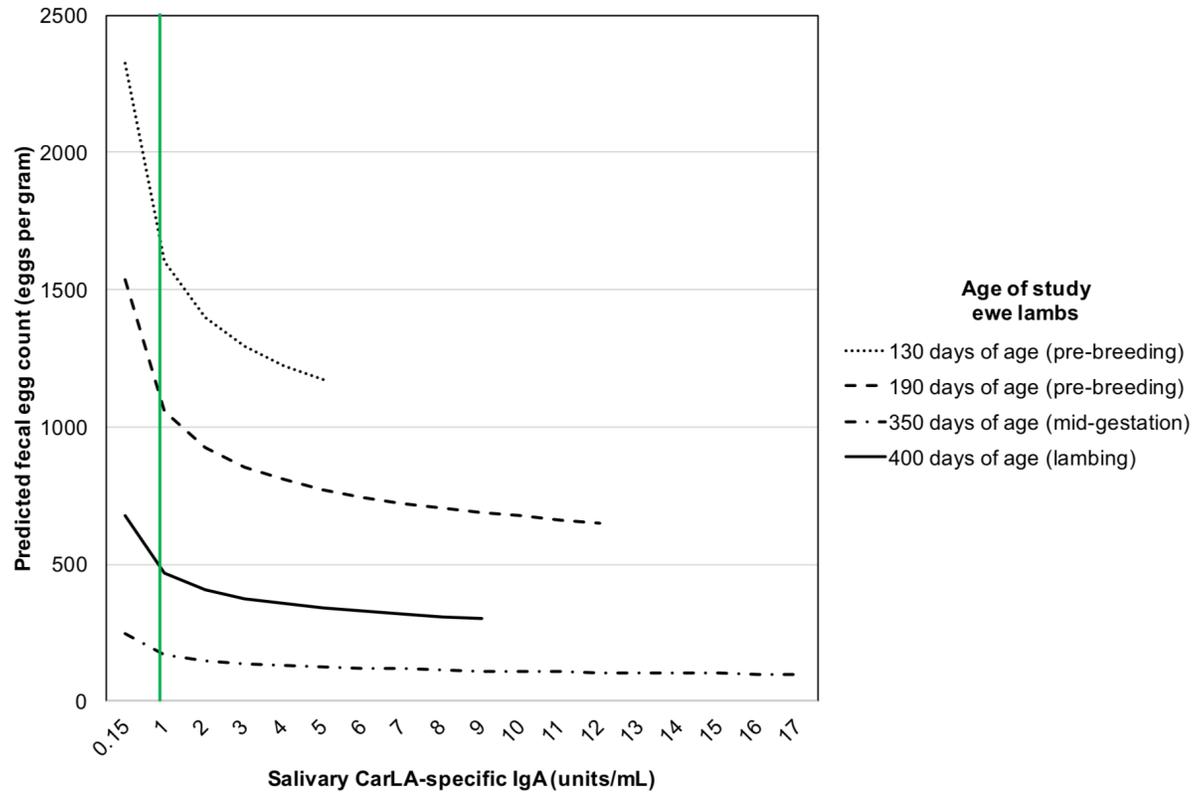


Figure 4.5. Predicted gastrointestinal nematode fecal egg count of Rideau-Dorset cross ewe lambs (n=140) based on salivary carbohydrate larval antigen (CarLA)-specific immunoglobulin A (IgA) levels, August-October 2017, in a commercial flock in central Ontario. Predictions are for study ewe lambs raised as triplets, with two-year flock average leukocyte count ($8.2 \times 10^9/L$) and serum globulins (36 g/L), that had not been treated with anthelmintics within the previous 6-8 weeks, and that gave birth to twin offspring at 400 days of age. Line length indicates the observed range of salivary CarLA-specific IgA at each age. The green line indicates salivary CarLA-specific IgA associated with strong GIN immunity (1.0 units/mL) per Shaw et al. (2013).

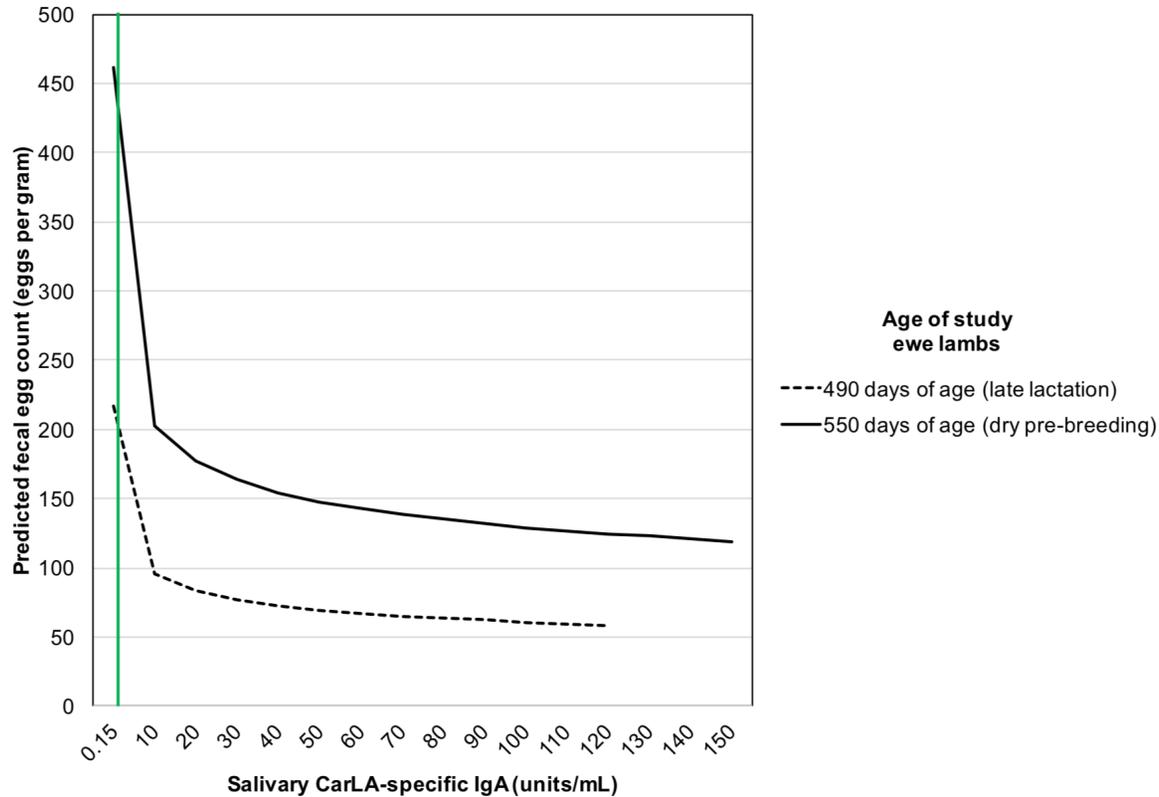


Figure 4.6. Predicted gastrointestinal nematode fecal egg count (FEC) of Rideau-Dorset cross ewe lambs (n = 140) based on previous anthelmintic treatment, August 2016-October 2017, in a commercial flock in central Ontario. Error bars indicate 95 % confidence intervals for predicted FEC, asterisks (*) indicate significant differences (p < 0.05). Predictions are for study ewe lambs raised as triplets, with two-year flock average leukocyte count (8.2 X 10⁹/L), serum globulins (36 g/L) and salivary carbohydrate larval antigen-specific immunoglobulin A (0.84 units/mL), and that gave birth to twin offspring at 400 days of age.

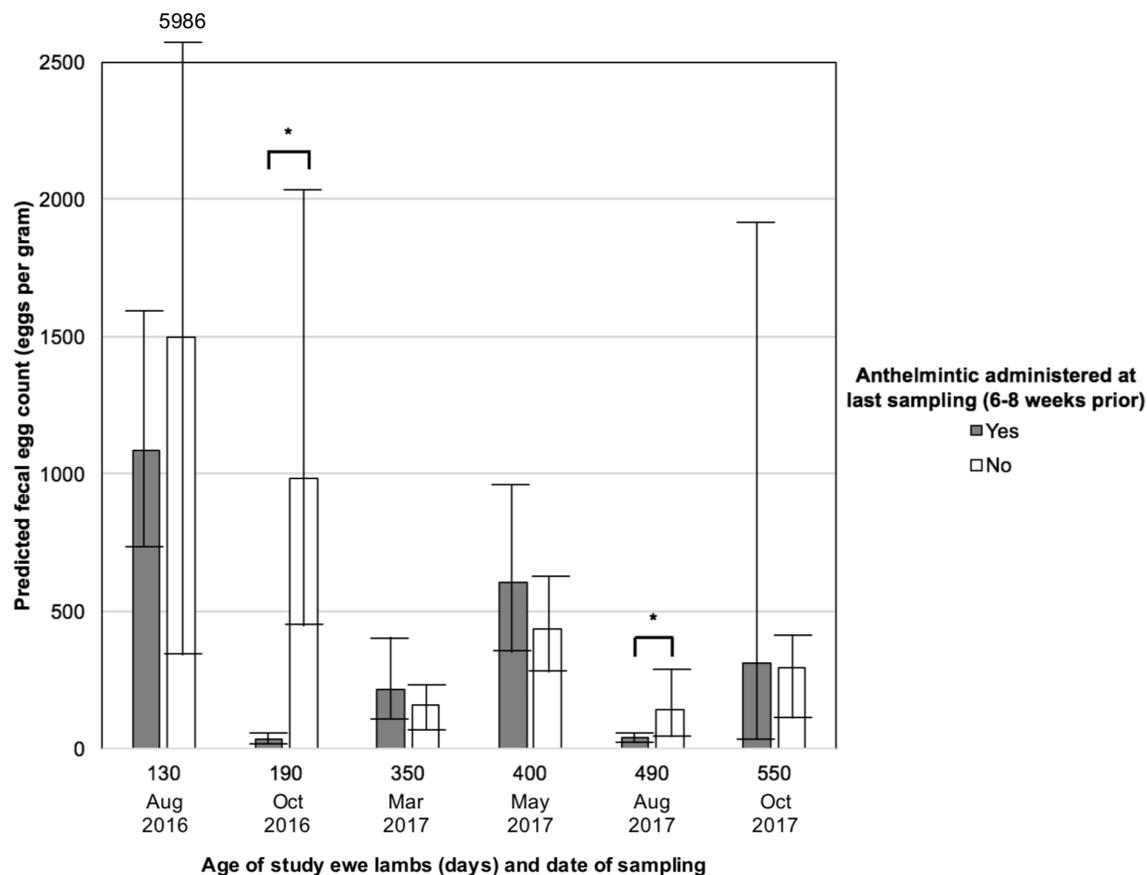
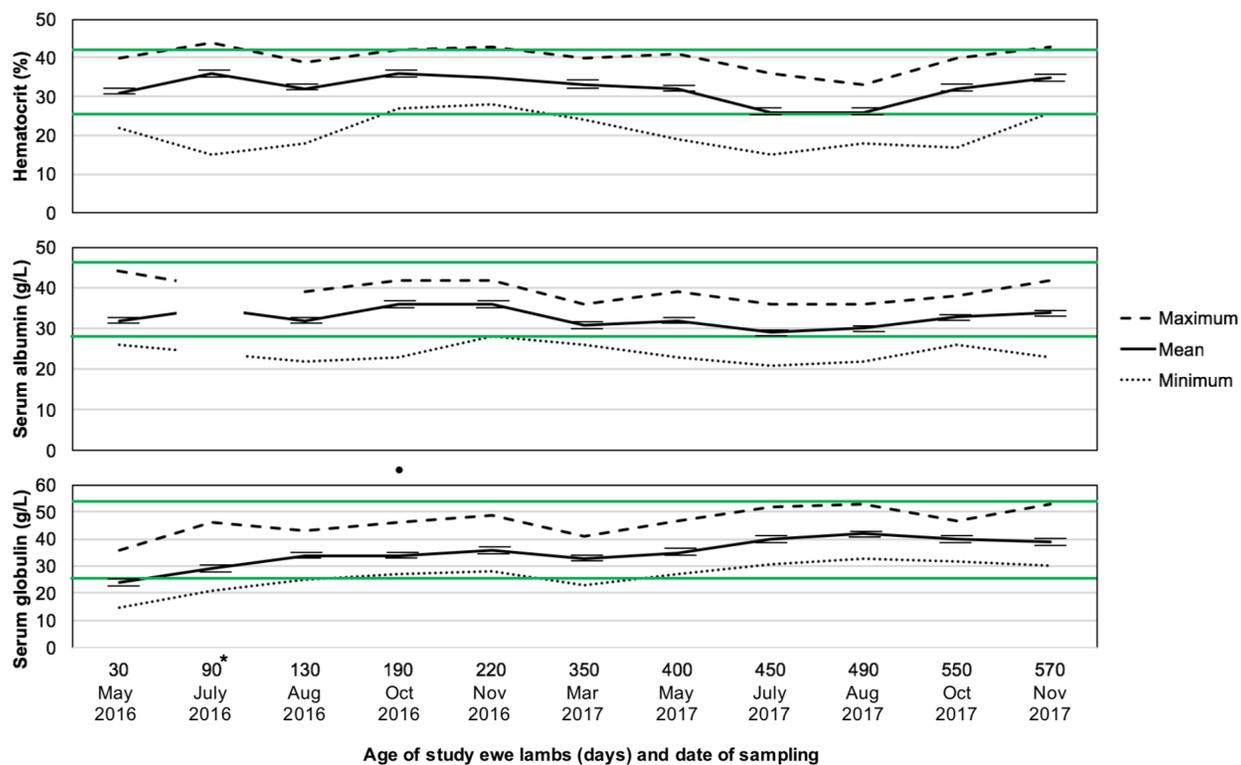


Figure 4.7. Mean, maximum, and minimum values of hematocrit, serum albumin and serum globulin of Rideau-Dorset cross ewe lambs (n = 140), 2016-2017, in a commercial flock in central Ontario. Error bars indicate 95 % confidence intervals for means, and green lines indicate reference intervals for healthy sheep (Animal Health Laboratory, 2019). An outlier excluded from analysis of serum globulin is indicated by the bullet point (•).

*Serum albumin levels at 90 days of age were not analyzed, as heat stress likely caused elevations in serum albumin unrelated to gastrointestinal nematode parasitism.



CHAPTER FIVE:

**THE EFFECT OF ACUTE STRESS RESPONSE ON
GASTROINTESTINAL NEMATODE PARASITISM IN SHEEP**

Based on a manuscript in preparation for submission to *Animal*.

Abstract

Glucocorticoid release in response to stress-induced activation of the hypothalamic-pituitary-adrenal axis is an important modulator of the immune response. Although prolonged glucocorticoid elevation in chronic stress is largely immunosuppressive, immunostimulatory effects of shorter-term glucocorticoid release in acute stress have been reported. In sheep, differential reactivity to acute stress is associated with variable cell- and antibody-mediated immune responses. The effect of variable reactivity to acute stress on mucosal immune responses in sheep has not been characterized and may have implications for genetic selection of sheep with immunity to gastrointestinal nematodes (GINs). This study compared indicators of GIN parasitism and associated clinical effects in sheep with high or medium reactivity to acute stress. Sheep with low stress reactivity have similar cell- and antibody-mediated immune responses to those with high stress reactivity, so were not assessed. Rideau-Dorset cross lambs naïve to GINs were challenged intravenously with bacterial lipopolysaccharide (LPS) to elicit an acute stress response. Peak serum cortisol levels were used to identify 15 lambs with high and 15 lambs with medium stress responses. These lambs were naturally exposed to GINs by

grazing on pasture with a commercial sheep flock. Five times during the grazing season, fecal egg counts were performed to assess GIN levels, blood was collected to determine hematocrit and serum albumin (clinical indicators of GIN parasitism), and pasture herbage was collected to assess exposure to infective GIN larvae. At the end of the grazing season, after spending 189 days on pasture, the study lambs were euthanized and GINs collected, counted, and speciated to determine parasite burden. General linear mixed models were generated to predict GIN fecal egg counts, hematocrit, serum albumin, and postmortem GIN burdens. No significant differences were identified between high and medium stress-responsive lambs (all p-values > 0.3). All but two study lambs developed a detectable GIN-specific salivary antibody response by 172 days on pasture, confirming that GIN exposure was sufficient to stimulate a mucosal immune response. However, fecal egg shedding was low throughout the study and clinical indicators of GIN parasitism remained within reference intervals for healthy sheep (Animal Health Laboratory, 2019), suggesting that GIN burdens were insufficient to cause a clinically relevant impact. Therefore, phenotypic variation in responsiveness to acute LPS stress does not appear to be associated with GIN parasitism in sheep with subclinical infections.

Keywords

Ovis aries, helminths, carbohydrate larval antigen, stress, cortisol

Implications

Gastrointestinal nematode (GIN) parasitism is an important economic and welfare disease of sheep. Chronic stress suppresses immunity to many disease agents, but acute stress can stimulate the immune system and its effect on immunity to GINs is poorly characterized. This study compared GIN infection in sheep with differing acute stress responsiveness. No significant differences in parasitism were identified between the stress response groups under field conditions; however, infection levels were low throughout the study. The results suggest that acute stress responses are not associated with subclinical GIN infection in sheep, but investigation in more heavily parasitized sheep is warranted.

5.1 Introduction

Gastrointestinal nematode parasites (GINs) are nearly ubiquitous on sheep farms, and commonly cause loss of productivity, morbidity, and mortality (Sutherland et al., 2019). Sheep are exposed to infective third stage larvae (L₃s) by grazing contaminated pastures, with a three-week prepatent period between ingestion of L₃s, and appearance of eggs in feces (Taylor et al., 2016). After these eggs hatch, the larvae moult twice to develop into L₃s, and then move away from the fecal material to facilitate ingestion (Taylor et al., 2016). Anthelmintics have traditionally been used as the primary means of GIN control, but widespread anthelmintic resistance is progressively limiting their utility (Kaplan and Vidyashankar, 2012). Genetic selection of sheep with a superior immune response to GINs is one management strategy under investigation as a complement to anthelmintic treatment (Shaw et al., 2012; Shaw et al., 2013; Sutherland et al., 2019). As in other mammals, the adaptive immune response to GINs in sheep is predominated by antibody-

mediated (type 2) immunity (McRae et al., 2015). In particular, sheep with increased salivary levels of secretory immunoglobulin A (IgA) specific to a carbohydrate larval antigen (CarLA) on L₃s have reduced GIN burdens and fecal egg shedding (Shaw et al., 2012; Shaw et al., 2013; Sutherland et al., 2019). However, exposed sheep display considerable variation in GIN immunity, some of which may be related to variability in reactivity of the hypothalamic-pituitary-adrenal axis (HPAA) to stress (You et al., 2008a). Glucocorticoids released following activation of the HPAA are an important modulator of immune responses in mammals, and their effects depend on the duration of the stressor and immune cell type (Dhabhar, 2009; Demas et al., 2011).

The immunosuppressive effects of glucocorticoid elevation in chronic stress are well-recognized in many species (Demas et al., 2011). However, the modulatory effects of acute stress on cell-mediated (type 1) and antibody-mediated (type 2) responses are more complex. Variability in responsiveness to acute stress can be reliably assessed in sheep by evaluating serum cortisol following immune challenge with lipopolysaccharide (LPS), which is used to simulate an acute bacterial infection (You et al., 2008a). This stress response phenotype is stable for at least 3 years and is associated with variation in type 1 and type 2 immune responses (You et al., 2008a; You et al., 2008b). Although glucocorticoids typically promote production of type 2 cytokines, acute stress also promotes cellular trafficking to the skin and lymph nodes, enhancing type 1 immune responses (Dhabhar 2009; Demas et al., 2011). Sheep with extremely high or low cortisol responses displayed increased antigen-specific cutaneous type 1 responses and reduced humoral primary type 2 responses when compared with medium cortisol responders, but

humoral secondary (anamnestic) type 2 responses were not different among the three different stress response phenotypes (You et al., 2008b). However, the effect of acute stress on mucosal type 2 responses is less clear, as both increased and decreased secretory IgA have been reported following acute stress in multiple species including sheep (Staley et al., 2018; Sutherland et al., 2019). Given that sheep mount short-term physiologic stress responses to routine management practices (Hemsworth et al., 2018), individual variation in HPA axis reactivity to different stressors may affect development and maintenance of the mucosal immune response to GINs, with secondary impacts on GIN burdens and clinical effects. The purpose of this study was to determine whether phenotypic variability in the acute stress response to LPS challenge is associated with variation in GIN burdens and clinical indicators of GIN parasitism in sheep. An improved understanding of the relationship between acute stress responsiveness and GIN parasitism may have practical implications for phenotypic assessment and genetic selection of sheep with optimal GIN immunity.

5.2 Materials and Methods

5.2.1 Lipopolysaccharide challenge

Ethics approval for all procedures involving animals was obtained from the University of Guelph Animal Care Committee (Animal Use Protocol Number 3380). Rideau-Dorset cross lambs were sourced from the University of Guelph Ponsonby Sheep Research Station, Ponsonby, Ontario, Canada. This flock is free of Maedi-Visna virus, *Coxiella burnetii*, and scrapie. Surveillance fecal egg counts on pooled fecal samples from the source flock have occasionally identified nonpathogenic *Nematodirus* spp., but no other

species of trichostrongylid GINs have ever been diagnosed in the source flock. Lambs born at the facility between April and November 2015 (n = 180) were challenged at 70 days of age with *Escherichia coli* 0111:B4 LPS (Sigma Chemical Co., St. Louis, MO, USA) according to a protocol reported by You et al. (2008a). Briefly, 0.4 µg/kg of *E. coli* LPS dissolved in 0.9 % sterile saline solution (Vétoquinol, Québec, Canada) was administered intravenously via the jugular vein to stimulate the HPA and induce cortisol release. Lambs were housed in individual pens with *ad libitum* access to hay and water throughout the challenge. Serum was obtained 4 hours post-challenge to measure peak serum total cortisol levels (You et al., 2008a). The cortisol concentration was determined within 24 hours of collection via chemiluminescent enzyme immunoassay using an Immulite 1000 Analyzer (Siemens Healthcare Diagnostics Products Ltd., Ontario, Canada), with an analytical sensitivity of 5.5 nmol/L.

5.2.2 Assessment of cortisol response

Individual peak cortisol concentrations are shown in Figure 5.1. Mean cortisol at 4 hours post-LPS challenge was 117.9 nmol/L (95 % confidence interval 104.2-131.7 nmol/L), with a standard deviation of 93.4 nmol/L. Lambs were excluded from consideration if 1) a medical condition was present that would interfere with the lamb's mobility or ability to graze (e.g. angular limb deformity, jaw malformation), or 2) the animal had been redirected to another study in the interval between LPS challenge and final selection. Comparison of lambs with high or medium stress responses was elected, as cell- (type 1) and antibody-mediated (type 2) immune responses were more similar between lambs with high and low stress response than between either high or low and medium stress

response in a previously reported study (You et al., 2008b). Fifteen lambs with peak cortisol concentrations greater than one standard deviation above the population mean (> 211.3 nmol/L) were selected as high stress response (HSR) sheep (cortisol range 242-494 nmol/L). The fifteen available lambs with the smallest absolute difference between the peak cortisol concentration and the population mean peak cortisol concentration (117.0 nmol/L) were selected as medium stress response (MSR) sheep (cortisol range 72-152 nmol/L). Preference was given to ewe lambs over rams when two individuals had similar peak cortisol concentrations, and only one lamb per full sibling litter was selected. The difference in age between the eldest and youngest lambs selected was 220 days in both the HSR and MSR groups, and in both ewe and ram lambs. Five of the six selected ram lambs were the HSR phenotype; all six were castrated at least 2 months prior to pasture turnout to prevent the permissive effect of testosterone on parasitism (Abuargob and Stear, 2014). Closed castration was performed via emasculator (Jorgensen Laboratories, Colorado, USA) following administration of 2 % lidocaine (Zoetis, Quebec, Canada) into the testicular cord. Testicular involution was assessed 3 weeks after castration to confirm efficacy. The lambs were also vaccinated for clostridial disease 8 weeks prior to pasture turnout.

5.2.3 Parasite challenge and monitoring

The selected lambs were turned out on pasture in central Ontario, Canada on April 28, 2016 (age range 160-380 days), with a group of commercial Rideau-Dorset cross ewes and their nursing lambs from a flock with a history of GIN parasitism. All of the study lambs had been housed exclusively indoors from birth until turnout. Fecal egg counts

(FECs) were performed on fecal samples obtained within 2 weeks of turnout, prior to completion of a 3-week prepatent period (Taylor et al., 2016), using a modified McMaster method with an analytical sensitivity of 8.33 eggs per gram (epg) (Zajac and Conboy, 2012). Low levels of nonpathogenic *Nematodirus* spp. were present in seven study lambs, consistent with exposure prior to turnout; however, no trichostrongylid-type eggs were identified. The lambs were naturally exposed to GINs by grazing with the commercial flock until November 3, 2016 (189 days). Water and mineral salt was provided *ad libitum* at all times.

The study lambs were weighed and sampled to evaluate direct and indirect indicators of parasitism at 12, 75, 117, 172, and 189 days on pasture. Each sampling included collection of whole blood via jugular venipuncture, feces per rectum, and saliva samples. Whole blood samples were refrigerated at 4 °C following collection, and fecal samples were stored at room temperature (20 °C) in airtight plastic bags. Both fecal and blood samples were processed within 48 hours of collection. Serum samples were also obtained and kept frozen at -80 °C until further processing. Saliva samples were obtained by swabbing the buccal space for 10 seconds with a cotton dental roll (Richmond Dental & Medical Inc., North Carolina, USA) clamped in hemostatic forceps, according to the method reported by Shaw et al. (2012). These samples were frozen at -80 °C until further processing.

5.2.4 Environmental monitoring

Climate data for May to November 2016 were obtained from the nearest weather station to the farm (26 kilometers distance). On each animal sampling day up to 500 g of herbage was collected from pasture the lambs had grazed for at least the previous 14 consecutive days to monitor numbers of L₃s, according to a previously described protocol (Ministry of Agriculture, Fisheries, and Food, 1984).

5.2.5 Laboratory methods

A modified McMaster method with an analytical sensitivity of 8.33 epg (Zajac and Conboy, 2012) was used to determine GIN FECs. In order to prevent morbidity or mortality due to GIN parasitism, study lambs with FECs > 500 epg at each sampling day were treated orally with 7.5 mg/kg levamisole (Huvepharma, Missouri, USA), the anthelmintic routinely used by the farm. Treatment was administered no less than 4 weeks prior to the next sampling to allow reinfection and completion of a prepatent period before the next evaluation of GIN parasitism (Taylor et al., 2016). As less than 4 weeks elapsed between the final two samplings, no treatment was administered after 172 days on pasture. Fecal samples from all lambs with GIN FECs > 200 epg were individually cultured to obtain first-stage GIN larvae (L₁s) for speciation using deep amplicon sequencing (Redman et al., submitted). A minimum threshold of 200 epg for culture was used to ensure at least 100 L₁s were recovered for ribosomal DNA sequencing from each 6 g sample cultured. Sequencing was performed using a method previously described by Redman et al. (submitted). Third-stage GIN larvae in pasture herbage samples were collected and counted using a method previously reported by the

Ministry of Agriculture, Fisheries, and Food (1984), and speciation performed using morphologic features described by van Wyk and Mayhew (2013).

Levels of CarLA-specific IgA were assessed in saliva samples obtained when the lambs had been on pasture for 12 (beginning of grazing season), 117 (middle of grazing season), and 172 days (end of grazing season). An enzyme-linked immunosorbent assay was performed according to the protocol described by Shaw et al. (2012), with a minimum detection limit of 0.3 units/mL (Shaw et al., 2013). Hematocrit, total erythrocyte count and total leukocyte count were determined on whole blood within 48 hours of collection using the Advia 2120 flow cytometry-based system (Siemens Healthcare Diagnostics Products Ltd., Ontario, Canada). Differential leukocyte counts were determined manually using blood smears. Total serum protein, albumin and globulin concentrations were measured via colorimetric assay using a Cobas 6000 C501 (Roche Diagnostics, Quebec, Canada), with a detection limit of 2.0 g/L.

5.2.6 Parasite collection and identification

All the lambs were slaughtered at the University of Guelph, Department of Animal Biosciences abattoir after 189 days on pasture. The lambs were transported from the farm to a holding facility less than 1 km from the abattoir and housed indoors as a group with *ad libitum* access to hay and water for 72 hours prior to slaughter to allow transport-associated stress to abate. Gastrointestinal nematodes were collected from the abomasum, small intestine, and cecum of each lamb using a modification of the protocol reported by the Ministry of Agriculture, Fisheries, and Food (1984). Briefly, each segment was

ligated and separated, and the contents collected in separate containers. The mucosal surface was washed vigorously in water, and all washings added to the contents to reach a total volume of 3.0 L. Mucosal washings were mixed evenly, then 1.0 L transferred to a flask and fixed via addition of 0.1 L of 100 % formalin. The washed mucosa was placed in 3.0 L of 0.9 % saline, incubated at 37 °C for 6 hours to release embedded immature stages, rinsed in the saline solution, and discarded. The resulting saline solution was mixed evenly and 1.0 L collected and preserved as described for mucosal washings. All GINs within 0.2 L (18.2 % of the 1.1 L formalin-fixed aliquot and 6.1 % of the 3.0 L total volume collected) of both the mucosal washings and saline bath were counted for each gastrointestinal segment; the first 100 adult and immature nematodes observed were speciated using criteria summarized in Tables 5.1 and 5.2. The total GIN burden was estimated by multiplying the number of GINs counted in 0.2 L by the minimum detection limit ($1 / 6.1 \% = 16.5$ worms in 3.0 L).

5.2.7 Statistical analysis

General linear mixed models were created for GIN FEC, hematocrit, serum albumin, and postmortem GIN burdens using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Normality of all outcome variables was evaluated using the Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises and Anderson-Darling tests (Ghasemi and Zahediasl, 2012). Natural logarithmic transformation was applied to GIN FEC, postmortem GIN burdens, and salivary CarLA antibody to normalize distribution of the data. Zero counts were adjusted to half the minimum detection limit of the respective

tests (4.16 epg for GIN FEC, 8.25 worms for postmortem GIN burden, and 0.15 units/mL for salivary CarLA antibody) prior to transformation (Schisterman et al., 2006).

Simple effects tested are listed and defined in Table 5.3. Variables with repeated measures (weight, salivary anti-CarLA antibody, total leukocyte count, total protein, serum globulin, serum albumin, FEC, total erythrocyte count and hematocrit) were assessed both as predictors of outcomes recorded at the same sampling point, and at the subsequent sampling point (lagged effects). All quadratic, two- and three-way interactions involving the listed effects and their lagged values were also tested. Stress phenotype and sex were nested within animal identification number and incorporated in the models as random effects. Backward elimination was used to eliminate effects that were neither significant ($p > 0.05$) nor involved in an interaction term; lack of significance or multicollinearity was confirmed by subsequent reintroduction of the removed effects. Simple effects involved in significant interaction terms were kept in the model regardless of significance to preserve model hierarchy. The Akaike information criterion was used to assess fit of random effects, autoregressive, heterogenous autoregressive, Toeplitz, heterogenous Toeplitz, and unstructured error structures (Dohoo et al., 2014). Residuals were plotted against predicted and explanatory variables to identify outliers and unequal variance, and to assess conformance to model assumptions. Outliers were examined for data error and retained in the model if data errors or confounding factors were not identified.

5.3 Results

5.3.1 Losses to follow-up, climate conditions and GIN challenge

One MSR lamb was euthanized immediately after sampling at 117 days on pasture due to progressive spinal ataxia, weakness, and impaired mobility over a course of 8 weeks. A postmortem examination was performed and no cause for the lamb's clinical signs was identified on gross examination; however, histopathology was not performed. Data collected prior to euthanasia were included in models of fecal egg count, hematocrit and albumin, but were excluded from analysis of postmortem GIN burdens due to the protracted interval (72 days) between euthanasia and slaughter of the remaining lambs.

Mean monthly temperatures during the 2016 grazing season gradually rose from 15 °C in May to a peak of 22 °C in August, and declined more sharply to 5 °C in November. The 2016 grazing season was characterized by moderate to severe drought (Agriculture and Agri-Food Canada, 2018), with a total of 319.5 mm of rainfall between May and November. Numbers of L₃s on pasture remained below 50 L₃s/kg dry matter through most of the grazing season, with two peaks; one at the start of the grazing season in May (12 days on pasture, 85 L₃s/kg dry matter), corresponding with the periparturient egg rise in lactating ewes co-grazed with the lambs (Mederos et al., 2010), and the other at the end of the grazing season in November (189 days on pasture, 148 L₃s/kg dry matter). Low pasture contamination through the summer likely reflected poor L₃ survival due to the low precipitation combined with warm weather. Increased pasture contamination in November was an unexpected finding and was possibly due to improved L₃ survival as

peak temperatures declined and daily minimum temperatures remained above 0 °C, though monthly precipitation remained low.

Mean FECs were not significantly different between the HSR and MSR lambs at any time point during the grazing season (see Figure 5.2). No GIN eggs were present in any fecal samples when the lambs had been on pasture for 12 days, and counts increased sharply between 75 and 117 days on pasture, peaking when the study lambs have been on pasture for 117 days. Mean FEC then rapidly decreased between 117 and 172 days on pasture, and remained low until the lambs were slaughtered after 189 days on pasture. Levamisole was administered ten days after sampling at 117 days on pasture to 14 study lambs with FEC > 500 epg (7 HSR and 7 MSR); none of the study lambs required anthelmintic treatment at any other sampling point. No significant differences in salivary CarLA antibody levels were identified between the HSR and MSR lambs. The three GIN species most commonly isolated from fecal cultures at all sampling points were *Haemonchus contortus*, *Teladorsagia circumcincta*, and *Trichostrongylus* spp., though only two lambs exceeded the minimum 200 epg cutoff for larval culture at 75, 172 and 189 days on pasture. Shedding of *Teladorsagia circumcincta* was highest when the study lambs had been on pasture for 75 days, then appeared to decrease during the grazing season, while shedding of *Haemonchus contortus* and *Trichostrongylus* spp. peaked at 117 days on pasture and declined thereafter. *Trichostrongylus* spp. contributed the largest proportion of eggs shed at all time points except 75 days on pasture. Low numbers (< 0.05 %) of *Cooperia* spp., *Chabertia ovina* and *Oesophagostomum venulosum* were

identified in three fecal samples obtained from three different animals at 117 (*Cooperia* spp. and *Oesophagostomum* sp.) and 189 days on pasture (*Chabertia* sp.).

5.3.2 Modeling variation in GIN fecal egg counts

Fecal egg counts were modeled using a general linear mixed model for repeated measures with an unstructured error structure. Fixed effects retained in the final model are summarized in Table 5.4. Stress phenotype was retained in the model as an effect of interest despite lack of significance ($p = 0.938$). Fecal egg counts measured at the first sampling were excluded from analysis as no study animals were detected shedding GIN eggs after 12 days on pasture. Sex and age of the study lambs were not significantly associated with FEC as independent effects ($p = 0.250$ and 0.429 , respectively), but were significant in an interaction term ($p = 0.012$). As discussed previously, the study lambs were predominately female ($n = 24$, wethers $n = 6$), and the eldest and youngest study lambs of both sexes were born 220 days apart. Although all of the study lambs were naïve to GINs when first exposed, increasing age was associated with higher FEC in wethers and lower FEC in ewes. Regardless of sampling date, FEC was predicted to be 234 epg lower in the eldest ewe lamb than in the youngest, compared with 1363 epg higher FECs in the eldest wether (see Figure 5.3). However, given the low number of wethers in the study group ($n = 6$), it is unclear whether this interaction term would remain significant if equal numbers of both sexes had been assessed. Day of sampling was also significantly associated with predicted FEC ($p < 0.001$), likely reflecting seasonal variation in FECs (see Figure 5.2). Despite allowing at least one prepatent period between treatment and fecal sampling, treatment with levamisole was significantly

associated with lower FECs at the subsequent measurement than were observed in untreated lambs ($p < 0.001$). This likely reflects continued accumulation of GINs in untreated animals.

5.3.3 Modeling variation in clinical indicators of parasitism

Hematocrit and serum albumin were modeled using general linear mixed models with a random effects error structure. Components of the final models are presented in Table 5.5. As in the model of FEC, stress phenotype was not associated with indirect indicators of GIN parasitism ($p = 0.396$ for hematocrit, $p = 0.784$ for serum albumin), but was retained as a parameter of interest. Mean hematocrit and serum albumin levels remained within the reference interval for healthy sheep throughout the study (Animal Health Laboratory, 2019; see Figure 5.4), with minimal variation between individual lambs regardless of HSR or MSR phenotype. Therefore, although several simple effects and interaction terms were significantly associated with hematocrit and serum albumin levels (see Table 5.5), none of these associations appeared to be biologically relevant.

5.3.4 Modeling variation in postmortem GIN burdens

Mean postmortem total and species-specific burdens of adult and immature GINs are presented in Table 5.6. No nematodes expected to produce strongyle-type eggs were identified in samples obtained from the cecum; low numbers of *Trichuris ovis* adults were observed in the cecum of one HSR lamb. Consequently, analysis of cecal nematode burdens was not performed. Greater total nematode burdens were present in the small intestine than in the abomasum, although larval GINs comprised a larger proportion of

total nematode burden in the abomasum. Within the abomasum, *Teladorsagia* spp. accounted for the majority of both adult and larval nematodes; lower numbers of adult *Trichostrongylus axei* and adults and larvae of *Haemonchus contortus* were also present. Within the small intestine, the majority of adult nematodes were *Trichostrongylus* spp., with *Capillaria* and *Nematodirus* spp. identified in much lower numbers. Conversely, although some *Trichostrongylus* spp. larvae were identified, *Nematodirus* spp. accounted for most of the small intestinal larval stages collected. Although low levels of *Cooperia* spp., *Chabertia ovina*, and *Oesophagostomum venulosum* were sporadically observed in fecal cultures during the grazing season, no larval or adult worms of these species were recovered postmortem.

Nematode burdens in the abomasum and small intestine were modeled using a general linear model with a random effects error structure. Effects retained within the final model are presented in Table 5.7. Stress phenotype was not significantly associated with gastrointestinal nematode burdens ($p = 0.796$). Increased FEC was a significant predictor of increased GIN burdens as both a lagged and unlagged term when these terms were introduced to the model separately ($p < 0.001$). However, unlagged FEC was not a significant predictor of GIN burden when both terms were introduced together ($p > 0.05$). As a result, lagged FEC was retained in the final model. Lagged total leukocyte count was not a significant predictor of GIN burden as a simple effect, but was involved in a highly significant interaction with worm maturity ($p < 0.001$) with opposite effects on adult versus larval GIN burdens. Increasing total leukocyte count was associated with increased burdens of larval GINs, but decreased burdens of adults (see Figure 5.5).

Differential leukocyte counts confirmed that eosinophils and lymphocytes were the leukocyte types involved in these associations. Similarly, lagged serum albumin was not significant as an independent effect ($p = 0.465$), but through its interaction with GI segment, lower serum albumin was significantly associated with higher abomasal and lower small intestinal GIN burden ($p < 0.001$). However, since all of the study lambs had serum albumin levels within the reference interval (29-47 g/L; Animal Health Laboratory, 2019) when assessed 17 days prior to slaughter, these associations were not likely clinically significant.

5.4 Discussion

This longitudinal study compared GIN parasitism over a single grazing season in central Ontario between sheep with high or medium stress response to challenge with *E. coli* LPS. Throughout the experimental period the predominant infecting GIN species and changes in FEC over the grazing season were consistent with patterns previously described in naïve lambs grazing on Ontario pastures (Mederos et al., 2010). In the work described here, mean FECs remained low following the sole anthelmintic treatment at 117 days on pasture. However, all but two of the study lambs (one HSR and one MSR) had detectable (> 0.3 units/mL) salivary IgA specific to CarLA by 172 days on pasture, indicating that the GIN challenge was sufficient to stimulate a mucosal immune response. This immune response may have been influenced by administration of levamisole, the anthelmintic routinely used on the farm, at 117 days on pasture. As indicated, 14 of the study lambs (7 HSR and 7 MSR) had FECs exceeding 500 epg at 117 days on pasture, and were treated with levamisole orally. Although this drug's immunostimulatory

properties are incompletely characterized, levamisole is capable of promoting both CMIR and AbMIR, unlike other anthelmintics used in ruminants (Stelletta et al., 2004; Plumb, 2011). For example, combining vaccination with subcutaneous administration of levamisole in sheep increased the humoral antibody titre to bluetongue virus relative to animals administered the vaccine alone (Stelletta et al., 2004). It is not clear whether use of oral levamisole for treatment of GINs, as described here, might have had a similar effect on the mucosal antibody response to GINs. However, as equal numbers of HSR and MSR sheep were treated and no significant phenotype-by-treatment interactions were identified in the models, any immunostimulatory effect appears to have been relatively equal between the two phenotype groups. Administration of a controlled GIN challenge infection under laboratory conditions could have allowed the interaction between acute stress response phenotype and GIN immunity to be assessed under more stable GIN challenge without the need for anthelmintic treatment. However, immune responses under such conditions may not accurately reflect those in sheep exposed to GINs by grazing on pasture.

Although reactivity of the HPAA to acute stressful stimuli is known to affect type 1 and type 2 immune responses in sheep (You et al., 2008a; You et al., 2008b), this stress response phenotype was not significantly associated with GIN FEC, hematocrit, serum albumin or postmortem GIN burdens. Peak cortisol levels following LPS challenge were considerably lower in the source population than levels reported for the same population by You et al. (2008b), indicating a decline in stress responsiveness in the Ponsonby flock over the past decade. Nevertheless, the HSR and MSR groups in the present study would

still have been classified as different stress response phenotypes by You et al (2008b), and as such would be expected to have differing immune responses. Instead, the lack of association between acute stress response phenotype and GIN parasitism may reflect the strong type 2 bias of the immune response to GINs (McRae et al., 2015). As discussed previously, HSR sheep displayed heightened type 1 and reduced primary type 2 immune responses compared with MSR sheep following antigen challenge (You et al., 2008b). However, secondary type 2 responses in previously sensitized sheep were not different between the two stress response phenotypes (You et al., 2008b). The mucosal immune response to GINs is known to develop more slowly than circulating antibody responses, but secondary type 2 immunity was shown to be detectable as CarLA-specific IgA in saliva after 8 weeks of GIN exposure under New Zealand grazing conditions (Shaw et al., 2013). Thus, a secondary rather than primary type 2 response would have been expected to contribute to variation in GIN parasitism in the study lambs from 75 days on pasture until the end of the study. The lack of significant associations between acute stress response phenotype and indicators of GIN parasitism may therefore be due to similar secondary AbMIR between the two phenotypes, and supports the comparatively minor role played by cell-mediated type 1 immune responses in GIN parasitism (McRae et al., 2015). The lack of significant differences in salivary CarLA antibody between the two phenotypes also suggests that secondary type 2 responses are similar in HSR and MSR sheep.

Although postmortem GIN burdens did not differ between the two stress response phenotypes, a significant association was identified between total leukocyte count and

postmortem GIN burdens. However, the direction of this association differed for adult versus larval GINs; numbers of adult GINs were predicted to decrease and numbers of larval GINs increase with increasing total leukocyte count. Leukocyte activation during the immune response to GINs typically leads to reduced adult and larval GIN burdens (McRae et al., 2015), so the positive association between total leukocyte count and larval GIN burden was unexpected. However, numbers of mucosal leukocytes are more strongly associated with reduced GIN burdens than numbers of circulating leukocytes (McRae et al., 2015). Moreover, although other disease conditions capable of causing an increase in circulating leukocytes were not detected in the study lambs, it is possible that circulating total leukocyte counts were due to inflammatory stimuli unrelated to GINs. Histopathologic examination of mucosal samples and comparison with samples from uninfected control animals might have clarified the relationship between circulating leukocytes, GIN burdens and the mucosal immune response.

In conclusion, no significant differences in GIN fecal egg counts, hematocrit, serum albumin levels or postmortem GIN burdens were identified between sheep with high and medium responsiveness to acute stress challenge following GIN exposure on pasture. However, both exposure to infective GIN larvae and resulting FECs were low throughout the study, and none of the study lambs displayed clinical signs of GIN parasitism. These results suggest that acute stress response phenotype is not associated with variability in GIN parasitism in sheep with subclinical GIN infections. However, the association between stress responsiveness and GIN parasitism merits further study under more intense parasite challenge.

5.5 Acknowledgements

This work was supported by the Canadian Agricultural Adaptation Program, the Ontario Agri-Food Innovation Alliance, Ontario Sheep Farmers, and a University of Guelph Undergraduate Research Assistantship. The authors wish to thank Stéphanie Bourgon, Rebecca Chant, Pam Hasson, Rebecca Fisher, Kevin Barbosa, Samantha Dixon, Mike Alcorn, Amanda Mansz, and members of Dr. Cánovas' lab (Department of Animal Biosciences, University of Guelph) for providing laboratory and field assistance, and Neil Moore and Dennis Inglis for providing climate data. Measurement of salivary anti-CarLA IgA was coordinated by Richard Shaw of AgResearch Inc., New Zealand. Special thanks to the sheep producer who participated in the study.

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5.7 Tables

Table 5.1. Features used in morphologic speciation of adult nematodes collected from the gastrointestinal tract of sheep.

Species	Size	Cuticular stria	Spicules ¹	Eggs ²	Other features
<i>Haemonchus contortus</i>	> 1 cm	Indistinct	Long Narrow Symmetric	Oval Morulated 65-80 um	Buccal lancet Cervical papillae Asymmetric dorsal ray ¹ Vulvar flap ²
<i>Teladorsagia circumcincta</i>	< 1 cm	Longitudinal	Long Narrow Symmetric	Oval Morulated 65-80 um	Cervical papillae Caudal annulus ² Vulvar flap ²
<i>Ostertagia trifurcata</i>	< 1 cm	Longitudinal	Short, Branching Symmetric	Oval Morulated 65-80 um	Cervical papillae Caudal annulus ² Vulvar flap ²
<i>Trichostrongylus colubriformis</i>	< 1 cm	Transverse	Short Barbed Symmetric	Oval Morulated 65-80 um	Prominent excretory notch
<i>Trichostrongylus vitrinus</i>	< 1 cm	Transverse	Short Tapered Symmetric	Oval Morulated 65-80 um	Prominent excretory notch
<i>Trichostrongylus axei</i>	< 1 cm	Transverse	Short Branching Asymmetric	Oval Morulated 65-80 um	Prominent excretory notch
<i>Capillaria</i> spp.	> 1 cm	Indistinct	Fused Protruding Blunt	Oval Operculated 50-70 um	Spines line spicule ¹
<i>Nematodirus</i> spp.	> 1 cm	Indistinct	Fused Protruding Tapered	Oval Morulated > 150 um	Cephalic vesicle Narrow neck Tail spine ²

Listed features are limited to species recovered from the study animals. Derived from

Zajac and Conboy (2012) and Taylor et al. (2016).

¹ Features that can only be used to speciate male gastrointestinal nematodes.

² Features that can only be used to speciate female gastrointestinal nematodes.

Table 5.2. Features used in morphologic speciation of immature parasitic nematodes collected from the gastrointestinal tract of sheep.

Species	Buccal capsule	Cephalic vesicle	Excretory pore	Other features
<i>Haemonchus contortus</i>	Deep Refractile walls	Absent	Absent	None
<i>Teladorsagia circumcincta</i>	Shallow	Absent	Absent	Anterior refractile bodies
<i>Trichostrongylus</i> spp.	Inconspicuous	Absent	Present	None
<i>Nematodirus</i> spp.	Inconspicuous	Present	Absent	Tail spine

Listed features are limited to species recovered from the study animals. Derived from Thomas and Probert (1993) and Taylor et al. (2016).

Table 5.3. Predictor variables evaluated in models of gastrointestinal nematode fecal egg count, hematocrit, albumin, and postmortem gastrointestinal nematode burden in sheep.

Variable	Definition	Model(s)
Stress	Stress response phenotype (high or medium)	All
Cortisol	Peak cortisol post-lipopolysaccharide challenge	All
Age	Age of study lamb	All
Sex	Study lamb sex (female or castrated male)	All
Weight ¹	Weight of study lamb	All
Ln CarLA ¹	Natural logarithmic transformation of salivary CarLA antibody	All
WBC ¹	Total leukocyte count	All
Day	Time spent on pasture	FEC, HCT, SA
Treatment	Whether lamb was treated with anthelmintic 4-8 weeks prior (yes/no)	FEC, HCT, SA
Protein ¹	Total protein	FEC, HCT, GINs
SG ¹	Serum globulins	FEC, HCT, GINs
SA ¹	Serum albumin	FEC, HCT, GINs
Ln FEC ¹	Natural logarithmic transformation of GIN fecal egg count	HCT, SA, GINs
RBC ¹	Total erythrocyte count	HCT, SA
HCT ¹	Hematocrit	SA, GINs
GI segment	Source of GIN (abomasum or small intestine)	GINs
Stage	Developmental stage of GIN (adult or immature)	GINs

Ln = natural logarithm; CarLA = carbohydrate larval antigen; WBC = white blood cell;

FEC = fecal egg count; HCT = hematocrit; SG = serum globulin; SA = serum albumin;

GINs = gastrointestinal nematode burden; RBC = red blood cell; GI = gastrointestinal.

¹ Evaluated as predictor effects of outcome variables measured at the same sampling time and at the subsequent time point (lagged effects).

Table 5.4. Predictors in a general linear mixed model of gastrointestinal nematode (GIN) fecal egg count (FEC) in Rideau-Dorset cross lambs with high (n = 15) or medium (n = 15) cortisol response to *E. coli* lipopolysaccharide challenge.

Predictor variable	p-value	Direction of association	NDF	DDF	F-value	Proportion of variance explained (%) ¹
Stress	0.938	NA	3	24	0.01	0.04
Day	< 0.001	Variable ²	1	24	67.36	73.73
Treatment	< 0.001	Negative	1	24	51.61	68.26
Sex	0.250	NA	1	24	1.39	5.47
Age	0.429	NA	1	24	0.65	2.64
Age*sex	0.012	See note ³	1	24	7.34	23.42

Direction of association is given for significant effects only. One medium cortisol

response phenotype lamb was euthanized at 117 days on pasture; data obtained from this animal prior to death were included in analyses. NDF = numerator degrees of freedom; DDF = denominator degrees of freedom; NA = not applicable.

¹ Estimated using the formulas described by Dohoo et al. (2014), page 330.

² Direction of association varies at different levels of a categorical variable.

³ Increasing age positively associated with GIN FEC in wethers and negatively associated with GIN FEC in ewes.

Table 5.5. Predictors in general linear mixed models of hematocrit (HCT) and serum albumin (SA) in Rideau-Dorset cross lambs with high (n = 15) or medium (n = 15) cortisol response to *E. coli* lipopolysaccharide challenge.

Model	Predictor variable	p-value	Direction of association	NDF	DDF	F-value	Proportion of variance explained (%) ¹
HCT	Stress	0.396	NA	1	27	0.74	2.67
	SG	< 0.001	Positive	1	95	16.95	15.14
	Ln FEC	0.031	Negative	1	95	4.79	4.80
	Day	0.003	Variable ²	4	95	4.33	15.42
	SG*SG	< 0.001	Negative	1	95	16.06	14.46
	SG*day	0.002	Variable ²	4	95	4.53	16.02
SA	Stress	0.784	NA	1	26	0.08	0.31
	Sex	< 0.001	See note ³	1	26	15.64	37.56
	Weight	0.013	Positive	1	24	7.27	23.25
	HCT	< 0.001	Positive	1	24	17.05	41.53
	WBC	0.048	Negative	1	24	4.36	15.37
	Lagged Ln CarLA	0.002	Negative	1	24	11.69	32.75
	HCT*sex	0.002	Positive	1	24	12.20	33.70

Direction of association is given for significant effects only. One medium stress response phenotype lamb was euthanized at 117 days on pasture; data obtained from this animal prior to death were included in analyses. Lagged effects were measured 6-8 weeks prior to outcome variables. NDF = numerator degrees of freedom; DDF = denominator degrees of freedom; NA = not applicable; SG = serum globulin; Ln = natural logarithm; FEC = fecal egg count; WBC = total leukocyte count; CarLA = carbohydrate larval antigen.

¹ Estimated using formulas described by Dohoo et al. (2014), page 330.

² Direction of association varies at different levels of a categorical variable.

³ Predicted serum albumin was higher in ewes than in wethers.

Table 5.6. Mean postmortem total and species-specific burdens of adult and immature gastrointestinal nematodes after 189 days on pasture in Rideau-Dorset cross lambs with high (n = 15) and medium (n = 14) cortisol response to lipopolysaccharide challenge.

Region	Stage	Species	Mean	Median	Range
Abomasum	Adult	All	87	17	0-462
		<i>Haemonchus contortus</i>	12	0	0-132
		<i>Teladorsagia</i> spp. ¹	62	0	0-413
		<i>Trichostrongylus axei</i>	11	0	0-116
	Immature	All	112	25	0-1007
		<i>Haemonchus contortus</i>	18	0	0-116
		<i>Teladorsagia</i> spp. ¹	93	0	0-974
Small intestine	Adult	All	2142	462	0-19,520
		<i>Trichostrongylus</i> spp. ²	1947	248	0-19,324
		<i>Nematodirus</i> spp.	2	0	0-56
		<i>Capillaria</i> spp.	49	0	0-528
	Immature	All	48	0	0-446
		<i>Trichostrongylus</i> spp. ²	5	0	0-132
		<i>Nematodirus</i> spp.	43	0	0-446
Cecum	Adult	<i>Trichuris ovis</i> ³	5	0	0-132

Cortisol response phenotype was not significantly associated with nematode burdens;

therefore, data from high and medium cortisol response lambs were combined.

¹ Includes *Teladorsagia circumcincta* and *Ostertagia trifurcata*, as females and larvae of these species cannot be differentiated based on morphology.

² Includes *Trichostrongylus colubriformis* and *Trichostrongylus vitrinus*, as females and larvae of these species cannot be differentiated based on morphology. No *Trichostrongylus axei* were isolated from the small intestine.

³ Adult *Trichuris ovis* were recovered from the cecum of one study lamb.

Table 5.7. Predictors in a general linear mixed model of gastrointestinal nematode burden in Rideau-Dorset cross lambs with high (n = 15) or medium (n = 14) cortisol response to lipopolysaccharide challenge.

Predictor variable	p-value	Direction of association	NDF	DDF	F-value	Proportion of variance explained (%) ¹
Stress	0.796	NA	1	24	0.07	0.3
GI segment	< 0.001	Variable ²	1	82	12.52	13.2
Stage	< 0.001	Variable ²	1	82	26.62	24.5
Lagged Ln FEC	< 0.001	Positive	1	82	18.72	18.6
Lagged WBC	0.613	NA	1	82	0.26	0.3
Lagged SA	0.465	NA	1	82	0.54	0.7
Stage*GI segment	< 0.001	Variable ²	1	82	50.20	38.0
Lagged WBC*stage	< 0.001	Variable ²	1	82	14.31	14.9
Lagged SA*GI segment	< 0.001	Variable ²	1	82	14.92	15.4

Direction of association is given for significant effects only. Lagged effects were

measured 6-8 weeks prior to outcome variables. NDF = numerator degrees of freedom;

DDF = denominator degrees of freedom; NA = not applicable; GI = gastrointestinal; Ln =

natural logarithm; FEC = fecal egg count; WBC = total leukocyte count; SA = serum

albumin.

¹ Estimated using formulas described by Dohoo et al. (2014), page 330.

² Direction of association varies at different levels of a categorical variable.

5.8 Figures

Figure 5.1. Cortisol concentrations in Rideau-Dorset cross lambs (n = 180), 4 hours after intravenous challenge with *Escherichia coli* lipopolysaccharide (LPS).

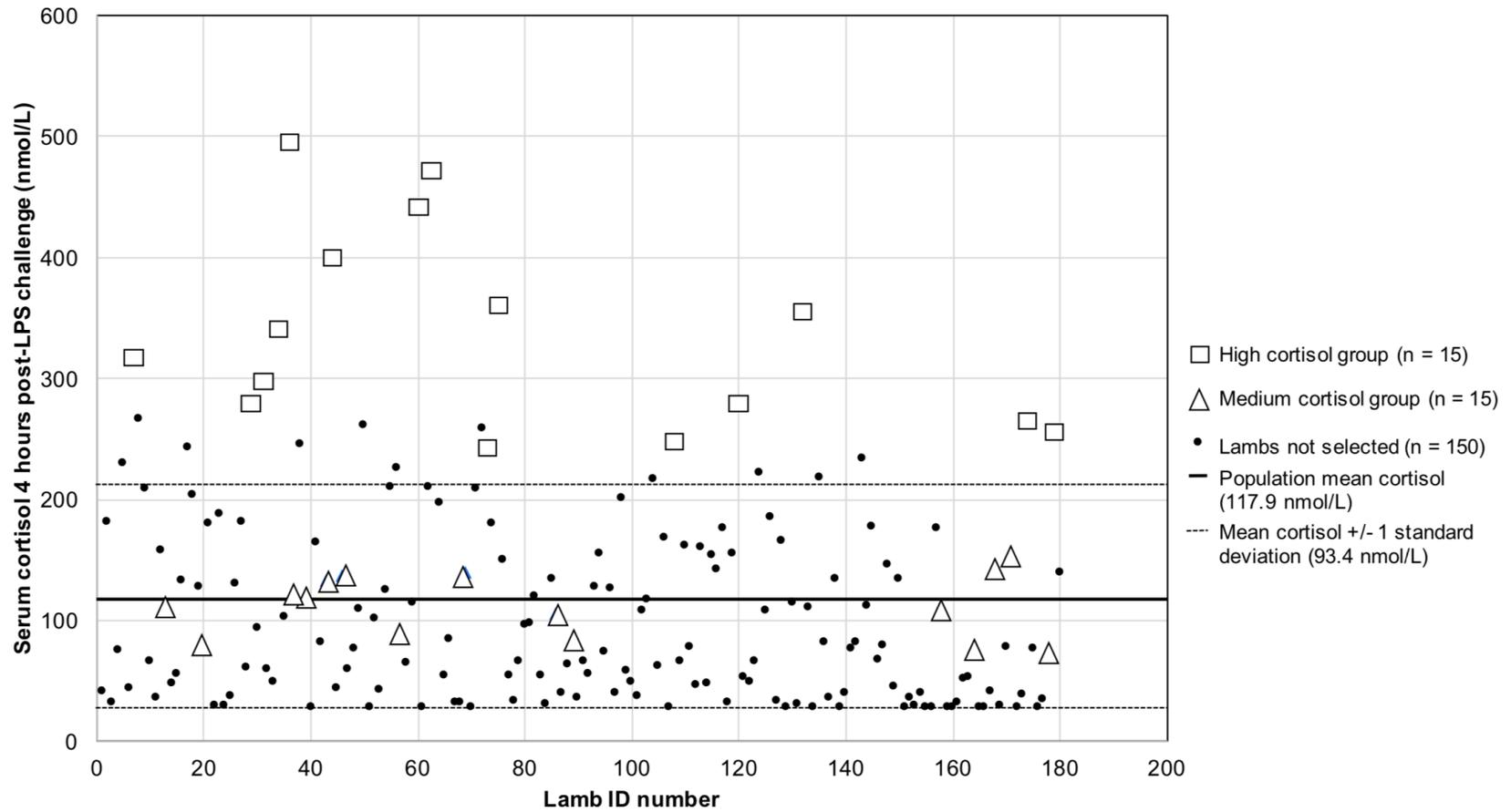


Figure 5.2. Mean total and species-specific gastrointestinal nematode (GIN) fecal egg counts (FEC) in Rideau-Dorset cross lambs with high (n = 15) and medium (n = 15) cortisol response to challenge with *Escherichia coli* lipopolysaccharide. MSR = medium stress response, HSR = high stress response. Error bars indicate 95 % confidence intervals for mean FEC in the two stress response phenotypes; wide bars indicate confidence intervals for MSR lambs; narrow bars indicate confidence intervals for HSR lambs. Anthelmintic treatment was administered 10 days after fecal sampling to 14 animals (7 high and 7 medium cortisol response) with FEC > 500 eggs per gram at 117 days on pasture; treatment was not administered to any other animals at any other time point. One medium cortisol response phenotype lamb was euthanized at 117 days on pasture; data obtained from this animal prior to death were included in analyses.

*No lambs exceeded the minimum egg count for culture in May 2016. Hence, species proportions and species-specific FEC could not be determined.

**Species-specific FECs are for both stress response phenotypes combined.

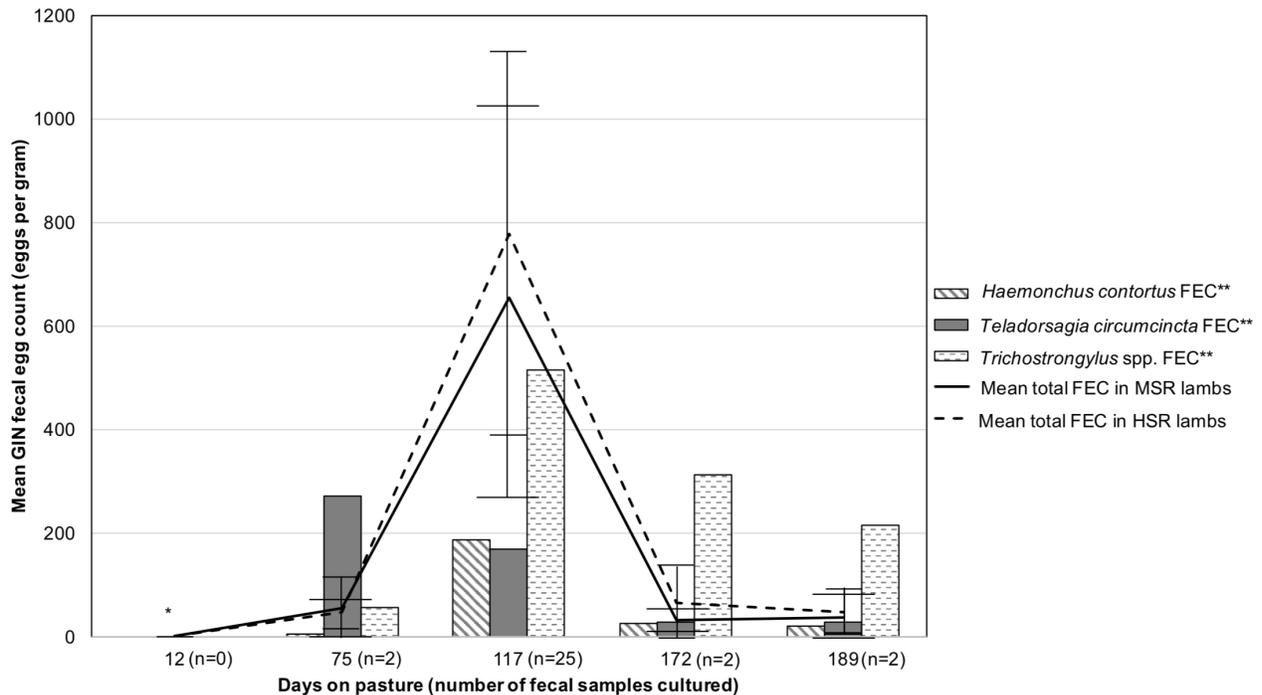


Figure 5.3. Predicted gastrointestinal nematode (GIN) fecal egg count of Rideau-Dorset cross lambs based on lamb age and sex.

Predictions are for lambs that had been exposed to nematode challenge on pasture for 117 days and had not been treated with an anthelmintic. Cortisol response phenotype was not significantly associated with GIN fecal egg counts; therefore, data from high and medium cortisol response lambs were combined. One medium cortisol response phenotype lamb was euthanized at 117 days on pasture; data obtained from this animal prior to death were included in analyses.

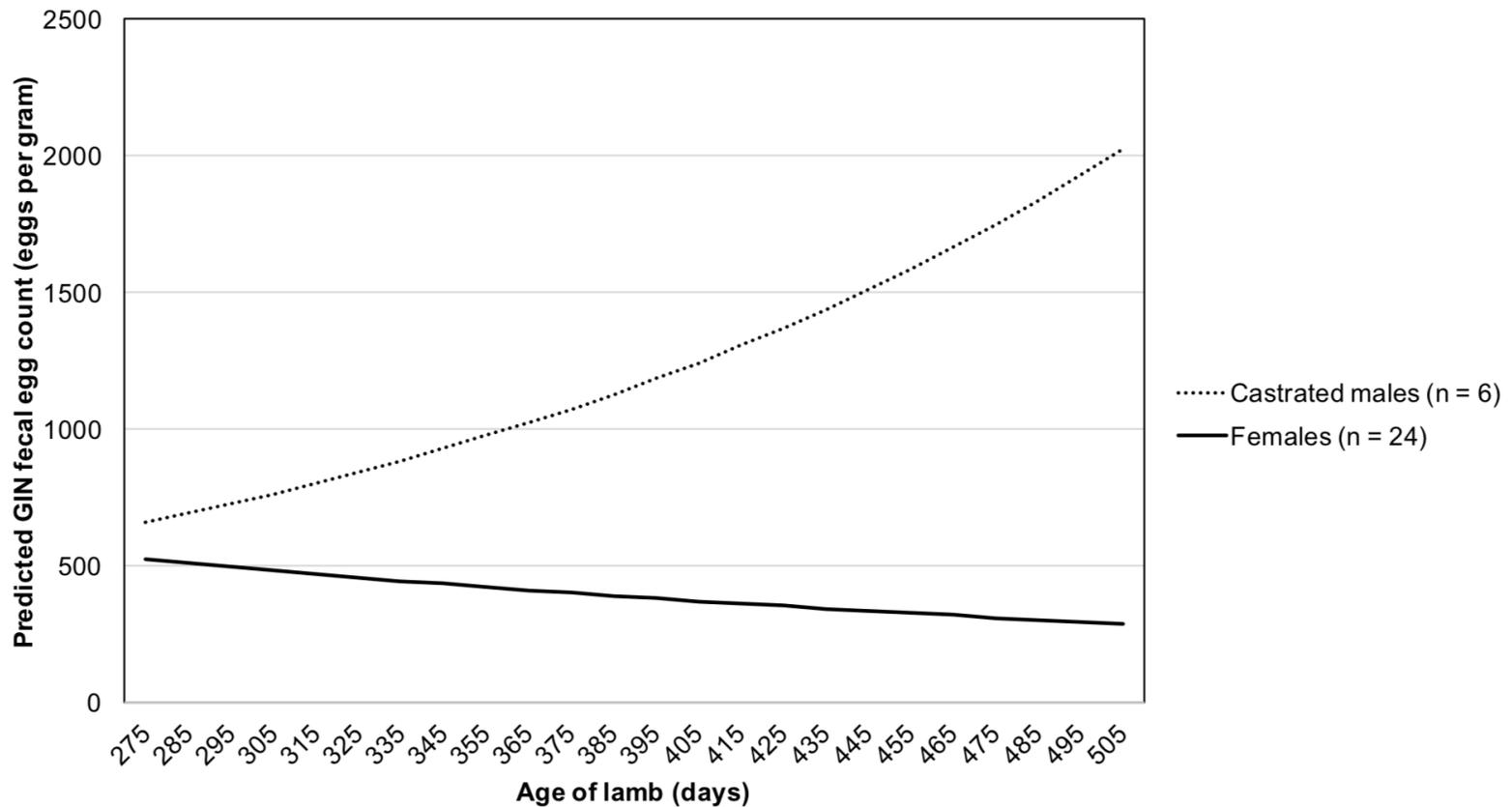


Figure 5.4. Mean, maximum, and minimum values of hematocrit and serum albumin levels in Rideau-Dorset cross lambs with high (n = 15) and medium (n = 15) cortisol response to challenge with *Escherichia coli* lipopolysaccharide. Error bars indicate 95% confidence interval for means; green lines delineate reference intervals for healthy sheep (Animal Health Laboratory, 2019). Cortisol response phenotype was not significantly associated with hematocrit or serum albumin; therefore, data from high and medium cortisol response lambs were combined. One medium cortisol response phenotype lamb was euthanized at 117 days on pasture; data obtained from this animal prior to death were included in analyses.

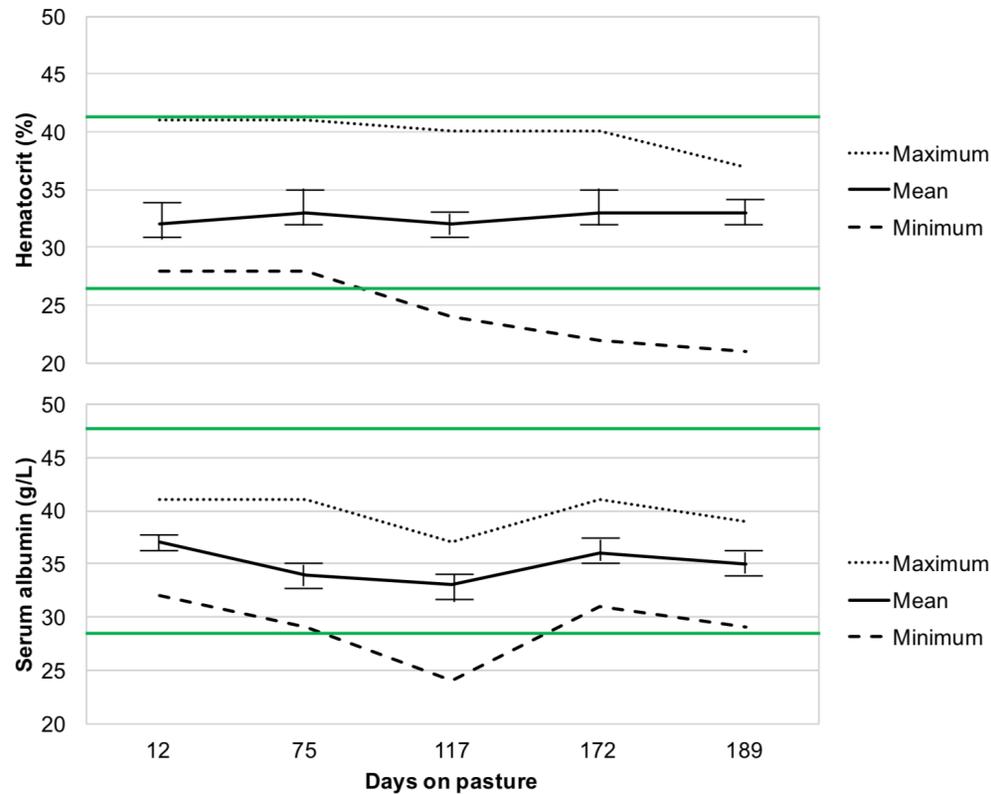
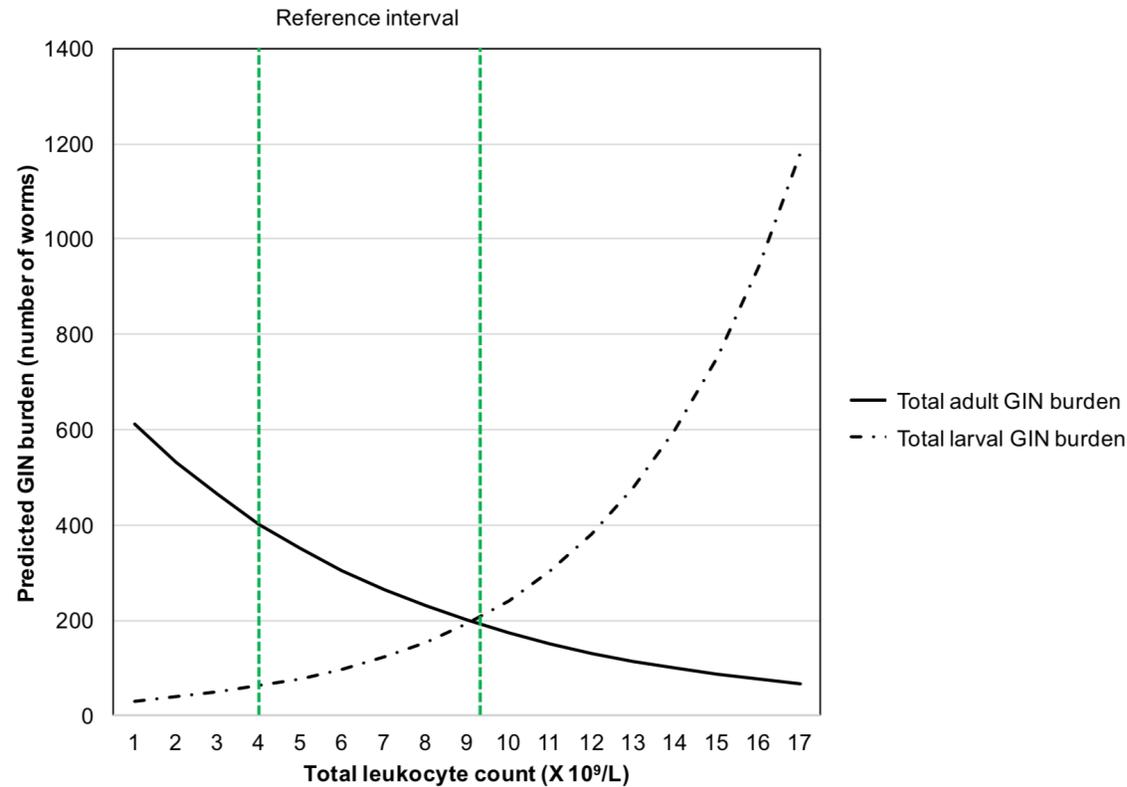


Figure 5.5. Predicted postmortem gastrointestinal nematode (GIN) burdens in Rideau-Dorset cross lambs with high (n = 15) and medium (n = 14) cortisol response to challenge with *Escherichia coli* lipopolysaccharide, based on total leukocyte count measured 17 days prior to slaughter. Predictions are for study lambs with flock average fecal GIN egg count (48.8 epg) and serum albumin (36.3 g/L), as measured 17 days prior to slaughter. Cortisol response phenotype was not significantly associated with GIN burdens; therefore, data from high and medium cortisol response lambs were combined. Vertical dashed lines indicate the reference interval for total leukocyte count in healthy sheep (4.0-9.3 X 10⁹/L; Animal Health Laboratory, 2019).



CHAPTER SIX:

ASSESSMENT OF ANTIBODY- AND CELL-MEDIATED IMMUNE RESPONSES IN SHEEP AND THEIR RELATIONSHIP WITH GASTROINTESTINAL NEMATODE PARASITISM

Based on a manuscript in preparation for submission to *Veterinary Immunology and Immunopathology*.

Abstract

Genetic selection of livestock with superior immune competence is an attractive strategy for mitigating the impact of common pathogens. Although a method to evaluate antibody-mediated (AbMIR) and cell-mediated (CMIR) immune responses is patented and proven to reduce the incidence of several diseases in cattle, including parasites, this technique has not been evaluated in sheep. Gastrointestinal nematodes (GINs) are important production-limiting pathogens of sheep with widespread anthelmintic resistance. Protective immunity to GINs is mediated by the mucosal AbMIR. Conversely, CMIR-bias is associated with susceptibility to GINs. This pilot study assessed AbMIRs and CMIRs in sheep using a challenge protocol modified from cattle. Rideau-Dorset cross lambs were challenged with *Candida albicans* antigen to stimulate a CMIR, and with hen egg-white lysozyme to stimulate an AbMIR. Fifteen animals with high AbMIR/low CMIR and fifteen with high CMIR/low AbMIR were selected to evaluate the association between phenotypic AbMIR or CMIR polarization and GIN parasitism. These lambs were naïve to GINs apart from low levels of nonpathogenic *Nematodirus* spp. in the source flock, and were

naturally exposed to GINs by grazing with a commercial flock in central Ontario, Canada, from April to November 2017, for a total of 196 days. Five times during the grazing season, fecal egg counts (FEC), total leukocyte counts (WBC) and clinical indicators of GIN parasitism (hematocrit and serum albumin levels) were assessed; herbage was collected from the pasture to confirm exposure to GIN larvae. The lambs were euthanized after 196 days on pasture, and GIN burdens determined by counting and identifying nematodes recovered from the gastrointestinal tract. The relationship between AbMIR/CMIR phenotype and GIN parasitism was evaluated using general linear mixed models for *in vivo* FEC, hematocrit, serum albumin, and postmortem GIN burdens. Polarization towards AbMIR or CMIR was not significantly associated with any assessed indicators of GIN parasitism (all p-values > 0.2). However, significant negative associations were identified between salivary GIN-specific antibody and FEC in the second half of the grazing season ($p = 0.027$), confirming development of a protective mucosal AbMIR, and between WBC and postmortem GIN burdens ($p = 0.032$), which may reflect a systemic AbMIR and/or CMIR to GINs. Despite evidence of a protective immune response to GINs in the study lambs, this preliminary study suggests that selection for AbMIR or CMIR bias in sheep may not significantly impact FEC or parasite burdens.

Keywords

Sheep, gastrointestinal nematode, cell-mediated immune response, antibody-mediated immune response

6.1 Introduction

Gastrointestinal nematodes (GINs) are important and widespread parasites of sheep, exerting a financial impact through clinical disease and subclinical loss of productivity (Mavrot et al., 2015; Sutherland et al., 2019). The life cycle of all trichostrongylid GINs is similar, with eggs shed in feces developing through two moults to the infective third stage larva (L₃), then moving into herbage to facilitate ingestion by grazing sheep (Taylor et al., 2016). Following ingestion, L₃s undergo two additional moults before maturing to egg-laying adults (Taylor et al., 2016). Several classes of anthelmintics are commonly used to control GIN parasitism. However, the prevalence of GIN resistance to one or more anthelmintic classes is now very high in most sheep-rearing countries (Kaplan and Vidyashankar, 2012; Falzon et al., 2013). As a result, complementary GIN management strategies are under investigation to reduce dependence on anthelmintics (Shaw et al., 2012; Shaw et al., 2013; Sutherland et al., 2019). One strategy that has been proposed is the identification and selective breeding of sheep with a superior immune response to GINs (Karrow et al., 2014).

The immune response of sheep to GINs is complex, involving interplay between the innate and adaptive immune system (Karrow et al., 2014; McRae et al., 2015). Production of interleukins (ILs) 4, 5, and 13 during the innate response polarizes the subsequent adaptive response towards an antibody-mediated immune response (AbMIR), and suppresses production of cytokines required to drive a cell-mediated immune response (CMIR) (Paul and Zhu, 2010; Venturina et al., 2013; Karrow et al., 2014; McRae et al., 2015). The AbMIR is generally considered protective against GIN infection and the CMIR permissive, as development of a CMIR-polarized response to GINs has been associated with improved GIN survival (Venturina et al., 2013;

McRae et al., 2015). Higher levels of circulating immunoglobulins (Igs) G and E specific to GIN antigens are associated with reduced fecal egg shedding due to impaired worm growth, but have also been associated with increased gastrointestinal inflammation resulting in diarrhea and reduced productivity (Shaw et al., 2013; Karrow et al., 2014; McRae et al., 2015). Levels of mucosal IgA specific to GIN antigens are not as strongly associated with these undesirable traits as circulating IgG and IgE. In fact, elevated salivary IgA specific to a carbohydrate larval antigen (CarLA) present on L₃s has been associated with improved growth of lambs, likely due to reduced GIN burdens (Shaw et al., 2012; Shaw et al., 2013). Consequently, methods developed to screen sheep for GIN immunity largely depend on identifying animals with a strong mucosal AbMIR (Shaw et al., 2012; Shaw et al., 2013; Sutherland et al., 2019). However, despite the generally antagonistic relationship between the AbMIR and CMIR, some CMIR cytokines such as IL-12 are produced during the AbMIR to GINs (Venturina et al., 2013). Thus, although the immune response to GINs is dominated by the AbMIR, the CMIR may also play a role in adaptive immunity to GINs. Moreover, as the CMIR is recognized to be an important contributor to host resistance to intracellular pathogens (McRae et al., 2015), selection methods that assess both antibody- and cell-mediated immune competence may have a greater potential to improve both overall health resilience of sheep and GIN immunity than selection for AbMIR alone.

Assessment of antibody- and cell-mediated immune competence has been used successfully to promote health in other species of livestock, including pigs and cattle (Heriazon et al., 2009; Thompson-Crispi et al., 2013). In dairy cattle, AbMIRs and CMIRs can be assessed via a patented immune challenge protocol, using vaccination with hen egg-white lysozyme (HEWL) to assess AbMIR and *Candida albicans* antigen (CAA) to assess CMIR (Mallard and Wagter, 2001;

Heriazon et al., 2009; Thompson-Crispi et al., 2013). A cell-mediated delayed-type hypersensitivity reaction occurs in response to intradermal challenge with CAA, while serum IgG specific to HEWL is used as a marker of AbMIR (Heriazon et al., 2009). The responses to these antigens can then be used to classify overall immune competence as below average, average, or high (Heriazon et al., 2009; Thompson-Crispi et al., 2013; Cartwright et al., 2017; Aleri et al., 2019). The high immune response (HIR) phenotype in cattle is associated with a lower incidence of mastitis, metritis, ketosis, and digital dermatitis (Thompson-Crispi et al., 2013; Cartwright et al., 2017). Furthermore, cattle with high AbMIR to a commercial vaccine for clostridial disease and leptospirosis had reduced fecal shedding of GIN eggs (Aleri et al., 2019). It is not known whether this association is also true for cattle with high AbMIR assessed using HEWL challenge. Although evaluation of AbMIRs through challenge with HEWL has been adapted for use in sheep by Stryker et al. (2013), it is also not known whether variable systemic antibody- and cell-mediated responses to HEWL and CAA challenge are associated with variability in the mucosal immune response to GIN parasitism in sheep. Therefore, the objective of this pilot study was to investigate the associations between AbMIR or CMIR-biased phenotype, GIN burdens, and clinical indicators of GIN parasitism in sheep. Lambs with AbMIR-biased immune responses were predicted to have reduced *in vivo* and postmortem GIN burdens and a lesser clinical impact of GIN parasitism on hematocrit and serum albumin levels than lambs with a CMIR-biased immune phenotype. The results of this study should provide insight into the efficacy of AbMIR and CMIR phenotyping as tools for selection of sheep with superior GIN immunity and health.

6.2 Materials and Methods

6.2.1 Assessment of cell- and antibody-mediated immune responses

Animal use approval was obtained from the University of Guelph Animal Care Committee (Animal Use Protocol Number 3380). Cell- and antibody-mediated immune responses were assessed in all Rideau-Dorset cross lambs born between January and November 2016 (n = 210) at the University of Guelph Ponsonby Sheep Research Station (Ponsonby, Ontario, Canada). The source flock is free of scrapie, Maedi-Visna virus, and *Coxiella burnetii*. Antigen challenge commenced when the lambs were 70 days of age, and was modified from a patented protocol (Mallard and Wagter, 2001; summarized in Figure 6.1). The first day of antigen challenge was designated day 0. On days 0 and 14, the lambs were vaccinated intramuscularly in the left neck with 0.5 mg of HEWL (Sigma-Aldrich, Missouri, USA) and 0.5 mg of Quil A® saponin adjuvant (Brenntag Biosector, Ballerup, Denmark) dissolved in 1.0 mL of 0.9 % sterile saline solution (Vétoquinol, Québec, Canada), and with 0.5 mg of CAA derived from whole cells (Stallergenes Greer, North Carolina, USA) and 0.5 mg of Quil A® dissolved in 1.0 mL of 0.9 % sterile saline solution in the right neck.

Both sides of the neck were shaved using an electric clipper with a size 40 blade (Newell Industries, New Jersey, USA) on day 20, and subsequent intradermal antigen challenge was performed 24 hours later (day 21) to allow skin irritation secondary to clipping to abate. On day 21, an intradermal injection containing 0.2 mg of CAA dissolved in 0.1 mL of 0.9 % sterile saline solution was administered in the right neck, and an intradermal injection of 0.1 mL of 0.9 % sterile saline solution (control) was administered in the left neck. Skin-fold thickness of the injection sites was measured in triplicate using a Harpenden skin fold caliper (Baty International,

Sussex, UK) prior to injection and at 2, 4, 6, 24, 48, and 72 hours post-intradermal injection to assess cell-mediated delayed-type hypersensitivity response as an indicator of CMIR (Heriazon et al., 2009). All measurements were performed by the same observer (E.B.) in order to ensure consistency in handling and interpretation of the caliper.

Mean skin-fold thickness at both intradermal injection sites was calculated from triplicate values for each lamb at each time point; differences between post-intradermal injection and baseline skin-fold thickness were calculated and plotted (see Figure 6.2). No significant change in skin-fold thickness was observed at the saline injection site between 0 and 6 hours post-intradermal injection ($p > 0.05$). Small significant ($p < 0.001$) increases in skin-fold thickness at the saline injection site were observed at 24, 48, and 72 hours post-intradermal injection, with the peak change (0.21 mm) occurring at 48 hours post-injection. Skin-fold thickness at the CAA injection site was significantly increased relative to baseline and greater than values obtained from the saline injection site at all measurements following intradermal injection ($p < 0.001$ for all comparisons). Peak change in skin-fold thickness at the CAA injection site occurred at 24 hours post-injection (mean 2.04 mm, see Figure 6.2). Therefore, the difference between skin-fold thickness at baseline and 24 hours post-intradermal injection was used to evaluate CMIR.

Serum samples were also collected on days 0 and 21 to assess baseline and secondary IgG antibody response to HEWL antigen, respectively, using an indirect ELISA modified from Stryker et al. (2013). Each well of a 96-well high-affinity microplate (PerkinElmer Inc., Massachusetts, USA) was coated with 100 μ L of 10 μ g/mL HEWL dissolved in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Wells were incubated, washed, and coated with 200 μ L of

blocking buffer BUF033B (BioRad Ltd., California, USA), then incubated and washed again. Serum samples were diluted to 1/6400 in wash buffer, and each individual diluted serum sample was loaded in triplicate at 100 μ L per well. The plate was then incubated for 2 hours at 37 °C, and washing was repeated three times. Rabbit anti-sheep IgG A130-101AP (Bethyl Laboratories Inc., Texas, USA) was diluted 1/5000 in buffer containing 0.82 g sodium chloride, 0.61 g Tris base, 50 μ L Tween 20 and 1.0 g bovine serum albumin (Sigma Aldrich, Ontario, Canada) in 100 mL Milli Q® (Millipore Sigma, Darmstadt, Germany), with a pH of 8.0. This antibody dilution was added to each well at 100 μ L/well, and incubated for 2 hours at 37 °C. Following three washes, 80 μ L of alkaline phosphatase yellow pNPP (Sigma Aldrich, Ontario, Canada) was added to each well and the plate was covered with foil and incubated at 20 °C for 30 minutes. Measurement of optical density was performed using a VICTOR 3 MULTILABEL COUNTER (Perkin Elmer, Massachusetts, USA). Serum samples collected on day 21 were pooled and serially diluted to 1/200, 1/400, 1/800, 1/1600, 1/3200, and 1/6400 to create standard curves; each plate included a control well without serum to assess nonspecific binding of the rabbit anti-sheep IgG antibody. Optical density was normalized across plates using the following correction factor:

$$\text{Correction factor} = \frac{\text{Overall mean (1/6400) of reference samples from all plates}}{\text{Actual mean (1/6400) of individual plate reference sample}}$$

Average intra- and inter-plate coefficients of variation were 3.4 % and 3.0 %, respectively. Mean corrected optical density was calculated across triplicate serum samples at day 0 and day 21 for each animal. At day 0, the flock mean corrected optical density (COD) value was 0.098, with a standard deviation of 0.039. The flock displayed more variation in COD values at day 21 (standard deviation 0.278); the flock mean value on this day was significantly higher than at day 0 (0.697, $p < 0.001$). Therefore, COD values at day 21 were used to evaluate AbMIR.

For each lamb, phenotypic residuals for AbMIR and CMIR were calculated using the following formulae:

$$\text{AbMIR residual} = \text{Individual day 21 COD value} - \text{Flock mean day 21 COD value}$$

$$\text{CMIR residual} = \text{Individual } \Delta \text{ skin thickness} - \text{Flock mean } \Delta \text{ skin thickness}$$

These residuals were plotted to identify 15 animals with high AbMIR and low CMIR, and 15 animals with high CMIR and low AbMIR (see Figure 6.3). Lambs with opposite AbMIR and CMIR phenotypes were chosen in order to investigate the effect of phenotypic bias towards type 1 or type 2 immune responses on GIN parasitism. Exclusion criteria included use of the animal in another study prior to final selection and pre-existing conditions, such as jaw or limb malformation, that would impair the lamb's ability to graze or its mobility. The selected lambs were born between January 25 and November 19, 2016, with a difference of 299 days between the oldest and youngest animal. In order to reduce sex differences in GIN parasitism (Abuargob and Stear, 2014), the 15 selected ram lambs (9 high AbMIR, 6 high CMIR) were castrated no less than 2 months prior to turnout on pasture. Prior to closed castration, 2 % lidocaine (Zoetis, Quebec, Canada) was administered into the testicular cord, and the cord was crushed using an emasculator (Jorgensen Laboratories, Colorado, USA). Successful castration was confirmed by assessing testicular involution 3 weeks later.

6.2.2 Parasite challenge and monitoring

The high AbMIR/low CMIR and high CMIR/low AbMIR lambs were housed indoors from birth until they were turned out on pasture in central Ontario, Canada on April 27, 2017 (age range 158-457 days). All of the lambs were vaccinated for clostridial disease 8 weeks prior to pasture

turnout. Low levels of infection with nonpathogenic *Nematodirus* spp. have been diagnosed sporadically in pooled fecal samples from the source flock. However, as no trichostrongyle-type eggs have been observed in the flock for more than two decades, the study lambs were considered naïve to other GIN species. The study lambs were naturally exposed to GINs by co-grazing with a commercial flock of primiparous Rideau-Dorset cross ewe lambs with a history of GIN parasitism, and remained on pasture with this flock until November 9, 2017 (a total of 196 days). Water and a commercial mineral supplement were supplied *ad libitum* at all times on pasture. The lambs were also supplemented with a ration of shelled corn provided at 0.45 kg per head per day from October 17 until completion of the study on November 9, 2017.

Samples were collected from the study lambs at five time points (26, 74, 116, 174, and 196 days on pasture), in order to assess indicators of GIN parasitism and its clinical effects. At each sampling the study lambs were weighed, fecal samples collected per rectum, whole blood and serum obtained via jugular venipuncture, and saliva samples collected by swabbing the buccal mucosa for 10 seconds with a cotton dental roll (Richmond Dental & Medical Inc., North Carolina, USA) held in hemostatic forceps (Shaw et al., 2012). Fecal samples were preserved in airtight plastic bags and held at room temperature (20 °C) for no more than 48 hours before processing. Blood samples were immediately chilled and maintained at 4 °C; whole blood was submitted for complete blood counts within 48 hours of collection, and serum was separated and frozen at -80 °C until subsequent analysis of protein levels. Saliva samples were held at -80 °C prior to laboratory analysis.

6.2.3 Environmental monitoring

In order to confirm exposure to L₃s, at each animal sampling date up to 500 g of herbage was collected from pasture the study lambs had grazed for the 14 days prior to sampling. Herbage was collected according to the protocol reported by the Ministry of Agriculture, Fisheries, and Food (1984). Weather patterns were monitored using data collected from the nearest weather station to the farm (26 kilometers distance).

6.2.4 Laboratory methods

Gastrointestinal nematode fecal egg counts (FECs) were determined using a modified McMaster method with an analytical sensitivity of 8.33 eggs per gram (epg) (Zajac and Conboy, 2012).

Eggs of gastrointestinal parasites shedding non-trichostrongylid type eggs, including *Nematodirus* spp., *Trichuris ovis*, and *Moniezia* spp., were also counted but were not included in GIN FECs. Given the low frequency of FEC monitoring during the study, a cutoff of ≥ 500 epg was selected for treatment with an anthelmintic to prevent GIN-related morbidity and mortality. All 30 study lambs were administered albendazole (Zoetis Canada Inc., Quebec, Canada) orally at 5 mg/kg if any lamb exceeded 500 epg in order to ensure consistency across the group.

Individual fecal egg counts only exceeded 500 epg at two sampling time points (26 and 74 days on pasture); treatment was administered at 36 and 84 days on pasture to allow the study lambs to become reinfected and a 3-week prepatent period to elapse prior to the next fecal sampling (Taylor et al., 2016). In order to assess species-specific GIN egg shedding, deep amplicon ribosomal DNA sequencing was performed on first-stage larvae (L₁s) cultured from individual fecal samples with >200 epg, according to protocols reported in Redman et al., (submitted). The

200 epg threshold was selected as the minimum FEC at which 100 or more L₁s could reliably be recovered from 6 g fecal samples for sequencing.

Pasture herbage samples were washed to extract L₃s according to the method described by the Ministry of Agriculture, Fisheries, and Food (1984). L₃s were speciated using morphologic criteria reported in van Wyk and Mayhew (2013).

Salivary CarLA-specific IgA antibody was assessed at the beginning (26 days on pasture), middle (116 days on pasture) and near the end (174 days on pasture) of the grazing season. Levels were determined using an enzyme-linked immunosorbent assay with a detection limit of 0.3 units/mL, as previously reported in Shaw et al. (2012, 2013). Complete blood counts were performed via flow cytometry on whole blood using an Advia 2120 (Siemens Healthcare Diagnostics Products Ltd., Ontario, Canada), and protein concentrations were determined via a colorimetric assay with a detection limit of 2.0 g/L, using a Cobas 6000 C501 (Roche Diagnostics, Quebec, Canada). Differential leukocyte counts were performed manually on blood smears.

6.2.5 Parasite collection and identification

Following the final sampling at 196 days on pasture, the study lambs were slaughtered at a provincially inspected abattoir. The lambs were transported from the farm to Ponsonby General Animal Facility (Ponsonby, Ontario, Canada), located approximately 40 km from the abattoir, and held for 72 hours prior to slaughter to allow transport-related stress to abate. Hay and water were provided *ad libitum* during the 72-hour holding period. Gastrointestinal tracts were

collected following slaughter and GINs recovered from the abomasum, small intestine, and cecum using a protocol modified from the Ministry of Agriculture, Fisheries, and Food (1984). Each segment was double ligated, separated, and its contents emptied into a separate container. The mucosa was then washed in water to detach remaining GINs, and the washings combined with the collected contents up to a total volume of 3.0 L. These washings were stirred, and a 1.0 L aliquot transferred to a flask and combined with 0.1 L of 100 % formalin for fixation. The washed mucosa was then immersed in 3.0 L of 0.9 % saline, incubated at 37 °C for 6 hours, and washed again in the saline solution to collect larval GINs. The saline solution was stirred and fixed as previously described. Adult and larval GINs in 0.2 L aliquots (18.2 % of the 1.1 L formalin-fixed aliquot and 6.1 % of the 3.0 L total volume collected) of the mucosal washings and saline solution were counted, and total GIN burden was estimated by multiplying the total number of GINs counted by the minimum detection limit ($1 / 6.1 \% = 16.5$ worms in 3.0 L). The first 100 adult and larval (L₃ and L₄) GINs collected were identified using criteria reported by Thomas and Probert (1993), Zajac and Conboy (2012), and Taylor et al. (2016).

6.2.6 Statistical analysis

SAS version 9.4 (SAS Institute Inc., North Carolina, USA) was used to generate general linear mixed models for GIN FEC, hematocrit, serum albumin and postmortem GIN burdens. These outcome variables were assessed for normality using the Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises and Anderson-Darling tests (Ghasemi and Zahediasl, 2012). Gastrointestinal nematode FEC, postmortem GIN burdens, and salivary CarLA antibody levels were subjected to natural logarithmic transformation prior to analysis to normalize their distributions; all zero values were corrected to half the assay minimum detection limit (4.16 epg for FEC, 8.25 worms

for postmortem GIN burden and 0.15 units/mL for salivary CarLA antibody) to allow transformation (Schisterman et al., 2006).

All variables assessed as fixed effects in the models are listed in Table 6.1. Those measured repeatedly were tested against outcome measures obtained at the same sampling date and at the next sampling date (lagged effects). Interaction terms involving up to three simple effects, including quadratic interactions, were also assessed. All four models included immune response phenotype and sex nested within individual animal identification number as random effects. Effects that were not significant ($p > 0.05$) were removed in order of decreasing p-value prior to reintroduction to confirm lack of significance and rule out multicollinearity. Simple effects that were not significant but were involved in a significant interaction term were retained in the model to maintain model hierarchy. Random effects, autoregressive, heterogenous autoregressive, Toeplitz, heterogenous Toeplitz and unstructured error structures were evaluated for best fit using the Akaike information criterion (Dohoo et al., 2014). Conformance to model assumptions, normality, and presence of unequal variance and outliers were evaluated by plotting residuals against predicted and explanatory variables. Outliers were removed to re-assess model fit, but were returned to the model if not found to be due to confounding or data error.

6.3 Results

6.3.1 Environmental conditions, GIN epidemiology, and losses to follow-up

Monthly rainfall was high during the first half of the 2017 grazing season, amounting to 505.5 mm between May and July, compared with 221.2 mm between August and November. Mean monthly temperature steadily rose from 12.5 °C in May to a peak of 20.9 °C in July, then

progressively declined to a minimum of 1.9 °C in November. Overnight minimum temperatures at or below freezing (0 °C) were observed in May, October, and November, while monthly maximum temperature exceeded 30 °C from June until September. Numbers of L₃s in pasture herbage samples were low (less than 25 L₃s/kg dry matter) in May and July, likely reflecting dilution of larvae by lush pasture growth due to high rainfall. Peak L₃ numbers (264 L₃s/kg dry matter) were observed in August after precipitation decreased and herbage growth slowed, then declined to 49 L₃s/kg dry matter in October as mean monthly temperatures decreased. No L₃s were extracted from pasture herbage collected in November.

Mean GIN FECs were not significantly different ($p > 0.05$) between the high AbMIR/low CMIR and high CMIR/low AbMIR phenotypes at any time point, and followed a similar pattern of change during the 2017 grazing season (see Figure 6.4). In both groups, mean FEC were between 100 and 225 epg when first sampled at 26 days on pasture (May), peaked at between 300 and 400 epg at 74 days on pasture (July), and decreased to low levels (< 50 epg) from 116 days on pasture (August) until the end of the study at 196 days on pasture (November). At least one study lamb exceeded the 200 epg cutoff for fecal culture at all time points except 174 days on pasture (October). The predominant GIN species cultured from fecal samples at all time points were *Teladorsagia circumcincta*, *Trichostrongylus* spp., and *Haemonchus contortus*. No other species that produce trichostrongylid-type eggs, including *Cooperia* spp., *Chabertia ovina*, and *Oesophagostomum* spp., were identified. *Nematodirus spathiger* was isolated from 56.7 % of fecal samples cultured, and accounted for 24 % of L₁s cultured from one study lamb at 74 days on pasture. However, levels of *N. spathiger* in the remaining study lambs were low (< 10 %) at all time points.

One high AbMIR/low CMIR phenotype study lamb developed progressive neurologic signs after 88 days on pasture, and was euthanized at 95 days on pasture. Although a postmortem examination was not performed on this lamb, it was presumptively diagnosed with listeriosis on the basis of clinical signs and a history of several confirmed cases of listeriosis in the commercial flock co-grazing with the study lambs in the month prior to its death. Data obtained from this animal prior to death were included in statistical analyses. None of the remaining study lambs developed clinical signs of listeriosis or other illness for the remainder of the study.

6.3.2 Modeling variation in GIN fecal egg counts

A general linear mixed model for repeated measures with an autoregressive error structure was generated for GIN FECs. Predictors in the final model are summarized in Table 6.2; immune response phenotype was not a significant predictor of FEC ($p = 0.257$), but was retained in the model as variable of interest. Lagged salivary IgA specific to CarLA was not a significant predictor of FEC as a simple effect ($p = 0.494$), but was involved in a significant interaction with day ($p = 0.027$). Since CarLA antibody levels were not determined for all sampling time points, inclusion of lagged CarLA antibody as a predictor in the model necessitated exclusion of FEC on days 26 and 116 from analyses, as these dates did not have a corresponding lagged CarLA antibody measurement. Higher CarLA antibody level at 26 days on pasture was significantly associated with higher FECs at 74 days on pasture, but higher CarLA antibody levels on days 116 and 174 on pasture were significantly associated with lower FECs on days 174 and 196, respectively. However, CarLA antibody levels were not significantly different between high AbMIR/low CMIR and high CMIR/low AbMIR lambs. Number of days on pasture was also

significantly negatively associated with predicted FEC as a simple effect ($p < 0.001$), consistent with the decrease in mean FECs after 74 days on pasture and consistently low FECs until the end of the study at 196 days on pasture (see Figure 6.4). Age was a significant predictor of FEC both as a positive linear and negative quadratic effect ($p = 0.036$ and 0.031 , respectively). The age at which the negative quadratic effect outweighed the positive linear effect of increasing age was determined using the following formula:

$$\text{Reflection} = [(-1) \times \text{linear FEC 450 estimate}] / [2 \times \text{quadratic FEC estimate}]$$

In animals under 340 days of age, FECs were predicted to be higher the older the animal. Lower FECs were predicted with increasing age in lambs older than 340 days of age.

6.3.3 Modeling variation in hematocrit and serum albumin levels

Variation in both hematocrit and serum albumin levels were modeled using general linear mixed models for repeated measures, with an autoregressive error structure fitted to the model of hematocrit and a random effects error structure applied to the model of serum albumin levels. Effects retained in the models are listed in Table 6.3. Although it was included in the models as the primary effect of interest, immune response phenotype was not significantly associated with either hematocrit or serum albumin ($p = 0.344$ and 0.570 , respectively). However, the study lambs displayed minimal variation in hematocrit and serum albumin levels during the grazing season (see Figure 6.5); the flock mean hematocrit and serum albumin levels consistently remained within reference intervals for healthy sheep (26-41 % and 29-47 g/L, respectively) (Animal Health Laboratory, 2019). Thus, although several effects were identified as significant predictors of hematocrit and serum albumin (see Table 6.3), these associations are likely of minimal clinical significance.

6.3.4 Modeling variation in postmortem GIN burdens

Species of GINs recovered from the gastrointestinal tracts of the study lambs at postmortem are listed in Table 6.4. All of the lambs were infected with at least one species of GIN, and there was considerable variation in GIN burdens between lambs. The highest burdens of immature nematodes, predominantly *Haemonchus contortus* with lower numbers of *Teladorsagia* spp., were observed in the abomasum; comparatively few adult GINs were present in the abomasum, despite no anthelmintic having been administered for 112 days prior to slaughter. Adult *Teladorsagia* spp. were more commonly recovered from the abomasum than adult *H. contortus*. *Trichostrongylus axei* adults were present in relatively low numbers. In the small intestine there were more adult than immature GINs. Adult *Trichostrongylus* spp. and *Nematodirus* spp. were present in similar numbers, though the larval burdens were primarily *Nematodirus* spp. Adult *Strongyloides* spp. were present in the small intestine of 10 study lambs; estimated *Strongyloides* spp. burdens in most of these lambs was low (< 150 worms), but one outlier had a burden of 620 *Strongyloides* spp. Low numbers of adult *Capillaria* spp. were also identified in the small intestine of 4 study lambs. *Trichuris ovis* was the only species identified in cecal samples and was present in low numbers in 10 of the 29 surviving study lambs. Cecal nematode burdens were not analyzed in the model of postmortem GIN burden due to the very low burdens.

A general linear mixed model with a random effects error structure was generated to predict total adult and immature GIN burdens in the abomasum and small intestine. Variables included in the final model are shown in Table 6.5. As in the models of GIN FEC, hematocrit, and serum albumin, immune response phenotype was not a significant predictor of postmortem GIN

burdens ($p = 0.263$). Lagged total leukocyte count ranged from 4.1 to 12.5 X 10⁹/L (mean 7.6 X 10⁹/L), and was significantly negatively associated with GIN burdens, both as a simple effect ($p = 0.032$) and in an interaction with GI segment ($p < 0.001$). These associations were primarily due to numbers of segmented neutrophils and lymphocytes. Predicted total GIN burden decreased more per unit increase in lagged total leukocyte count with small intestinal GIN burdens than with abomasal burdens. In addition to its interaction with lagged total leukocyte count, GI segment was also significantly associated with GIN burdens as a simple effect ($p < 0.001$). However, in contrast to the burdens that were observed, this simple effect predicted higher total GIN burdens in the small intestine than the abomasum (see Table 6.4). GIN maturity was also a significant predictor of postmortem GIN burdens as both a simple effect and an interaction term with lagged FEC ($p < 0.001$ and $p = 0.003$, respectively). The simple effect of GIN maturity predicted higher overall burdens of larval nematodes, consistent with the postmortem findings; predicted GIN burdens were positively associated with FECs for both larval and adult nematodes. However, GIN burden increased more rapidly per unit increase in FEC for adult nematodes than for larvae. Unlagged measurements of total leukocyte count and FEC were involved in similar significant interactions to the lagged measurements, but became non-significant when the lagged terms were introduced. Thus, the unlagged terms were omitted from the final model.

6.4 Discussion

In this study, lambs were selected for high AbMIR/low CMIR or high CMIR/low AbMIR phenotype, then naturally exposed to GIN parasites on pasture to investigate possible associations between type 1 and type 2 immune competence and GIN parasitism in sheep.

Weather patterns during the study were consistent with conditions previously described for the province of Ontario (Mederos et al., 2010), with the exception of high monthly precipitation between May and July. As discussed previously, the high rainfall early in the grazing season caused rapid growth of pasture herbage, likely contributing to low numbers of L₃s per herbage dry weight recovered at the first two samplings, when the animals had been on pasture for 26 (May) and 74 days (July). However, these low numbers of L₃s coincided with the highest mean FECs, indicating sufficient GIN challenge to lead to infection.

The four species of GINs cultured from fecal samples during the study (i.e *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus* spp., and *Nematodirus spathiger*) are common on Ontario sheep farms, but the pattern of change in FECs observed in the study lambs was more similar to reported trends for previously exposed mature ewes than for naïve lambs, in that FECs peaked in early summer and declined to low levels by late summer (Mederos et al., 2010). However, the magnitudes of the FECs observed in the study lambs were much lower than those in the commercial flock with which they were co-grazed. Metabolic stress associated with lambing and lactation in the commercial flock was likely a primary contributor to this difference, though effects of differing ages, genetic background, and immune responses cannot be ruled out. Since the highest numbers of L₃s were observed in herbage samples collected at 116 days on pasture (August), 4 weeks after the second group treatment with albendazole, anthelmintic treatment did not likely contribute to the persistently low FECs observed from 116 days on pasture until the end of the study at 196 days on pasture (November). Provision of supplemental feed from 174 days on pasture until the end of the study may have affected grazing behaviour and reduced the numbers of L₃s ingested, but does not explain the low FECs at 116 and 174 days

on pasture. The low FECs observed at this time more likely reflected a robust immune response to GIN challenge than insufficient exposure to L₃s or artificial suppression of FEC by anthelmintics. This hypothesis is supported by the fact that all of the study lambs had detectable salivary CarLA antibody by 116 days (approximately 4 months) on pasture. However, since *Nematodirus* spp. had been sporadically diagnosed in the source flock and the study lambs were not treated with an anthelmintic prior to pasture turnout, it is possible that the study lambs were not GIN-naïve. Indeed, 20 of the 30 study lambs were shedding *Nematodirus* spp. eggs in their first fecal samples, though these FECs were performed after the study lambs had been on pasture with the commercial flock for 26 days (more than a 3-week prepatent period). Thus, it is not possible to differentiate pre-existing GIN infection present at pasture turnout from infection acquired between turnout and the first sampling at 26 days on pasture. Nevertheless, pre-existing GIN infection other than *Nematodirus* spp. in the study lambs prior to turnout is considered unlikely for several reasons: first, GIN species that produce trichostrongylid-type eggs have not been diagnosed in the source flock for more than twenty-nine years; second, the study lambs had been housed exclusively indoors without access to other livestock from birth to turnout; and lastly, 25 of the 30 study lambs had salivary CarLA antibody below the detection limit (0.3 units/mL) when first sampled at 26 days on pasture. The nature of the stimulus driving detectable salivary CarLA antibody in five lambs at 26 days on pasture is unclear. These lambs may have mounted a rapid immune response to the GINs on pasture, as detectable salivary CarLA antibody has been reported in weaned lambs as soon as one month after GIN exposure (Shaw et al., 2013). Alternatively, since CarLA is known to be present on the surface of *Nematodirus spathiger* L₃s (Harrison et al., 2003), the CarLA antibody response could have been stimulated by previous exposure to *Nematodirus* spp. Regardless of the stimulus, higher CarLA antibody at 26 days on

pasture was positively associated with FEC at the subsequent sampling point at 74 days on pasture. This suggests that CarLA antibody had not yet reached protective levels in the five lambs with detectable CarLA antibody at 26 days on pasture, or that these lambs had simply ingested more L_3 s between turnout and 74 days on pasture than other study lambs.

Regardless of whether the study lambs were naïve to GINs at turnout, an anamnestic immune response would be expected in all of the animals after exposure to GINs for 8 weeks (Falzon et al., 2013; Shaw et al., 2013; Venturina et al., 2013). Thus, any effects of differential GIN exposure prior to the study on the immune response would be expected to be limited to the earliest sampling at 26 days on pasture. Given the strong bias of the immune response to GINs towards type 2 antibody-mediated responses (Paul and Zhu, 2010; Venturina et al., 2013; Karrow et al., 2014; McRae et al., 2015), lambs selected for high AbMIR/low CMIR response were hypothesized to have lower FECs and GIN burdens than lambs with a high CMIR/low AbMIR phenotype. Resilience to clinical effects of GIN parasitism on hematocrit and serum albumin levels was also predicted to be better in high AbMIR/low CMIR lambs than high CMIR/low AbMIR lambs. However, the AbMIR- and CMIR-biased phenotypes evaluated were not significantly associated with either the direct (FEC and postmortem burden) or indirect (hematocrit and serum albumin) indicators of GIN parasitism assessed. This lack of association may have been due to the method used to evaluate AbMIR in the lambs (measurement of circulating IgG following HEWL challenge). Since GINs of sheep do not migrate beyond the gastrointestinal tract, the antibody-mediated immune response to GINs is primarily driven by mucosal antibody rather than circulating immunoglobulins (Shaw et al., 2012; Shaw et al., 2013; McRae et al., 2015; Sutherland et al., 2019). Sheep with high levels of circulating

immunoglobulins do not necessarily have high mucosal IgA levels specific to GIN antigens (Shaw et al., 2012; Shaw et al., 2013). However, evidence of reduced GIN FEC has been reported in cattle with high AbMIR (Aleri et al., 2019), suggesting that a positive association between circulating AbMIR to HEWL and mucosal antibody responses may exist in other ruminants, including sheep.

Although considerably higher mean FECs were observed in the commercial flock, mean FECs in the study lambs were consistently low (< 400 epg), and no individual lamb exceeded a FEC of 900 epg at any sampling point. Higher GIN infection pressure could have been achieved by administering an artificial challenge under laboratory conditions, though this would not have been reflective of the variable levels of GIN exposure seen in pastured sheep. However, mean FECs in the study lambs were higher than or similar to FECs in the population of heifers in which high AbMIR was associated with reduced FECs, although the infecting GINs in the heifers consisted entirely of three species of *Cooperia* (Aleri et al., 2019). Conversely, the most common GIN species in the study lambs were *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus* spp., and *Nematodirus spathiger*; no *Cooperia* spp. were identified in the study lambs. Nevertheless, these results suggest that any association between AbMIR/CMIR phenotype and FEC should be independent of the magnitude of FEC. The association between AbMIR and FEC reported in cattle was weak ($r = -0.108$) and the tested population consisted of 393 heifers (Aleri et al., 2019). It is therefore likely that, if a similarly weak association was present between AbMIR and FEC in sheep, the study population of 30 lambs provided insufficient power to detect significance. The significant negative associations between salivary CarLA antibody levels measured on days 116 and 174 on pasture and the

subsequent FECs at 174 and 196 days on pasture, respectively, support the importance of mucosal antibody in the immune response to GINs, even in lambs with low FECs. Therefore, the relationship between AbMIR to HEWL challenge and mucosal AbMIR to GINs merits further characterization in sheep.

Mean hematocrit and serum albumin levels remained within reference intervals for sheep at all time points (Animal Health Laboratory, 2019); there was relatively little variation among individual animals (see Figure 6.5). This suggests that, although the level of GIN challenge was sufficient to stimulate an immune response, infection pressure was insufficient to cause a clinical impact on the study lambs. Treatment of the whole study group with albendazole at 36 and 84 days on pasture was necessary to prevent morbidity or mortality given the long intervals (6-8 weeks) between FEC monitoring, but likely contributed to the lack of clinical disease. It remains unclear whether immune response phenotype may be associated with clinical disease in more heavily parasitized sheep.

In contrast to the clinical effects of GIN parasitism, anthelmintic treatment did not appear to affect postmortem GIN burdens, likely due to the long interval (112 days) between the last group treatment with albendazole and slaughter. Indeed, postmortem GIN burdens, particularly of larval abomasal GINs, were surprisingly high given the low FECs and numbers of L₃s on pasture throughout most of the grazing season. The high numbers of larval GINs relative to adults likely reflect onset of hypobiosis, an expected occurrence in late fall in Ontario (Mederos et al., 2010). Although AbMIR/CMIR phenotype was not significantly associated with postmortem GIN burdens, higher circulating total leukocyte count was associated with lower burdens of GINs in

both the abomasum and small intestine. The precise branch of the immune system involved in this association (innate, type 1 or type 2) remains unclear.

6.5 Conclusions

This pilot study assessed the relationship between GIN parasitism in sheep and antibody- and cell-mediated immune response phenotypes assessed using a challenge protocol adapted from cattle. Although the lambs displayed variation in antibody- and cell-mediated immune responses, polarization towards antibody- or cell-mediated responses was not associated with significant differences in FEC, postmortem GIN burdens, or clinical indicators of GIN parasitism in the study lambs. However, the study lambs displayed minimal variation in clinical indicators of GIN parasitism. This suggests that the GIN burdens did not cause a biologically relevant clinical impact, and that assessment of the interaction between immune responses and clinical indicators of GIN parasitism in sheep may require a higher parasite challenge. Nonetheless, significant negative associations were identified between salivary CarLA antibody level and FEC, and between circulating total leukocyte count and postmortem GIN burdens, confirming the role of mucosal antibody, and possibly circulating type 2 and/or type 1 effector cells, in the immune response to GINs. Moreover, since a weak negative association between high AbMIR phenotype and GIN FECs has previously been reported in a large population ($n = 393$) of cattle, it is likely that the sample size evaluated in this study ($n = 30$) was too small to detect similar weak associations in sheep. Therefore, the relationship between AbMIR and CMIR phenotype and GIN parasitism in sheep warrants further investigation using larger numbers of animals.

6.6 Acknowledgements

This work was supported by the Canadian Agricultural Adaptation Program, the Ontario Agri-Food Innovation Alliance, the Ontario Ministry of Agriculture, Food, and Rural Affairs Highly Qualified Personnel program, Ontario Sheep Farmers, and a University of Guelph Undergraduate Research Assistantship. The authors wish to thank Stéphanie Bourgon, Pam Hasson, Samantha Dixon, Karen Carlton, and members of the Cánovas lab (Department of Animal Biosciences, University of Guelph) for providing laboratory and field assistance. Climate data were graciously provided by Neil Moore and Dennis Inglis. Measurement of salivary anti-CarLA IgA was coordinated by Richard Shaw of AgResearch Inc., New Zealand. Special thanks to the sheep producer who participated in the study.

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6.8 Tables

Table 6.1. Predictor variables evaluated in models of gastrointestinal nematode fecal egg count (FEC), hematocrit (HCT), serum albumin (SA), and postmortem burden (GINs) in sheep.

Variable	Definition	Model(s)
Immunity	Immune response phenotype (high cell/low antibody or high antibody/low cell)	All
AbMIR	Antibody-mediated immune response ¹	All
CMIR	Cell-mediated immune response ²	All
Age	Age of study lamb	All
Sex	Study lamb sex (female or castrated male)	All
Weight ³	Weight of study lamb	All
Ln CarLA ³	Natural logarithmic transformation of salivary antibody to carbohydrate larval antigen	All
WBC ³	Total leukocyte count	All
HCT ³	Hematocrit	FEC, GINs, SA
Ln FEC ³	Natural logarithmic transformation of fecal egg count	GINs, HCT, SA
Day	Time spent on pasture	FEC, HCT, SA
Protein ³	Total protein level	FEC, GINs, HCT
SG ³	Serum globulin level	FEC, GINs, HCT
SA ³	Serum albumin level	GINs, HCT
RBC ³	Total erythrocyte count	GINs, SA
GI segment	Source of GIN (abomasum or small intestine)	GINs
Stage	Developmental stage of GIN (adult or immature)	GINs

GI = gastrointestinal.

¹ Serum immunoglobulin G specific to hen egg white lysozyme 21 days after antigen challenge.

² Change in skin-fold thickness following intradermal challenge with *Candida albicans* antigen.

³ Evaluated as predictor effects of outcome variables measured at the same sampling time and at the subsequent time point (lagged effects).

Table 6.2. Predictors in a general linear mixed model of gastrointestinal nematode fecal egg count in Rideau-Dorset cross lambs with high cell/low antibody (n = 15) or high antibody/low cell (n = 15) immune responses.

Predictor variable	p-value	Direction of association	NDF	DDF	F-value	Proportion of variance explained (%)¹
Immunity	0.257	NA	1	26	1.34	4.90
Day	< 0.001	Negative	2	53	35.05	56.95
Age	0.036	Positive	1	26	4.91	15.88
Lagged Ln CarLA	0.494	NA	1	53	0.47	0.88
Lagged Ln CarLA*day	0.027	Variable ²	2	53	3.86	12.71
Age*age	0.031	Negative	1	26	5.21	16.69

Direction of association is given for significant effects only. One high antibody/low cell phenotype lamb was euthanized at 95 days on pasture; data obtained from this animal prior to death were included in analyses. NDF = numerator degrees of freedom; DDF = denominator degrees of freedom; NA = not applicable; Ln = natural logarithm; CarLA = carbohydrate larval antigen.

¹ Estimated using formulas described by Dohoo et al. (2014), page 330.

² Direction of association varies at different levels of a categorical variable.

Table 6.3. Predictors in general linear mixed models of hematocrit (HCT) and serum albumin (SA) in Rideau-Dorset cross lambs with high cell/low antibody (n = 15) or high antibody/low cell (n = 15) immune responses.

Model	Predictor variable	p-value	Direction of association	NDF	DDF	F-value	Proportion of variance explained (%)¹
HCT	Immunity	0.344	NA	1	27	0.93	3.33
	Day	< 0.001	Variable ²	2	50	37.81	60.20
	Ln CarLA	0.574	NA	1	50	0.32	0.64
	Sex	0.118	NA	1	27	2.61	8.81
	SA	0.021	Negative	1	50	5.72	10.27
	Day*sex	0.003	Variable ²	2	50	6.48	20.58
	Ln CarLA*sex	0.009	See note ³	1	50	7.43	12.94
	SA*SA	0.018	Positive	1	50	5.95	10.63
SA	Immunity	0.570	NA	1	28	0.33	1.16
	Lagged Ln CarLA	0.008	Positive	1	55	7.49	11.99
	HCT	< 0.001	Positive	1	55	37.50	40.54
	Ln FEC	0.017	Negative	1	55	6.09	9.97

Direction of association is given for significant effects only. One high antibody/low cell phenotype lamb was euthanized at 95 days on pasture; data obtained from this animal prior to death were included in analyses. NDF = numerator degrees of freedom; DDF = denominator degrees of freedom; NA = not applicable; Ln = natural logarithm; CarLA = carbohydrate larval antigen; FEC = gastrointestinal nematode fecal egg count.

¹ Estimated using formulas described by Dohoo et al. (2014), page 330.

² Direction of association varies at different levels of a categorical variable.

³ Higher salivary antibody to CarLA was associated with lower hematocrit in ewe lambs and higher hematocrit in wethers.

Table 6.4. Postmortem total and species-specific burdens of adult and immature gastrointestinal nematodes after 196 days on pasture in Rideau-Dorset cross lambs with high cell/low antibody (n = 15) or high antibody/low cell (n = 14) immune responses.*

Region	Stage	Species	Mean	Median	Range
Abomasum	Adult	All	55	33	0-248
		<i>Haemonchus contortus</i>	18	0	0-182
		<i>Teladorsagia</i> spp. ¹	29	16.5	0-159
		<i>Trichostrongylus axei</i>	8	0	0-99
	Immature	All	4849	3465	446-18,117
		<i>Haemonchus contortus</i>	4676	3417	314-17,579
Small intestine	Adult	All	750	462	0-3432
		<i>Trichostrongylus</i> spp. ²	384	281	0-1860
		<i>Nematodirus</i> spp.	326	83	0-3432
		<i>Capillaria</i> spp.	7	0	0-66
	Immature	<i>Strongyloides</i> spp.	33	0	0-620
		All	337	198	0-1304
		<i>Trichostrongylus</i> spp. ²	1	0	0-17
		<i>Nematodirus</i> spp.	336	198	0-1304
Cecum	Adult	<i>Trichuris ovis</i>	6	0	0-33
	Immature	<i>Trichuris ovis</i> ³	1	0	0-17

*Immune response phenotype was not associated with nematode burden. Therefore, data from both phenotypes were combined.

¹ Includes *Teladorsagia circumcincta* and *Ostertagia trifurcata*, as females and larvae of these species cannot be morphologically distinguished.

² Includes *Trichostrongylus colubriformis* and *Trichostrongylus virtrinus*, as females and larvae of these species cannot be morphologically distinguished. No *Trichostrongylus axei* were collected from the small intestine.

³ Immature *Trichuris ovis* were collected from the cecum of two study lambs.

Table 6.5. Predictors in a general linear mixed model of postmortem gastrointestinal nematode (GIN) burden in Rideau-Dorset cross lambs with high cell/low antibody (n = 15) or high antibody/low cell (n = 14) immune responses.

Predictor variable	p-value	Direction of association	NDF	DDF	F-value	Proportion of variance explained (%)¹
Immunity	0.263	NA	1	25	1.31	4.98
GI segment	< 0.001	See note ²	1	54	38.01	41.31
Stage	< 0.001	See note ³	1	54	140.79	72.28
Lagged Ln FEC	0.079	NA	1	54	3.22	5.63
Lagged WBC	0.032	Negative	1	54	4.84	8.23
Lagged Ln FEC*stage	0.003	Positive	1	54	9.88	15.47
Lagged WBC*GI segment	< 0.001	Negative	1	54	15.23	22.00

Direction of association is given for significant effects only. NDF = numerator degrees of freedom; DDF = denominator degrees of freedom; NA = not applicable; GI = gastrointestinal; Ln = natural logarithm; FEC = gastrointestinal nematode fecal egg count; WBC = total leukocyte count.

¹ Estimated using formulas described by Dohoo et al. (2014), page 330.

² Predicted GIN burdens were higher in the small intestine than the abomasum.

³ Predicted larval GIN burdens were higher than predicted adult GIN burdens.

6.9 Figures

Figure 6.1. Antigen challenge protocol used to evaluate cell- and antibody-mediated immune responses in 70-day-old Rideau-Dorset cross lambs (n = 210). Modified from Heriazon et al. (2009) and Stryker et al. (2013). CAA = *Candida albicans* antigen; HEWL = hen egg white lysozyme.

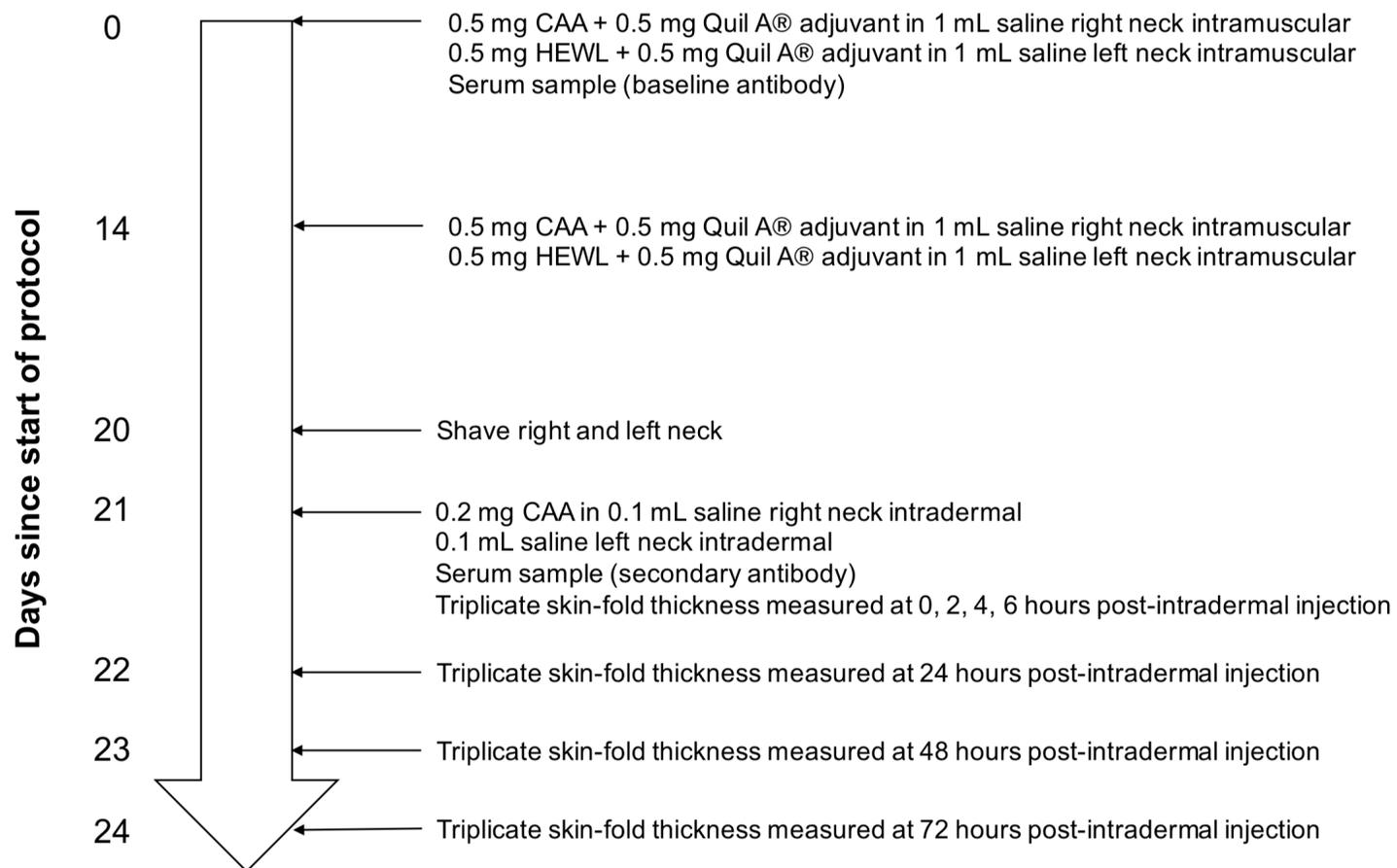


Figure 6.2. Mean change in neck skin-fold thickness following intradermal injection of 0.2 mg *Candida albicans* antigen (CAA) dissolved in 0.1 mL sterile saline in previously sensitized Rideau-Dorset cross lambs (n = 210). Change in skin thickness at a distant control site injected with 0.1 mL sterile saline is provided for comparison. Dotted lines indicate 95 % confidence interval for the respective means.

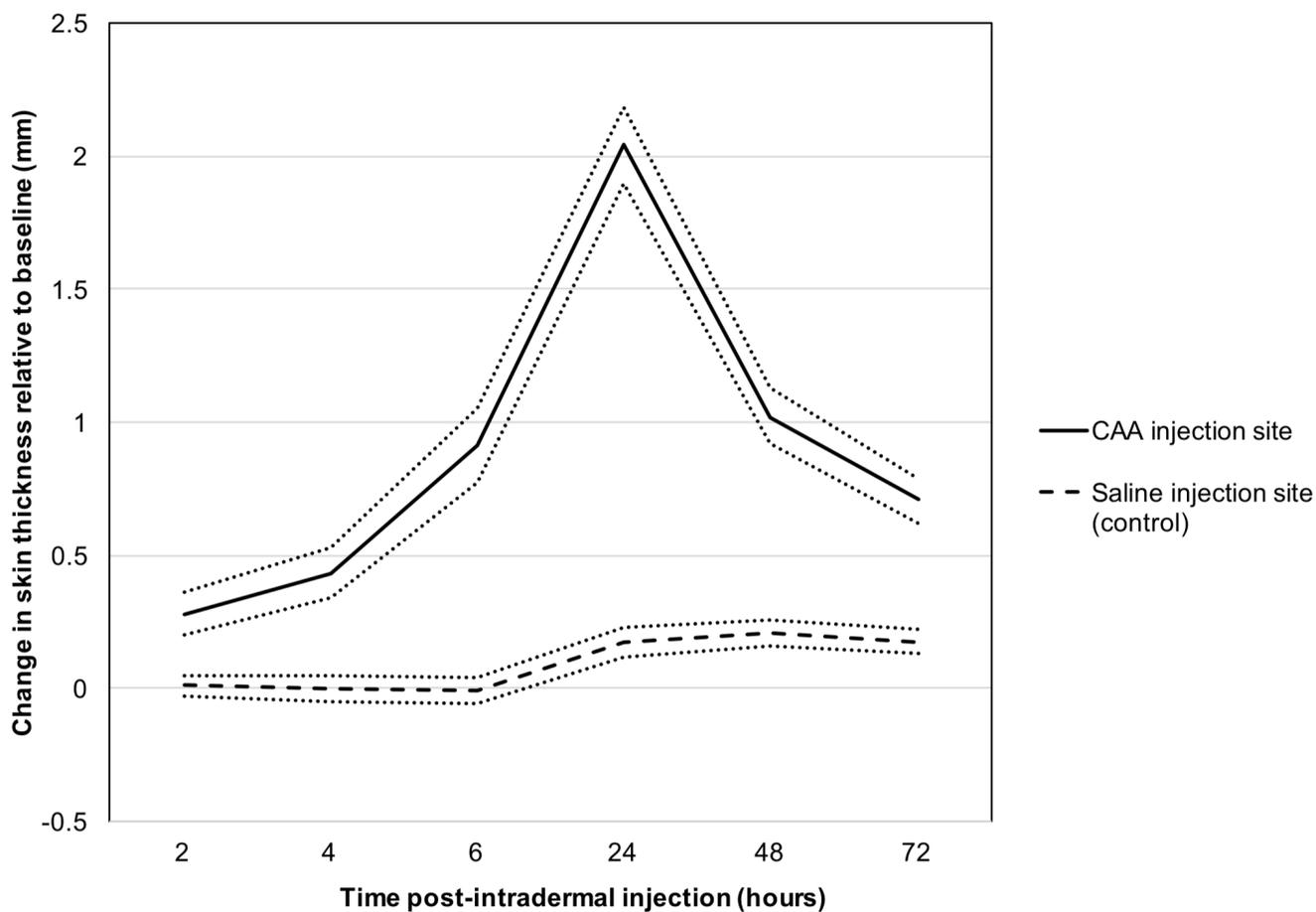


Figure 6.3. Phenotypic residuals for antibody- and cell-mediated immune responses in Rideau-Dorset cross lambs selected as high antibody/low cell responders or high cell/low antibody responders. The 15 lambs with the highest residuals for antibody-mediated response, assessed by challenge with hen egg white lysozyme, and low to average cell-mediated response, assessed by challenge with *Candida albicans* antigen, were selected as high antibody/low cell responders (right lower quadrant). The 15 lambs with the highest residuals for cell-mediated response and low to average antibody-mediated response were selected as high cell/low antibody responders (left upper quadrant). Residuals for unselected lambs (n = 180) are not shown.

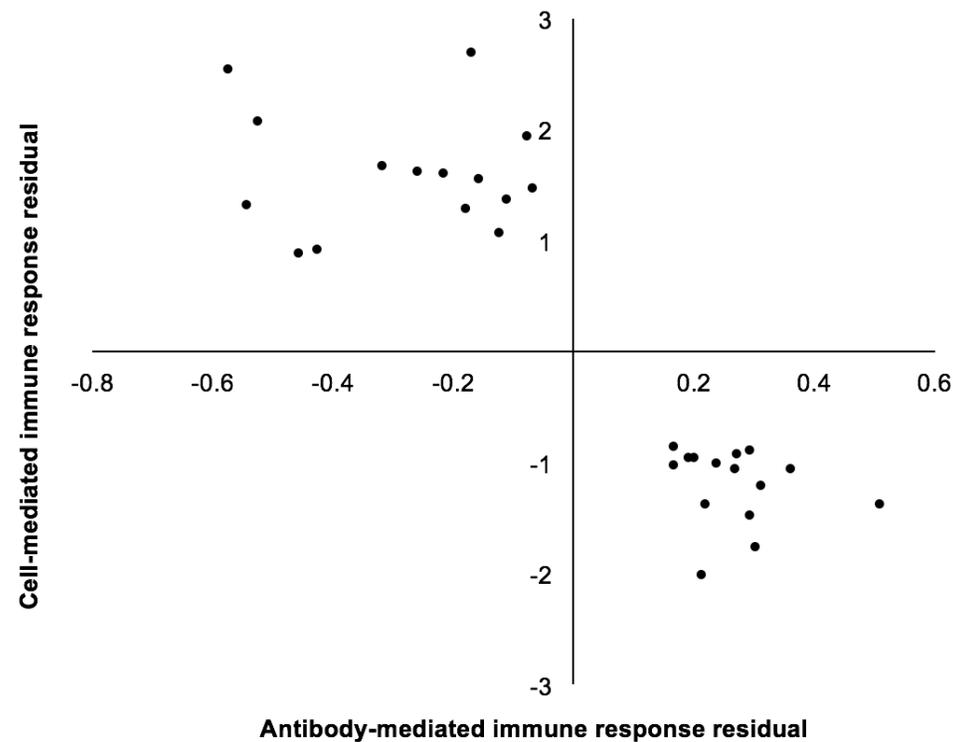


Figure 6.4. Mean total and species-specific gastrointestinal nematode (GIN) fecal egg counts (FEC) in Rideau-Dorset cross lambs with high antibody/low cell (n = 15) or high cell/low antibody (n = 15) immune responses, May-November 2017. CMIR = cell-mediated immune response; AbMIR = antibody-mediated immune response. Error bars indicate 95 % confidence intervals for mean FEC in the two immune response phenotypes; narrow bars indicate confidence intervals for high AbMIR/low CMIR lambs, and wide bars indicate confidence intervals for high CMIR/low AbMIR lambs. One high antibody/low cell phenotype lamb was euthanized at 95 days on pasture; data obtained from this animal prior to death were included in analyses.

* No study lambs exceeded the minimum FEC for fecal culture in October 2017 (200 eggs per gram). Hence, species proportions and species-specific FEC could not be determined.

** Species-specific FECs are for both immune response phenotype groups combined.

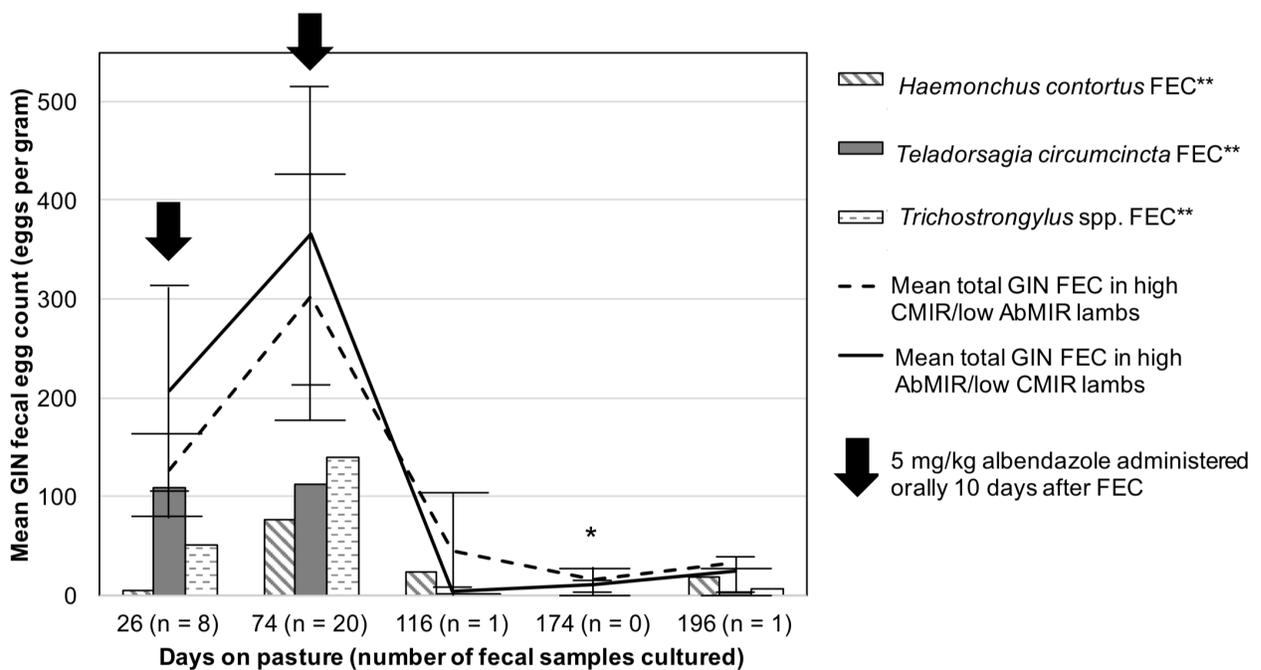
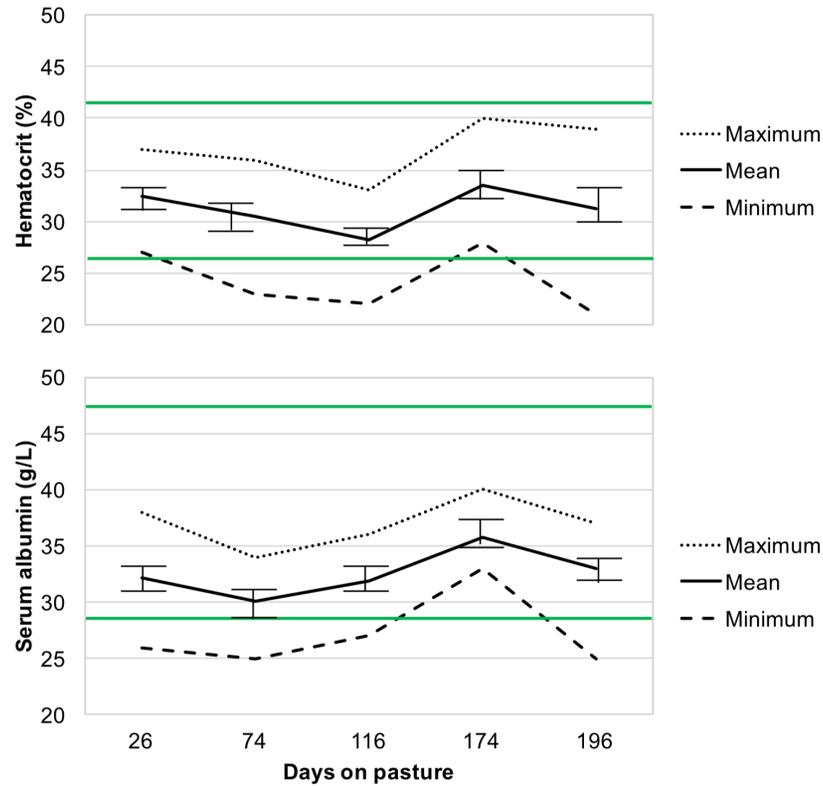


Figure 6.5. Mean, minimum, and maximum values of hematocrit and serum albumin levels in Rideau-Dorset cross lambs with high antibody/low cell (n = 15) or high cell/low antibody (n = 15) immune responses, May-November 2017. Error bars indicate 95 % confidence interval for means. Green lines delineate reference intervals for healthy sheep (Animal Health Laboratory, 2019). Immune response phenotype was not significantly associated with hematocrit or serum albumin level; therefore, data from both phenotypes were combined. One high antibody/low cell phenotype lamb was euthanized at 95 days on pasture; data obtained from this animal prior to death were included in analyses.



CHAPTER SEVEN:

DISCUSSION AND CONCLUSIONS

Successful control of parasitism by gastrointestinal nematodes (GINs) is one of the greatest challenges faced by sheep producers globally, particularly given the rapid rise of widespread anthelmintic resistance (Falzon et al., 2013; Lamb et al., 2017). Reports of decreased efficacy and resistance to novel anthelmintics arising within a few years of product release or approval (Sales and Love, 2016; Lamb et al., 2017) underscore the importance of combining anthelmintic treatment with complementary management strategies, including genetic selection of sheep for improved immune response to GINs. Extensive research has begun to clarify the complex immune responses underlying individual variation in susceptibility to GIN infection, with the goal of leveraging the immune response to reduce reliance on anthelmintics. However, much of this work has been performed in countries with humid warm temperate climates allowing year-round exposure to GINs on pasture (World Maps of Köppen-Geiger Climate Classification, 2019). Comparably, little is reported about development of immunity to GINs in sheep under the shorter grazing seasons and long, cold winters observed in boreal climates, such as in Ontario, Canada. Thus, the primary goals of this thesis were to characterize the immune response of sheep to GINs under Ontario grazing conditions, and to assess possible selection criteria for use in identification of Ontario sheep with superior immune responses to GIN infection.

A wide variety of mechanisms have been implicated in the immune response of sheep to GINs; however, these can vary in importance for different GIN species depending on where

development from larva to adult occurs (i.e. lumen versus mucosa) and on GIN feeding behaviour. For example, circulating immunoglobulin (Ig) G is more strongly associated with immunity to *Haemonchus contortus* than other GINs, since these antibodies are ingested by *H. contortus* as it feeds on blood, but not by other species that feed on epithelial cells (Knox et al., 2003; Ekoja and Smith, 2010). Since mixed infection with several GIN species is typical in grazing sheep (Mederos et al., 2010), correlation of an immune response mechanism with burdens of a given GIN species requires knowledge of the species composition infecting the host. In live sheep, this has traditionally been performed by culturing eggs shed in feces to third-stage larvae (L₃s) and performing morphologic speciation (Ministry of Agriculture, Fisheries, and Food, 1984); this method is time-consuming, requires specialized expertise, and is error-prone. Thus, speciation of GIN eggs shed in feces has been largely underutilized in studies of the immune response to GINs.

Deep amplicon sequencing of L₃s cultured from feces was recently validated in both cattle and sheep (Avramenko et al., 2015; Avramenko et al., 2017; Redman et al., submitted), and offers numerous advantages to morphologic speciation, including higher throughput capacity and reduced risk of misclassification. Results of deep amplicon sequencing have not been previously compared with species proportions determined by morphologic speciation. Moreover, culture from egg to L₃ requires at least 8 days (Ministry of Agriculture, Fisheries, and Food, 1984), and mortality of larvae during this interval is high. Although culture to first-stage larvae (L₁s) requires only two days (Redman et al., submitted) and should theoretically mitigate the risk of differential mortality rates in different GIN species during culture, the proportion of recovered larvae relative to eggs cultured has not been compared between L₁ and L₃ culture. Therefore, the

goal of Chapter Two was to compare GIN species composition and proportion of recovered larvae relative to eggs cultured using morphologic speciation of L₃s and deep amplicon sequencing of L₁s. Significant differences between species proportions were identified for only one pooled sheep fecal sample that was later determined to have been refrigerated between collection and culture. In all samples, a significantly higher proportion of GIN eggs were successfully cultured to larvae when cultured to L₁s than L₃s.

The results of the study described in Chapter Two indicate that L₁ deep amplicon sequencing yields comparable results to L₃ morphologic speciation, provided samples are not chilled prior to culture, and that the shorter culture duration to L₁s is associated with markedly improved larval recovery proportion. Although the pooled fecal samples originated from a small number of farms (n = 5), the GIN species proportions were similar on all but one farm (Farm 4), and the most prevalent species on all of the sheep farms (*H. contortus*, *Teladorsagia colubriformis* and *Trichostrongylus* spp.) were consistent with those reported by Mederos et al., 2010. Another advantage of L₁ deep amplicon sequencing is that only 6 g of feces is required for L₁ culture, which may permit speciation at the individual animal level. Comparison of the two speciation methods in individual sheep may determine whether or not deep amplicon sequencing of L₁s will allow for more detailed investigation of the association between individual variability in immune responses and GIN species composition.

Although clinical disease due to GIN parasitism in sheep is easily recognized, loss of productivity is less readily apparent and often has a greater financial impact on producers. A recent meta-analysis confirmed that GIN infection is associated with reduced lamb growth,

reduced milk production in ewes, and reduced wool production, but the studies used in this meta-analysis reported conflicting results and often failed to reach clinical significance (Mavrot et al, 2015). Reproductive performance, another important indicator of productivity, was not assessed, likely due to the small number of studies reporting associations between GIN parasitism and reproduction in sheep. Moreover, as with research into the immune response to GINs, few of these studies were conducted under North American climate and management conditions. In order to address these knowledge gaps, Chapter Three evaluated the relationship between GIN parasitism, growth, and reproductive performance in a group of replacement ewe lambs in central Ontario, Canada. A commercial farm raising Rideau-Dorset cross sheep was recruited, and a study group of 140 nursing ewe lambs was enrolled and followed for their first two grazing seasons, including their first lambing and lactation. Weights were recorded every 6-8 weeks during both grazing seasons (May to November in 2016 and 2017), and at mid-gestation in March 2017. Fecal samples were also collected at these same time points to assess GIN fecal egg counts (FECs). Complete lambing records and offspring weaning weights were obtained to assess reproductive performance of the study ewe lambs.

No significant associations were identified between FEC and weight change, or between FEC measured before lambing and litter size. The significant positive association between FEC at lambing and litter size was consistent with previous reports of higher periparturient egg rise due to higher metabolic stress in ewes bearing larger litters (Kerr et al., 2017). However, an unexpected significant negative linear and a positive quadratic relationship was identified between FECs in late lactation and offspring weaning weights. The positive quadratic effect predominated when FECs exceeded 361 eggs per gram (epg), counter to the conventional theory

that increasing GIN burden is associated with a metabolic cost to the host and diverts energy and nutrients from productivity (Mavrot et al., 2015). Several elements of the study design could have affected the observed associations between FECs and productivity. First, this study was performed in one flock, which may not be representative of the impact of GINs on production in other flocks raising other breeds of sheep. However, farm selection criteria included management practices and breeds representative of Ontario flocks (Kennedy, 2012); thus, the results are likely representative of conditions on other farms in Ontario raising Rideau or Rideau-crossbred sheep. Nonetheless, the Rideau breed, particularly when crossed with Polled Dorset sheep, are recognized for their superior maternal traits, including prolificacy and milk yield (Kennedy, 2012; Oklahoma State University, 2019). It is possible that these traits reflect an ability to efficiently partition energy reserves into productivity under pasture GIN challenge. Thus, evaluation of the relationship between GINs and productivity in multiple Ontario flocks raising other common breeds and with different management practices is warranted. Anthelmintic treatment may also have affected the relationship between GIN parasitism and productivity. Any study ewe lamb with a FEC greater than 500 epg was selectively treated with anthelmintic to prevent morbidity or mortality due to GINs during the study. This treatment may have minimized the clinical effects of GIN parasitism and any relationship between parasitism and productivity, but was necessary to preserve animal welfare given the infrequent monitoring of FEC (every 6-8 weeks). More frequent monitoring of FECs in future studies may allow a less conservative treatment cut-off to be used such that animals can be safely exposed to higher GIN infection pressure. However, this approach may prove difficult to apply in field studies on commercial farms, as intentional exposure to higher GIN infection pressure is unlikely to be favourably received by sheep producers.

Multiple clinical indicators of GIN parasitism have been investigated as means of genetic selection of sheep with a strong immune response to GINs, including FECs (Douch et al., 1996; Woolaston and Baker, 1996; Saddiqi et al., 2012), Faffa Malan Chart (FAMACHA©) scores (Saddiqi et al., 2012; Pereira et al., 2016), and fecal breech soiling (dag) scores (Douch et al., 1996; Pickering et al., 2012; Williams and Palmer, 2012; Pickering et al., 2015). However, all of these are indirect indicators of GIN immunity, and have significant limitations that render them more useful for selective treatment than for genetic selection. In particular, FAMACHA© and dag scores can be elevated by disease conditions unrelated to GINs, and the significant variability in FECs requires counts to be performed repeatedly to accurately assess GIN immunity (Douch et al., 1996; Woolaston and Baker, 1996; Saddiqi et al., 2012). Levels of circulating IgE and IgG specific to GINs are a more direct means of evaluating the immune response and are heritable, but have been associated with impaired productivity, suspected to be due to heightened gastrointestinal inflammation (Douch et al., 1996; Shaw et al., 1999; Williams, 2011). Conversely, selection of sheep with high salivary IgA specific to GIN antigens, such as carbohydrate larval antigen (CarLA), is not associated with impaired productivity and has proven promising as a tool for genetic selection in both New Zealand (Shaw et al., 2012; Shaw et al., 2013), and the United Kingdom (Fairlie-Clarke et al., 2019). Assessment of salivary CarLA IgA (sCarLA) levels and their potential utility in genetic selection for GIN resistance has not been previously evaluated in boreal climates with cold snowy winters, as are observed in Ontario and much of North America (World Maps of Köppen-Geiger Climate Classification, 2019). As such, it was unclear what effect the prolonged lack of exposure to L₃s for several months during winter may have on development and maintenance of this response. Therefore, the goals of Chapter

Four were to characterize sCarLA levels in Ontario sheep, and to evaluate the association between sCarLA levels and FEC under Ontario grazing conditions.

Development of an sCarLA response was monitored from 2016 to 2017 in the same group of 140 Rideau-Dorset cross replacement ewe lambs enrolled in the longitudinal study of productivity and GIN parasitism described in Chapter Three. *In vivo* GIN burdens were monitored via FECs as described previously, and sCarLA levels were measured at the beginning (May), middle (August), and end (October) of each grazing season, and at mid-gestation in March 2017. In addition, clinical indicators of GIN infection (hematocrit, serum albumin level, and serum globulin level) were determined with fecal sampling at 6- to 8-week intervals. Most of the flock (68.3 %) developed detectable sCarLA levels by the end of their first grazing season, and all but one animal had detectable sCarLA levels by the middle of the second grazing season. Mean sCarLA levels in the flock declined between October 2016 (1.2 units/mL) and May 2017 (0.5 units/mL), corresponding with the interruption in L₃ exposure during winter, but increased sharply to much higher levels by August 2017 (13.9 units/mL). Spearman's rank correlation coefficients between the sCarLA measurements at all time points were positive and almost all were significant ($p < 0.05$); the two non-significant comparisons approached significance ($p = 0.081$ and $p = 0.060$). A significant ($p < 0.0001$) negative association was identified between sCarLA levels and FECs at all time points. No significant associations were identified between sCarLA levels and the assessed clinical indicators of GIN parasitism. However, the flock displayed little variation in these parameters, and flock mean hematocrit, serum albumin level, and serum globulin level remained within the reference intervals for healthy sheep throughout most of the study (Animal Health Laboratory, 2019).

The results of the study in Chapter Four highlight the high potential of using the sCarLA level expressed in an individual animal as a tool for genetic selection of Ontario sheep for superior immune responses to GINs, and thus lower risk of disease due to GIN and lower pasture contamination with GIN eggs due to lower FECs. The end of a lamb's first grazing season, prior to breeding, appears to be a suitable time in Ontario to assess sCarLA levels. sCarLA levels could then be used by producers as a criterion for selection of breeding stock. This could offer a dual benefit of reduced parasitism in replacement ewe lambs, and reduced pasture contamination to infect their offspring and other sheep without the trait. However, as discussed previously, the results are derived from a single flock and it remains unclear how well these results might apply to other breeds or management systems in Canada. Therefore, evaluation of the relationship between sCarLA and GIN parasitism in a larger number of Ontario flocks raising different breeds is a logical next step.

Although sCarLA is known to be moderately heritable (Shaw et al., 2012), sCarLA levels were only described in replacement ewe lambs for a single generation in the present study and were not used in a genetic selection program. As a follow up, pedigree analysis will be performed on the flock of replacement ewe lambs studied in Chapter Four to address the possible influence of pedigree on sCarLA levels, but prospective data are needed to evaluate the longitudinal effect of selection based on high sCarLA levels. Thus, investigation of the relationships between sCarLA and FECs in successive generations of Ontario sheep is needed to provide more conclusive evidence of the potential utility of the sCarLA phenotype for genetic selection. This analysis could also be combined with genome-wide association studies to identify genetic polymorphisms

that may be associated with variation in sCarLA levels; these could subsequently then be used for genomic, as opposed to phenotypic, selection.

Currently, a major barrier to use of sCarLA testing on sheep farms in Ontario and elsewhere in North America is the necessity to ship frozen saliva samples to New Zealand for analysis, and the associated cost. Should sCarLA values prove a useful method of selection in larger studies conducted over multiple generations, licensing agreements that permit the sCarLA assay to be offered in North America may mitigate these costs.

The decline in sCarLA levels while the replacement ewe lambs were not exposed to L₃s between the end of their first grazing season (2016) and the beginning of their second (2017) is consistent with previous reports that sCarLA levels decline quickly when GIN exposure is not continuous (Harrison et al., 2008; Shaw et al., 2012). The markedly higher sCarLA levels in 2017 partly reflect the increased L₃ challenge compared with that recorded in 2016. However, mean sCarLA levels in the lambs selected from the University-owned flock for immune responses and co-grazed with the commercial flock in 2017 were approximately half the sCarLA levels in the replacement ewe lambs, which suggests that increased L₃ challenge, alone, does not explain the magnitude of the difference in sCarLA levels. These observations suggest that an anamnestic immune response also contributed to this observation. Although involvement of invariant natural killer T cells in response to glycolipids such as CarLA may allow for some immunologic memory, antibody responses to non-protein antigens were traditionally believed to lack robust memory (Harrison et al., 2008; Shaw et al., 2012; Avci et al., 2013; Jaurigue and Seeberger, 2017). Evidence of immunologic memory to CarLA has important implications for vaccine

development, as the currently available GIN vaccines, or ones being developed, typically require frequent booster doses to maintain immunity (Nisbet et al., 2013; Bassetto and Amarante, 2015; Nisbet et al., 2016). Since sheep are naturally exposed to CarLA by ingesting L₃s on pasture, it may be possible to select animals with higher sCarLA levels, then supplement the immune response with a CarLA vaccine during intervals when sheep are not exposed to GINs. Therefore, investigation of the utility of CarLA as a vaccine antigen is warranted.

Whilst chronic stress is typically immunosuppressive, glucocorticoids released in the response to acute stress can have variable immunostimulatory effects, depending on individual stress responsiveness (You et al., 2008b; Dhabhar 2009; Demas et al., 2011). Previous research has shown that sheep with high acute stress responsiveness (HSR) or low stress responsiveness (LSR) have heightened cell-mediated immune responses (CMIRs) and lower primary antibody-mediated immune responses (AbMIRs) than medium stress-responsive (MSR) sheep, but the anamnestic AbMIR was similar in all three phenotypes (You et al., 2008b). Since sheep are frequently exposed to short-term stress through routine handling and management practices (Hemsworth et al., 2018), variation in stress responsiveness may have an effect on their immune response to a variety of pathogens, including GINs, but this relationship has not previously been investigated. Chapter Five described a pilot study in which 15 HSR and 15 MSR lambs were exposed to GINs by co-grazing with the flock of replacement ewe lambs described previously, in order to evaluate the effect of acute stress responsiveness on GIN parasitism. The stress responsiveness phenotype was assessed using an intravenous *Escherichia coli* lipopolysaccharide challenge that was previously optimized (You et al., 2008a; You et al., 2008b). No LSR lambs were selected for this study because reported immune responses in LSR lambs were similar to

the HSR lambs (You et al., 2008a), and because logistical constraints allowed for only 30 animals to be included in the study. The selected lambs grazed from May to November 2017 and were euthanized in November to allow collection of postmortem GIN burdens. In the end, the stress response phenotype was not found to be significantly associated with FEC, postmortem GIN burden, or clinical indicators of GIN parasitism (hematocrit and serum albumin levels), though the two stress response groups displayed minimal variation in clinical parameters during the study.

These HSR and MSR study lambs were managed as a group and exposed to the same GIN challenge on pasture. Thus, variation in GIN parasitism in the HSR and MSR lambs most likely reflected variation in GIN immunity rather than GIN exposure. A secondary anamnestic mucosal AbMIR was considered the primary contributor to this variation for two reasons. First, the immune response to GINs is known to be strongly biased towards an AbMIR, with the CMIR playing a smaller role (Venturina et al., 2013; McRae et al., 2015). Second, although it is unclear precisely how long lambs must be exposed to GINs to develop a primary mucosal AbMIR to GINs, the interval between GIN exposure and peak IgA production is approximately 9 days in previously exposed sheep mounting a secondary AbMIR (Halliday et al., 2007). Since the study lambs were exposed to GINs on pasture for several months during the study, and assessment of GIN parasitism was performed at 6- to 8-week intervals, a secondary AbMIR could have developed by as early as the second measurement time point in July 2016. Therefore, the lack of an association between the acute stress response phenotype and GIN parasitism may have been due to the similar secondary AbMIR in MSR and HSR lambs (You et al., 2008b); more frequent assessment of GIN parasitism at the start of the study, shortly after the lambs were turned out on

pasture and exposed to GINs for example, would have been necessary to investigate the possible effect of differential primary AbMIRs on GIN parasitism.

Any MSR or HSR study lambs with a FEC greater than 500 epg were treated with an anthelmintic to prevent morbidity or mortality, as was performed in the commercial flock described previously. In order to minimize disruption to routine management protocols, the commercial farm on which these study lambs grazed was permitted to use the same anthelmintic in the study lambs as was used in the rest of the flock. However, the anthelmintic used in 2016 was levamisole, which has immunostimulatory properties not shared by other classes of anthelmintics (Plumb, 2011). Levamisole was only administered once during the study in August 2016, and equal numbers of HSR and MSR lambs were treated ($n = 7$ of each phenotype). Nonetheless, administration of levamisole may have stimulated the immune response in lambs with weaker immunity and biased the results towards the null hypothesis (Cabaj et al., 1995; Stelletta et al., 2004). Therefore, use of levamisole as a treatment should be avoided in studies of GIN immunity, though clarification of the possible effect of levamisole on the immune response to GINs is warranted. Alternatively, assessment of GIN immunity without the requirement for anthelmintics could be performed under laboratory conditions in lambs administered a controlled GIN challenge infection. However, such conditions are not representative of those encountered on most sheep farms.

The limited variability in clinical indicators of GIN parasitism in the HSR and MSR lambs was likely to be due to a combination of low GIN burdens and anthelmintic control. Comparison of hematocrit and serum albumin levels in the different stress response groups may have yielded

different results under more intense GIN challenge. However, as discussed for Chapter Three, more frequent monitoring of FECs would be necessary to allow safe exposure to higher GIN challenge and a higher anthelmintic treatment cutoff. The timing of assessment of postmortem GIN burdens may have also obscured a difference between the stress response groups, particularly in regards to adult GIN burden, as this assessment was performed in late fall (November). At this time of year in Ontario, most newly ingested larvae undergo hypobiosis and low burdens of adult GINs are expected (Mederos et al., 2010). Since a lower GIN burden was expected in both phenotypic groups, collection of worm burdens at the end of the grazing season could have biased the results towards the null hypothesis (no difference between HSR and MSR lambs). The timing of euthanasia was a compromise to provide sufficient power to compare FECs and clinical indicators between the two phenotype groups for the remainder of the grazing season, whilst still allowing postmortem GIN burdens to be assessed. However, it remains unclear whether GIN burdens differed between the HSR and MSR lambs when peak burdens of adult GINs are expected, for example at mid-summer. Thus, postmortem GIN burdens should be compared in lambs with different stress response phenotypes at other time points during the annual GIN infection cycle.

As discussed previously, immune responses to GINs are typically predominated by antibody-mediated mechanisms. However, since the cytokines that promote AbMIRs or CMIRs are generally mutually inhibitory (Venturina et al., 2013), genetic selection that favours one response over the other may lead to increased susceptibility to other pathogens. A method to assess both CMIRs and AbMIRs is patented in cattle and can be used to identify animals with high overall immune responses (Heriazon et al., 2009; Thompson-Crispi et al., 2013), and these cattle have

lower incidences of multiple common diseases (Thompson-Crispi et al., 2013; Cartwright et al., 2017). Moreover, a high AbMIR was recently associated with reduced GIN FECs in cattle, though this association did not hold for animals with high overall immune response (Aleri et al., 2019). Although genetic selection for high immune responses has proven promising in cattle, this method had not been evaluated in sheep. Therefore, in Chapter Six, AbMIRs were assessed using a method reported in sheep by Stryker et al. (2013), and the method for assessing the CMIR in cattle was adapted for use in sheep. Groups of sheep with either a high AbMIR/low CMIR or a high CMIR/low AbMIR phenotype were exposed to GINs by grazing on pasture with the flock of commercial replacement ewe lambs described previously. Sheep with opposite AbMIR and CMIR phenotypes were selected to evaluate the relationship between systemic AbMIR or CMIR bias and GIN parasitism. These lambs were grazed with the commercial flock for approximately seven months in 2017 and were euthanized for assessment of postmortem GIN burden at the end of the grazing season in November.

Although the AbMIR is considered more important than a CMIR in the immune response to GINs in sheep, no significant associations were identified between high AbMIR/low CMIR or high CMIR/low AbMIR phenotypes and FECs, clinical indicators of GIN parasitism (hematocrit and serum albumin levels), and postmortem GIN burdens. However, the association between FEC and AbMIR reported in cattle was weak (Aleri et al., 2019), and the bovine test population was much larger ($n = 393$) than the current pilot study of 30 lambs. If the association between AbMIR/CMIR phenotype and GIN parasitism is similarly weak in sheep, a larger number of sheep might be required to provide sufficient power to detect an association. Moreover, the selected lambs in Chapter Six were either high AbMIR/low CMIR or high CMIR/low AbMIR, in

order to assess the relative contributions of each branch of the immune system to GIN parasitism. As discussed previously, selection of animals with both high AbMIR and high CMIR appears to confer optimal health in cattle (Thompson-Crispi et al., 2013; Cartwright et al., 2017). Since high AbMIR/high CMIR phenotype and low AbMIR/low CMIR phenotype lambs were not included in the study, it remains possible that high overall immune competence may also be associated with improved health in sheep. This possibility merits further characterization of health outcomes in lambs with a wider variety of AbMIR/CMIR phenotypes.

Similar to the stress phenotype lambs described in Chapter Five, there was limited variation in clinical indicators of GIN parasitism amongst the high AbMIR/low CMIR and high CMIR/low AbMIR lambs. Unlike the stress phenotype lambs, anthelmintic treatment was administered to all lambs in the AbMIR/CMIR study when individuals exceeded a FEC of 500 epg. This was elected to reduce the risk of bias introduced by differential treatment, but likely kept GIN burdens below clinically significant levels. Nonetheless, all of the study lambs developed detectable sCarLA levels during the grazing season, indicating that GIN challenge was sufficient to stimulate a mucosal immune response. However, antibody levels at mucosal surfaces are not necessarily reflected by circulating antibody levels (Shaw et al., 2012; Shaw et al., 2013). Therefore, it remains unclear whether the systemic AbMIR phenotype assessed by the challenge protocol used in Chapter Six also reflects gastrointestinal mucosal AbMIR. Investigation of the relationship between systemic AbMIR/CMIR phenotype and mucosal antibody levels in sheep exposed to GIN challenge may help clarify this uncertainty.

The studies in this thesis present much-needed information regarding the immune response to GIN parasitism in sheep raised in a boreal climate with annual interruption of GIN exposure for several months during winter. In particular, Chapter Four reports the first evaluation of salivary CarLA IgA levels in sheep raised in North America. Additionally, the data reported in Chapters Five and Six are currently being used to correlate variation in the gastrointestinal transcriptome with variation in GIN parasitism. Collectively, the results will hopefully be used to direct validation of CarLA antibody and phenotyping of antibody, cell, and stress responses in different breeds of sheep raised in a wider geographic area. Ultimately, the goal will be to support integrating selection for GIN immunity into current genetic selection programs, offering sheep producers a valuable complementary strategy to manage GIN infections whilst preserving the efficacy of available anthelmintics.

7.1 Key findings

- Deep amplicon sequencing of L₁S yields comparable speciation results to morphologic speciation of L₃S cultured from small ruminant feces.
- The proportion of small ruminant GIN eggs that develop to larvae is much higher when cultured to L₁S than when cultured to L₃S.
- Subclinical GIN infection (flock mean FEC < 3,000 epg) appears to have limited impact on growth and reproductive performance of Ontario replacement ewe lambs.
- Rideau-Dorset cross lambs grazing in central Ontario develop a detectable sCarLA response by the end of their first grazing season.
- Individual sCarLA levels are consistent over time, and higher sCarLA level is consistently associated with lower GIN FECs in Ontario sheep.
- Acute stress response phenotype does not appear to be associated with GIN parasitism in sheep with subclinical GIN infections (peak FEC < 900 epg).
- Antibody-biased or cell-biased immune responses do not appear to significantly affect GIN parasitism in sheep.

7.2 References

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