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COMPARISON OF IMMUNE RESPONSES DURING GASTROINTESTINAL HELMINTH SELF-CURE EXPULSION BETWEEN RESISTANT GULF COAST NATIVE AND SUSCEPTIBLE SUFFOLK SHEEP

A Dissertation

Submitted to the Graduate Faculty of
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Pathobiological Sciences
in the School of Veterinary Medicine

by
Javier Jesus Garza
B.S. University of Texas Pan-American, 2006
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Abstract

The immune response to the self-cure phenomenon seen during gastrointestinal nematode (GIN) parasitism of small ruminants was compared between sheep breeds that are resistant or susceptible to *Haemonchus contortus* infection. Fifty-four Gulf Coast Native (Native, resistant) and Suffolk (susceptible) lambs were allowed to acquire a natural GIN infection on pasture and were then randomly allocated into 4 groups. After being moved to parasite free housing for 2 months, lambs were given a challenge infection of 20,000 *H. contortus* infective larvae . Fecal egg counts (FEC) were monitored throughout the study and animals were necropsied at 0, 1, 3, and 7 days post infection (DPI). FEC decreased beginning at 3 DPI in both breeds, with Native lambs having a higher percent reduction in FEC at 3 and 7 DPI compared to Suffolk lambs. Both Native and Suffolk lambs were able to expel their existing adult population. However, while Native lambs also successfully cleared the larval challenge, Suffolk lambs did not. The numbers of eosinophils within the abomasal mucosa reflect the magnitude and timing of FEC reductions in both breeds. Additionally, the elevated levels of eosinophils within the abomasal mucosa of Native lambs at 3 DPI are likely to be involved with the clearance of the larval burden via eosinophil mediated larval killing. Suffolk lambs displayed a delayed cellular response that resulted in larvae entering the mucosa before sufficient eosinophilic response could be established. Elevated mast cells within the abomasal mucosa coincide with the clearance of adult GIN and along with elevated levels of IL-13 seen in both breeds suggest their involvement. The results suggest that self-cure is an immune mediated response that can occur in both resistant and susceptible breeds of sheep while differences in the magnitude and time course of immune responses may prevent susceptible sheep from fully clearing the infection.

Chapter 1: Literature Review

1.1 Parasitism in Ruminants

Ruminants, like all organisms are plagued with a variety of maladies and diseases ranging from nutritional to pathogenic in origin. Physical manifestations resulting from these maladies can vary from non-clinical to debilitating and can become problematic from a production standpoint. Production losses due to parasitism are a significant problem, and are responsible for substantial economic losses worldwide. Parasitism in ruminants can include both exogenous and endogenous parasites, the former including organisms such as ticks, flies, mites and lice, while the later including various protozoa and nematodes. Of all of these parasites, gastrointestinal nematodes (GIN) are perhaps the most damaging parasites of all.

1.2 Gastrointestinal Nematodes

Gastrointestinal nematodes are of significant concern in small ruminant production systems worldwide. As ruminants are an important source of nutrition and income in much of the world, GIN parasitism also represents a major socioeconomical problem (McLeod, 1995). In ruminants the majority of GIN are members of the Phylum Nematoda, Class Secementia, Subclass Rabditia, Order Strongylida, and the Family Trichostrongylidae. These nematodes are often collectively referred to as "trichostrongyles" and include members of the genera *Haemonchus*, *Teladorsagia*, *Trichostrongylus*, *Cooperia*, and *Oesophagostomum*. All trichostrongyles are oviparous and polyandrous which provides them with significant genetic diversity. The life cycle of a trichostrongyle nematodes is simple and direct. Eggs are shed by gravid females within the gastrointestinal (GI) tract and are passed into the environment via defecation. Larvae develop within the eggs and after 1-2 days hatch and emerge as 1st stage larvae (L1). The L1 feed on bacteria and other organic matter within the fecal pat and

subsequently molt to 2nd stage larvae (L2) 2-3 days later. After approximately 2 days, the L2 molt to the 3rd stage larvae (L3) and migrate within the fecal pat where moisture content is highest to prevent desiccation. The L3 will then leave the fecal pat and enter the environment as the moisture within the fecal pat decreases (Soulsby, 1982). L3 retain the L2 cuticle which is a sheath to provide some protection from the environment but also covers their buccal cavity rendering them unable to feed. Thus, the L3 is dependent upon the energy and nutrition acquired and stored from their previous two molts. The L3 is the infective stage of trichostrongyle nematodes and gain entry into the host ruminant via ingestion. The free-living stages of trichostrongyle nematodes are highly influenced by environmental factors such as temperature, humidity and light availability (Soulsby, 1982). Free-living stages grow optimally at 27°C with temperatures below 10°C generally inhibiting growth. Temperature is important for larvae, as temperature greatly influences metabolism and environmental humidity. Temperatures at or exceeding 36°C have a negative influence on trichostrongyle development. As temperature increases, the larvae become more motile at the expense of their finite energy stores and have less than 10 percent survival on grass at 45°C after 24 hours (Veglia, 1915). The ingested L3 passes through the gastrointestinal tract and exsheath in the rumen and travel to their respective host niche within the GItract. Once there, the larvae will molt twice more, first to the 4th stage larvae (L4) and then to the young adult 5th stage (L5) which grow to sexual maturity. Adult male and females find each other and copulate and eggs are shed within the GI tract and exit the host upon defecation completing the life cycle (Veglia, 1915).

1.2.1 *Haemonchus*

Haemonchus contortus is a trichostrongyle nematode known to infect the abomasum of small ruminants (sheep and goats) and wild ruminants (deer, giraffe, etc.) worldwide (Davidson

et al., 1980; Garretson et al., 2009). The life cycle of *H. contortus* favors warm humid environments, and as such is a major problem in the subtropical area of the southeastern United States where conditions allow the parasite to persist year round. When conditions are unfavorable for growth in the external environment, the L4 is able to migrate within the abomasal mucosa and enter a state of arrested development or "hypobiosis" until external conditions improve, thus it is able to persist even in cold areas. Adult *H. contortus* are much larger than other trichostrongylids, measuring approximately 10-20mm in length for males and 18-30mm for females (Soulsby, 1982). Female *H. contortus* are also much more fecund than other trichostrongyles, with a single female laying several thousand eggs per day (Levine, 1968). Additionally, *H. contortus* is hematophagous and feeding is facilitated via a chitinous scalpel-like appendage called a stylet that is located within the buccal cavity of the parasite that is present on parasitic stages (L4, L5, Adult). The ovaries of the female are intertwined with the alimentary tract which encompasses approximately ¾ of the length of the worm and when fed the alimentary tract becomes filled with blood giving it a barber's pole-like appearance which is where the common name "the barberpole worm" is derived from (Dunn, 1978). The majority of the pathology during infection is due to the feeding of the parasite as a single adult female consumes approximately 50µl of blood a day which can quickly exsanguinate an infected animal during heavy infections (Baker et al., 1959). In addition to this, *H. contortus* secretes a calreticulin, that binds to clotting factors inhibiting clotting and allows blood flow to continue after the parasite has left the site of incision (Suchitra and Joshi, 2005). Because of these factors, heavy infections of *H. contortus* results in severe anemia, hyperproteinemia, hyperglobinemia and submandibular edema often referred to as "bottle jaw". Clinically, infected animals will present with general signs of weakness, unthriftiness and failure to thrive as they often have loss

of appetite and weight loss. These symptoms can be followed by death of the infected animal (Soulsby, 1982) . Young animals under 8 months of age (immune system is still under development) are most susceptible to *H. contortus* and infections can be classified into hyperacute, acute, and chronic types. Hyperacute infections are a result of a massive influx of L3 from pasture and may result in death as early as one week after infection. Acute haemonchosis is characterized by the onset of anemia and edema accompanied by many of the clinical signs listed above. While chronic infections are often due to the low numbers of parasites present within the abomasum is it of high economic importance due to the very high level of morbidity in this type of infection. Animals suffering from chronic haemonchosis often suffer from a progressive loss of weight, are generally unthrifty and anemic. Death, while not common, may still occur due to persistent anemia and a resulting depletion of iron and protein stores (Abbott et al., 1984) (Soulsby, 1982). While adult animals are generally refractory to infection, *H. contortus* is of considerable concern to pregnant ewes due to the decrease in immune resilience during and around parturition. This relaxation of immunity and increase in infection results in an increase in environmental egg shedding by pregnant ewes and is known as the "periparturient rise" in infection and results in increased levels of pasture contamination that becomes a primary source of infection for lambs. (Gibbs and Barger, 1986; Salisbury and Arundel, 1970).

1.2.2 *Teladorsagia*

Teladorsagia circumcincta also parasitizes the abomasum of small ruminants. With adult males measuring approximately 8mm in length, and females measuring 12mm, *T. circumcinta* is smaller than *H. contortus*. Additionally, in contrast to *H. contortus*, the larvae of *T. circumcincta* are much more resilient to cold temperatures and thus are able to persist in colder climates

(Soulsby, 1982). Adult females are also less fecund, laying approximately 200 eggs per day (Dunn, 1978). Whereas *H. contortus* reaches peak pasture infectivity in the summer months, the highest pasture infectivity of *T. circumcincta* is seen in the fall and winter months. The major cause of pathogenesis within the host is due to direct morphological changes to the abomasal mucosa by the larval stages. After ingestion, exsheathed L3 enter the gastric glands of the abomasum and remain for 18-21 days. Their presence within the gastric mucosa induces morphological changes within the infected glands, resulting in the replacement of the specialized mucous, zomogenic, and parietal cells with an undifferentiated, mucous secreting columnar epithelial cell type (Soulsby, 1982). As L4 emerges from infected glands 7-14 days later, an undifferentiated cellular hyperplasia occurs among the surrounding gastric glands, resulting in the inability to secrete HCL by non-functional parietal cells and an increase in abomasal pH. As pH increases within the abomasum, pepsinogen is unable to convert to pepsin which causes a disruption in digestion. Additionally, a breakdown of cellular junctions causes serum hypoproteinemia as serum proteins begin to leak into the abomasum. The end result is severe diarrhea and anorexia which can ultimately lead to death of the infected animal (Murray et al., 1970; Soulsby, 1982). Clinical signs also include severe malabsorption syndrome, malaise, dehydration, weight loss and decreased yields.

1.2.3 *Trichostrongylus*

Members of the genus *Trichostrongylus* can be found in both the abomasum (*T. axei*) and the small intestine (predominantly *T. colubriformis*). Females are approximately 5.5mm in length, with males being slightly smaller. The lifecycle follows similar seasonality to *H. contortus* but the L3 are slightly more resilient to cooler temperatures. The pathology associated with *T. colubriformis* infections is a result of the migrations of parasitic life stages beneath the

cells of the intestinal epithelia (Soulsby, 1982). This causes inflammation of the lamina propria, along with an increase in vascular permeability, hypoalbuminaemia, villous atrophy, and malabsorption. The result is reduced feed intake by infected animals which leads to anorexia and stunted growth (Ross et al., 1971; Ross et al., 1967; Taylor and Pearson, 1979).

1.2.4 *Cooperia*

Cooperia spp. are found in the small intestine and share similar approximate size, seasonality and pathogenesis to *T. colubriformis*.

1.3 Control Methodologies

1.3.1 Anthelmintic Treatment

Prior to the 1940's, treatments for GIN parasites in livestock involved the use of varied substances that worked by either antagonizing the nematodes and forcing their departure from within the GI tract, or removing the niche within the host (such as causing sloughing on the GI mucosa!) to inhibit or prevent parasite establishment (McKellar and Jackson, 2004). In 1940-50, phenothiazine, and piperazine ushered in the era of chemical treatment for GIN that had direct effects on the parasites (Habermann et al., 1940; Shorb and Habermann, 1940; Whitehurst and Swanson, 1942; Whitten, 1948). These were not without their faults; both phenothiazine and piperazine required large doses for treatment, and piperazine had a very narrow spectrum of efficacy with limited activity against GIN in sheep (Conder et al., 1991). In 1961, the introduction of thiabendazole marked the first of the traditional broad spectrum chemical anthelmintics used today (Brown et al., 1961). Traditional anthelmintics are classified into four groups based on their method of action. Benzimidazoles were some of the first to be discovered and work by binding tubulin, which is essential for the formation of microtubules that are involved with intracellular transport of various proteins and enzymes. The inhibition of this

intracellular transport ultimately results in cell lysis and death of the nematode (Lacey, 1988). Organophosphates are acetylcholinesterase inhibitors and interfere with muscular contractions by blocking the inactivation of acetylcholine, a potent neuromuscular stimulator, by acetylcholinesterase leading to buildup of the active neurotransmitter and subsequent paralysis (Martin, 1997). Imidithiazoles are nicotinic agonists and include levamizole and the tetrahydropyrimidies (pyrantel and its derivatives). They were approved for use in the 1970s. Nicotinic agonists affect muscular contraction by inducing the opening of nicotinic acetylcholine receptors. This results in an increase in membrane depolarization and a subsequent increase in muscular contraction which causes the parasite to become unable to maintain its position within the GI tract and become swept away by smooth muscle peristalsis (Aubry et al., 1970). Macrocyclic lactones were introduced in the 1980s and include ivermectin and other derivatives that function by affecting glutamate-gated chloride channels within the parasites. This leads to an unregulated influx of chloride ions into muscle tissue, particularly pharyngeal muscle causing an impairment in feeding and subsequent passage of the parasite due to starvation (Shoop et al., 1995).

Wide-spread and intensive use of broad-spectrum anthelmintics became a mainstay in control programs, but it was only a few years before anthelmintic resistance began to appear. Resistance of *H. contortus* against thiabendazole was first reported in 1964, only three years after its approval for use in sheep (Drudge et al., 1957). Within the next decade, wide spread reports of benzimidazole resistance was seen in multiple species of GIN (Berger, 1975; Hotson et al., 1970). The first documented case of resistance to ivermectin was reported in 1988, only seven years after its approval for use in sheep (Van Wyk and Malan, 1988). Despite the increasing reports of anthelmintic resistance by various GIN around the world, the 1980s and 1990s brought

wide spread reports of GIN with resistance to multiple anthelmintic classes (Green et al., 1981; Jackson et al., 1992; Mwamachi et al., 1995; Waruiru et al., 1998; Watson and Hosking, 1990). The current state of anthelmintic resistance in small ruminant production systems is best described as grave. While the dominant genera of GIN may vary by geographical location, resistance to most, if not all, anthelmintic classes in now widespread, with reports from almost all continents including Australia, Europe, Africa and the Americas. In Brazil, where one of the earliest cases of anthelmintic resistance was recorded, a survey assessing levels of benzimidazole resistance on 182 farms within the state of Rio Grande do Sul found resistance on 97% of all farms tested (Echevarria et al., 1996). Additionally, 90% of farms showed resistance to benzimidazoles, 84% to levamisole and 73% resistance to a combination of the two. Resistance was seen in multiple genera of GIN including *T, circumcincta* which showed a level of resistance to benzimidazoles on 84% of farms tested, 72% resistance to levamisole, and a resistance to combination treatment using both benzimidazole and levamisole on 81% of tested farms. Fewer farms had resistant *H. contortus* (68%, 19%, and 15% respectively). In Argentina, a survey of 73 farms from central and northeastern provinces, showed that 46% of all farms tested had GIN resistant to at least one type of anthelmintic class (Eddi et al., 1996). Furthermore, in Paraguay 73%, 68% and 73% of farms surveyed for resistant GIN populations had benzimidazole, levamisole, and ivermectin resistance, respectively (Maciel et al., 1996). A similar trend can be seen in Europe where a survey of over 40 sheep farms (primarily *T. circumcincta)* reported 39 farms had benzimidazole resistant GIN (Taylor et al., 2009). Additionally, 14 farms had GIN with resistance to multiple anthelmintic classes (benzimidazoles, macrocyclic lactones, and imidithiazoles) (Taylor et al., 2009). In Scotland, a survey of 38 sheep flocks was conducted to access ivermectin resistance and found less than a 95% efficacy on 35% of farms (Bartley et al.,

2006). In The Netherlands decreased efficacy to macrocyclic lactones (15%) has been shown with *H. contortus* and benzimidazoles (87%) for both *H. contortus* and *T. circumcincta* (Borgsteede et al., 2007). Reports of anthelmintic resistance have come from Africa where *H. contortus* is the primary GIN of concern (Van Wyk et al., 1999). A series of surveys performed on 80 farms in various provinces in South Africa showed that approximately 95% of *H. contortus* on all farms had less than 95% efficacy to at least one anthelmintic class and up to 75% had a susceptibility of less than 40% (Van Wyk et al., 1999). In New Zealand, a survey showed that 64% of 112 randomly selected farms from both the North and South Islands were unable to a achieve a reduction of 95% in FEC using at least one type of anthelmintic class (Waghorn et al., 2006). The southeastern United States provides favorable conditions for the year-long maintenance of the life cycle of GIN and producers have used anthelmintics heavily during the hot summer months when GIN infections peak on pasture. A recent study was performed to determine the prevalence of anthelmintic resistance in small ruminant farms in the southeastern United States, including the states of Alabama, Arkansas, Florida, Georgia, Kentucky, Louisiana, Maryland, Virginia, Puerto Rico, and the Virgin Islands (Howell et al., 2008). The results showed that the majority of the 46 farms had benzimidazole resistant GIN (98%) while levamisole, ivermectin and moxidectin resistance was found on 54%, 76%, and 24% of all farms, respectively.

While surveys may not always represent the population as a whole, the data presented by these surveys portray the seriousness on the current state of anthelmintic resistance. Indeed, it seems that resistance is becoming the norm, rather than incidental findings. Furthermore, it is evident that the efficacy of anthelmintics is negatively correlated to their usage, as increased usage and overuse, combined with the genetic diversity and fecundity, has resulted in repeated

development of resistance shortly after the introduction of new anthelmintics. Given the lack of new anthelmintics over the past couple of decades, it has become evident that GIN control and treatment can no longer rely primarily on the use of anthelmintics, lest we continue to contribute to the global issue of anthelmintic resistance in GIN populations. We must look toward the development and implementation of alternatives to current treatment protocols.

1.3.2 Nematophagous Fungi

With the advent of anthelminthic resistance, the use of biological control methods has become an essential component of GIN control strategies. Nematophagous fungi are comprised of over 200 species and can be divided into three groups: nematode trapping, endoparasitic, and those that parasitize nematodes within cysts and root knots of plants. Nematophagous fungi are soil dwelling and saprophytic in nature, with the ability to enter a parasitic stage in which they can utilize nematodes as an additional source of energy. Cyst and root knot parasitic fungi infect their hosts via vegetative hyphae which penetrate eggs within a cyst or root knot (Vianene and Abawi, 2000). Endoparasitic fungi rely on the host nematode to ingest crescent shaped spores which subsequently attach to the musculature of the esophagus. In some species, spores can also adhere to the cuticle of the host and gain entry via cuticular penetration. As time progresses and the fungi disseminates throughout the body of the nematode, conidophores develop which penetrate the cuticle and once again form spores (Jansson et al., 1984). Nematode trapping fungi form specialized structures that ensnare the host. The morphology of these trapping structures is diverse and includes vegetative hyphal nets, adhesive knobs, constricting rings, and adhesive spores. (Grønvold et al., 1996; Grønvold et al., 1993).

While there are many fungi that parasitize nematodes, not all nematophagous fungi are effective as a biological control for GIN in ruminants. A successful candidate for biological

control must first be able to access the fecal pat in order to reach the larvae within. Second, the fungus must be able to develop into the parasitic phase within the fecal pat. Over 100 fungi with known nematophagous activity have been examined for their potential to control GIN in ruminants and it was determined that *Arthrobotrys* spp. consistently yielded reductions in L3 within feces (Larsen et al., 1994; Waller and Faedo, 1993). However, when subjected to the harsh abomasal environment, *Arthrobotrys* spp. displayed stunted growth due to the thin chlamydospore walls that rendered the spores non-viable after passage through the GI tract (Larsen et al., 1994). Thus, a fungi which produced a thick walled chlamydospore was needed. *Duddingtonia flagrans* was isolated from the feces of grazing livestock in Austrailia and assessed for its ability to survive the GI tract as well as its predatory activity. It was found that the number of L3 recovered from feces was reduced by over 80% (Larsen et al., 1998). *D. flagrans* is a member of the Family Orbilaceae in the Division Ascomycota. Like *Arthrobotrys* spp., *D. flagrans* uses hyphal nets consisting of several hyphal rings which constrict and ensnare L3. The hyphae then penetrate the larval cuticle and digest it from within. Subsequent research in horses (Baudena et al., 2000; Fernández et al., 1997; Larsen et al., 1995), swine (Ferreira et al., 2011; Nansen et al., 1996; Petkevicius et al., 1998), goats (Ojeda-Robertos et al., 2005; Terrill et al., 2004; Wright et al., 2003), and sheep (Knox and Faedo, 2001; Mendoza de Gives et al., 1998), have determined that *D. flagrans,* is an effective method of biological control against GIN parasites.

1.3.3 Nutritional Supplementation

It is known that the nutritional status of the host is important in host-parasite interactions (Van Houtert and Sykes, 1996),and as such, supplementation of nutrients can help reduce the use of anthelmintics. GIN infections result in losses of nutrients via various means, such as anorexia,

malabsorption, and loss of endogenous protein (blood loss, shed epithelia) (Van Houtert and Sykes, 1996). Additionally, nutritional requirements are altered not only directly by parasite induced damage, but by the increased nutritional cost for the activation of the immune response reported as high as 15% (Sykes, 1994). These are the primary reasons for the losses in production seen during GIN infections.

Reduced food intake (anorexia) is a large factor in the state of health of an infected animal, as an animal that does not eat is unlikely to recover adequately from infection. The establishment of an immune response places increased nutritional requirements on the host and increased utilization of protein due to an increase in protein synthesis in the GI tract (Brown et al., 1991; Sykes and Greer, 2004).

It is believed that animals follow a framework of nutrient partitioning whereby food recourses are allocated via stratified levels of priority, with host parasite regulation mechanisms considered less of a priority over reproduction, growth and general body protein maintenance (Coop and Kyriazakis, 1999). While this is a hypothetical system yet to be proven concretely, evidence of this mechanism at work can be seen in the decrease of immune competency by pregnant and lactating ewes and the accompanying peri-parturient rise in FEC (Taylor et al., 1990). Indeed, nutritional supplementation in the form of increased protein has been shown to partially abrogate the rise in FEC in infected pregnant and lactating ewes (Houdijk et al., 2006). Additionally, supplementation with vitamin E in an experimental *H. contortus* infection in lambs has been shown to result in a decrease in worm burden and FEC when compared to control animals that had received no additional supplementation (De Wolf et al., 2014). The increased eosinophilia present in the abomasal regions of vitamin E supplemented animals as well as the strong negative correlation seen between worm burden and eosinophilia suggest an increase in

immune function; however, the role that vitamin E plays in this increase in effector cell recruitment requires further study.

Supplementary feeding with minerals can also have direct anti-parasitic effect. Copper oxide wire particles (COWP) are commercialized as a ruminant supplement for use in areas where copper levels in forages are insufficient. It was later discovered that COWP also resulted in a decrease in FEC (Bang et al., 1990a). The effects of COWP can last for over a week, with concentration peaking at 4 days and persisting as long as 10 days before decreasing (Bang et al., 1990b). Sheep are highly susceptible to copper toxicity and the margin between the recommended intake level of copper and the level at which copper toxicity occurs is very narrow, thus the regulation copper supplementation is important. Various studies have been conducted in both sheep and goats to evaluate the efficacy of COWP in GIN infections. The results have shown that COWP effectively reduced FEC in *H. contortus* infected sheep and goats as high as 80% (Burke and Miller, 2006) (Burke et al., 2004; Burke et al., 2007; Soli et al., 2010). However, limited efficacy was seen in GIN located in the small and large intestine. Still, in areas where *H. contortus* is present in high numbers, COWP may be an effective means of controlling infection while reducing the need for anthelmintics.

1.3.4 Bioactive Forages

Usage of bioactive forages is another means to aid in the control of GIN infection. Condensed tannin (CT) containing forages have been shown to directly affect GIN by preventing incoming L3 from establishing an infection, by reducing fecundity in adult females in an established infection, and by reducing the viability of eggs shed into the environment (Martínez-Ortíz-de-Montellano et al., 2010).

Several different CT containing forages have been examined for their anti-parasitic activity in small ruminants including trefoil (*Lotus* spp.) (Marley et al., 2003; Niezen et al., 1998), sainfoin (*Onobrychis vicifolia*) (Manolaraki et al., 2010), and *Lysiloma latisiliquum* (Martínez-Ortíz-de-Montellano et al., 2010). Additionally, several efficacy studies have been performed in the southeastern United States using Chinese Bush Clover (*Lespedeza cuneata*), also known as sericea lespedeza (SL), in both sheep and goats. When fed as hay to goats, SL was shown to reduce FEC and the number of *H. contortus* L3 recovered from feces (Shaik et al., 2006). The number of adult nematodes present within both the abomasum and small intestines were also reduced. Reductions in FEC and worm burden were also seen in lambs fed SL hay (Lange et al., 2006). Additionally, pelleted SL hay showed an increased efficacy against GIN in goats (Burke et al., 2010; Gujja et al., 2013; Kommuru et al., 2014; Shaik et al., 2006; Terrill et al., 2007).

While the exact mechanism by which CT affect GIN within the GI tract is not known, CT have been shown to bind to dietary protein and form complexes which prevent protein degradation within the rumen (which, has an added benefit that can reduce the risk of bloat) (Waghorn et al., 1987). The low pH of the abomasum causes dissociation of these complexes and allows for digestible protein to become biologically available for processing within the lower GI tract, possibly providing additional amino acids for the production of an enhanced immunological response (Waghorn et al., 1987). It is also possible, that CT may bind to the proteinaceous outer cuticle of GIN directly, specifically near the buccal cavity and genital pore of females, causing stress and thereby reducing fecundity (Martínez-Ortíz-de-Montellano et al., 2010).

1.3.5 Refugia and Pasture Management

In a population of animals that are infected with GIN, the majority of infection, i.e., the highest worm burdens are present in a small percentage of the population. It is this small number of animals that is largely responsible for the majority of contamination that can be found on a particular pasture (Sreter et al., 1994). This implies that in a given population of infected animals, only a few may require anthelmintic intervention. Upon administration of anthelmintics using selective treatment protocols in which only those animals with the highest levels of infection are treated, the population of GIN within those animals that remain untreated are said to be "in refugia" or unexposed to the selective pressure of treatment which promotes the development of resistance (Soulsby, 2007; Van Wyk, 2001). Furthermore, in the absence of anthelmintic treatment, the unexposed susceptible population is able to confer their susceptibility to their progeny, thereby perpetuating a susceptible phenotype that will help dilute resistant phenotypes. Thus, the exploitation of refugia is not necessarily a standalone means to decrease dependence on anthelmintics, but rather a way to limit, or even reverse the trend of increasing anthelmintic resistance (Martin et al., 1981). Over the past decade, there has been a push towards finding ways to manage refugia which has led to changes regarding the recommended pasture management practices of the 1990's and earlier. Several studies have been performed to attempt replacement of a resistant population of nematodes with a susceptible population and the results have been fairly successful (Bird et al., 2001; Sissay et al., 2006; Van Wyk and Van Schalkwyk, 1990). This method, while successful when resistant populations can be decreased to low levels in pasture, becomes less so as resistance increases. Indeed, the dilution of resistant parasites would be problematic if the resistant population on pasture is unable to be reduced prior to seeding with a susceptible population because said population would need to be much higher

than the resistant population in order for successful dilution to occur . Another method of utilizing refugia is via the selective treatment of only those individual animals within a population with the highest levels of infection. The monitoring of FEC provides a means to find and target animals for treatment, however the majority of producers do not have access to the equipment necessary to perform a fecal exam and must rely on rounding up, collecting, and submitting fecal samples to a laboratory after which animals must be subsequently rounded up again for selective treatment. This can be a potentially expensive and labor intensive task that producers may not be willing to do; however, an alternatives does exist in the form of the FAMACA scoring system.

1.3.6 FAMACHA

The FAMACHA system was developed in South Africa for the purpose of easily determining the level of *H. contortus* infection in sheep (Van Wyk et al., 1997). The system consists of a color chart ranked on a scale reflecting the blood packed cell volume based on the color of the lower eyelid inside mucous membrane ranging from 1, which is a healthy red, to a 5, indicating a highly anemic (and highly parasitized individual). Animals with a score of 4 or 5 are considered to be anemic and therefore require treatment while scores of 3 are treated based on the discretion of the producer. Studies performed in both South Africa and the southeastern United States have shown that the FAMACHA system shows a high sensitivity with moderate specificity when determining levels of anemia caused by *H. contortus* in sheep and goats (Kaplan et al., 2004; Vatta et al., 2001). The FAMACHA system is not without its faults, as it is limited to identifying only animals infected with *H. contortus* that need treatment, meaning that in areas where the GIN of significance is non-hematophagous, this system is ineffective. Additionally, the process can be labor intensive, requiring producers to gather and examine the

entire population. However, in production systems where *H. contortus* is the nematode of primary importance, the FAMACHA system provides immediate onsite results that the producer can use to determine treatment.

1.3.7 Breeding for Resistance

Animals within a given population have varying levels of resistance to GIN infection. This is due to normal genetic variation. Additionally, it is known that levels of resistance, defined as the ability of an animal to prevent the establishment of an infection, and resilience, defined as the ability of an animal to tolerate an infection without showing clinical signs of disease can vary within breed. Similarly, breeds of sheep are known to have varying levels of resistance or susceptibility to GIN infection. This is due to within-breed and between-breed genetic variation. Breeds such as the Barbados black belly (Courtney et al., 1985; Yazwinski et al., 1980), Red Massai (Abbott et al., 1985; Altaif and Dargie, 1978; Preston and Allonby, 1978), Florida and Gulf Coast Native (Amarante et al., 1999; Bahirathan et al., 1996b; Bradley et al., 1973; Miller et al., 1998; Radhakrishnan et al., 1972), St. Croix (Gamble and Zajac, 1992) , Katahdin, and Dorper (Burke and Miller, 2002; Vanimisetti et al., 2004), have classically been shown to be relatively resistant to *H. contortus* infection. Studies have also shown that the basis for resistance is genetic in origin; therefore, selective breeding for resistance may be a feasible option for decreasing reliance on anthelmintics (Albers et al., 1987; Stear and Murray, 1994). The classical trait for selecting for resistance is FEC, based on the assumption that the animals within a flock that have the lowest FEC have a lower level of infection, and are therefore genetically superior in terms of resistance to infection (Bishop and Morris, 2007). However, other traits have been examined as well, including measures of anemia via FAMACHA or

immunological parameters such as eosinophil counts and immunoglobulin levels (Stear et al., 2002; Strain et al., 2002).

1.3.8 Vaccines

Another means to control GIN infection without relying on anthelmintics may be by altering the host's immune response directly via the utilization of vaccines. The history of finding an effective vaccine has been long and somewhat unrewarding. Beginning in the 1960s, studies with irradiated attenuated larvae of the lungworm *Dictyocaulus viviparous*, showed protection against a normal infection.(Jarrett et al., 1959; Jarrett et al., 1960) Irradiated larvae retained their immunogenic potential but failed to mature within the lungs of the animal resulting in a lack of pathology typically involved in lungworm infection (Jarrett and Sharp, 1963). This groundbreaking discovery lead to the development of Dictol™ a live attenuated oral vaccine, and the first commercial vaccine against a nematode parasite. Its success is such that it is still available and used today in the ruminant industry. A successful vaccine starts with an antigen that is a foreign protein that can illicit an immune response. In the context of parasitic helminthes, antigens can come from a variety of places. Cuticle and excretory/secretory products are of particular interest in the development of vaccines against several helminthes of animals and man. Antigens derived from the alimentary tract of blood feeding parasites have become increasingly popular, with the development of the tick gut antigen vaccines (de la Fuente et al., 1998; Fragoso et al., 1998; Willadsen et al., 1995). For *H. contortus*, the hidden gut antigens H11 and H-gal-GP have shown promise as vaccine candidates, however these antigens provide little to no protection when produced via genetic recombination due to improper folding of the recombinant proteins (Smith, 2008). Vaccination with *H. contortus* cysteine proteases have also resulted in protection against the parasite, however, similar problems were encountered

when trying to produce the recombinant protein antigens via genetic recombination (Redmond and Knox, 2004, 2006).

1.4 Gulf Coast Native and Suffolk Sheep

1.4.1 Gulf Coast Native

Native sheep are believed to be descended from Spain and brought to the Americas by the explorers of the 1500s. They also brought their GIN population with them. The hot and humid environment of the southeastern United States was ideal for the survival of the GIN population (especially *H. contortus)* which forced the ancestors of the Native to adapt via survival of the fittest natural selection. The result was a robust sheep that could survive in such an environment. Adult sheep can way between 100 to 190 pounds and ewes are excellent mothers that are able to breed and lamb all year (Fernandez and Bixby, 1995). The Native has been shown to be relatively resistance to GIN infection, specifically *H. contortus* (Miller et al., 1998; Pena et al., 2004).

1.4.2 Suffolk

Originating in England from the crossing of Southdown rams and Norfolk Horned ewes in the 1700s, Suffolk sheep are a quintessential meat breed highlighted by their heavy carcass weight and quick finishing potential (Palsson, 1939). Suffolk sheep are fairly large, with rams weighing 200 to 350 pounds and ewes weighing 180 to 250 pounds. In contrast to Native sheep, Suffolk sheep are relatively susceptible to GIN infection, specifically *H. contortus*, and as such are often used as representative for a susceptible breed in infection studies (Bahirathan et al., 1996a; Miller et al., 1998).

1.5 The Immune System

The immune system is a robustly complex series of cells and molecules whose purpose is the identification and destruction of pathogens within a larger organism while simultaneously recognizing the self. This section will provide a basic introduction of the individual components of the immune system as well as their generalized interactions.

The innate immune system is the first line of defense against pathogens and is composed of mechanisms that result in an immediate response against foreign invaders. This defense contains both physical and soluble components as well as resident and migrating inflammatory cells. The skin is an essential part of the innate immune system because it serves as a physical barrier that must be overcome by many pathogens to gain access into a host. The skin is several layers of cells with the portion exposed to the environment consisting of multiple layers of keratinocytes (a type of stratified squamous epithelial cell). Undamaged skin normally produces antimicrobial peptides that kill potential microbes by binding to cell membranes and promoting pore formation. Wounds to the skin allow microorganisms that are ubiquitous in the environment, to gain access to the living layer of keratinocytes and the complement system.

1.5.1 Complement

The complement system is a group of proteins whose main function is the destruction of pathogens by pore formation via three possible pathways. The classical complement pathway is activated by the binding of the antibodies IgG or IgM to the surface of a pathogen. The complement factor C1 is comprised of two subunits, of which C1q recognizes and binds to antibodies while C1r and C1s bind to form C1r2s2 which then bind to C1q to form activated C1. The proteolytically active C1 then cleaves inactive C4 into C4b, which binds to C1, and C4a which remains unattached and serves as a chemo-attractant for other immune cells as well as an

opsonin (a molecule that coats foreign invaders and "marks" them for destruction by phagocytic cells such as macrophages). C2 is activated in a similar manner, with C2b binding to C2b to form C4b2b (C3 convertase) and C2a filling the same role as C4a. C3 convertase then cleaves the inactive C3 to form C3a and C3b (Tizard, 1992). The latter binds to the complex to form C4b2b3b (C5 convertase), while the former floats away and serves as an opsonin and inflammatory chemo-attractant. C5 convertase cleaves C5 to its active forms C5a and C5b. C5a acts in a function similar to C3a, while C5b binds to the surface of the pathogen and is the initiator of the membrane attack complex (MAC) formation. C5b is then bound by C6, C7, and C8. The binding of multiple C9 molecules to the C5b678 complex serve to form the complement pore which spans the length of the microbe membrane resulting in the exposure of the microbe cytoplasm to the environment that results in subsequent lysis of the cell. While the alternative and lectin-dependent complement pathway both differ in their activation, the latter half of the cascade (C5-C9) is conserved (Tizard, 1992). Unlike the classical pathway which requires immunoglobulin to begin the reaction, the alternative pathway is initiated via the spontaneous transformation of C3 to an active form via hydrolysis into C3H20. Factor B then binds to C3H20 and is activated by factor D to create a C3 convertase which cleaves an inactive C3 into C3a and C3b. The lectin-dependent pathway is initiated in a method similar to the classical pathway but with pathogen derived mannose serving as the molecule to which a mannose binding lectin attaches which facilitates the cleavage of C4 and C2 for the formation of C3 convertase (Götze and Müller-Eberhard, 1976).

1.5.2 Innate Recognition of Non-Self

Rather than detect specific antigens in an event of foreign challenge, the innate immune system relies on pathogen recognition receptors (PRR) that detect pathogen associated molecular

patterns (PAMPs), a series of patterns that are highly conserved amongst microbes and other non-self-organisms and particles. PRRs were first discovered in *Drosophila* and named Toll (Lemaitre et al., 1996). Since their discovery, several toll-like receptors have been found in various locations on or within mammalian cells of immunologically related origin and detect different PAMPs depending PPR type and location. Toll like receptors represent the largest group of PRRs. Indeed, to date, 10 TLRs have been identified in a variety of mammals including humans, cattle, sheep and pigs, while 12 have been identified in mice (Chang et al., 2009; Medzhitov et al., 1997; Uenishi and Shinkai, 2009). TLR2 recognizes lipoproteins and lipotechoic acid of gram positive bacteria such as *Listeria*, while TLR3 recognizes double stranded RNA. TLR4 recognizes lipopolysaccharide found on the surface of gram negative bacteria while TLR5 has an affinity for bacterial flagellin. TLR9 recognizes unmethylated CpG DNA while TLR7 and 8 recognize single stranded RNA. TLR2, 4, and 9 are found on the outer membrane of cells, while TLR7, 8, and 9 are localized within endosomes to detect pathogens that have been phagocytized. Activation of TLRs result in profound changes in immune responses by promoting the release of cytokines that activate inflammatory responses as well as modulatory cytokines that affect cells of the innate immune system such as dendritic cells, as well as T cells of the adaptive immune system (Roses et al., 2008).

1.5.3 Acute Phase Response

The subsequent production and secretion of inducible antimicrobial peptides that both actively affect the invading microbe and serve as chemotactic factors for the recruitment of cells and the onset the acute phase response (APR). The APR is a series of systemic changes that occur during inflammation. Indeed, these changes occur within the body in places distal to the site of inflammation and involve changes in excess of 200 proteins all with varying levels of up-

regulation or down-regulation (Kaneko et al., 1997; Parra et al., 2006). These proteins work to induce changes within the body which can range from fever induction to tissue repair. The APR is largely regulated and activated by the pro-inflammatory cytokines IL-6, IL-1 and TGF-β. Through the use of IL-6 knockout mice models, it has been shown that IL-6 is essential to the establishment of APR (Kopf et al., 1994). Additionally, IL-1β deficient mice exhibit an impaired APR when challenged by a localized inflammatory agent, implicating its importance in APR induction as well (Zheng et al., 1995).

1.5.4 Dendritic Cells

Dendritic cells are the most diverse cell type in the innate immune system and perhaps the most diverse in the immune system as a whole. Dendritic cells are known to play an essential role in linking the innate and adaptive immune systems by activating T cell responses within immunological organs via the presentation of antigen (Steinman and Cohn, 1974; Steinman et al., 1979; Steinman et al., 1974). Dendritic cells can be found in most all tissues and it has become evident that their role is much greater than what was previously believed.

1.5.5 Macrophages

Macrophages are amoeboid-like phagocytic cells of the innate immune system and share the same progenitor as dendritic cells; the monocyte which comprise approximately 3% of white blood cells found in circulation (Duque and Descoteaux, 2014). Monocytes that leave circulation and enter tissue differentiate into macrophages and serve as first responders in the event of immune challenge. Macrophages classically play many roles including the phagocytosis of foreign invaders and are important in the induction of inflammation via the release of cytokines (Hume, 2006). Macrophages involved in inflammatory responses are known as classical or M1 macrophages and are involved in responses against bacteria and protozoa

(Mechnikov, 1908). These classically activated macrophages require priming via IFN-γ and LPS (Dalton et al., 1993). Pathogens that are phagocytosed are broken down in phagolysosomes and processed for presentation to helper T cells via MHC class II receptors (Harding et al., 2003). Classically activated macrophages secrete a variety of chemokines that serve as chemo-attractants for other leukocytes including neutrophils, natural killer cells and t-cells (Duque and Descoteaux, 2014).

Macrophages can also be alternatively activated via IL-4 or IL-13 stimuli (Stein et al., 1992). In addition, activated macrophages secrete factors that promote the control of inflammation and support the induction of wound repair via increased production of collagen and other components of the extra cellular matrix (Song et al., 2000). These macrophages are also heavily involved in the expulsion of GIN by stimulating the recruitment of eosinophils to the site of infection via up-regulating the production of eotaxin and monocyte chemotactic protein 1, both strong chemotactic cytokines of eosinophils (Voehringer et al., 2007; Zhu et al., 2004). It is also speculated that alternatively activated macrophages play a more direct role in worm expulsion via the secretion of acidic mammalian chitinase (AMcase) that may damage the chitinous cuticles of the parasites (Nair et al., 2005).

1.5.6 Eosinophils

Eosinophils are leukocytes that are involved in parasitic infections and allergies. These specialized cells contain granules, which upon stimulation release a variety of cytotoxic granules including monocyte basic protein (MBP) eosinophil cationic protein, eosinophil peroxidase, and eosinophil derived neurotoxin (Ackerman et al., 1983; Archer and Hirsch, 1963; Gleich and Adolphson, 1986). Additionally, stimulation of eosinophils also promotes the secretion of various cytokines including IL-4, IL-5, IL-6, IL-10, and IL-13, and the chemokines, Eotaxin-1,

RANTES, and MIP-1α (Lacy and Moqbel, 2000; Rothenberg and Hogan, 2006). Eosinophils also regulate the function of mast cells via MBP (Piliponsky et al., 2002), and promote B cell IgE isotype switching via IL-5 secretion (Behm and Ovington, 2000).

1.5.7 Neutrophils

Neutrophils are the most abundant leukocyte in circulation and are an important innate cell in the immune response against bacterial and fungal pathogens, where they serve as one of the first lines of defense. Like eosinophils, neutrophils contain granules within their cytoplasm. However, the secretory granules of neutrophils are released into phagosomes containing phagocytized microorganisms which result in pathogen degradation (Borregaard et al., 2007). Additionally, studies have shown that neutrophils are able to expel their cellular contents and form complexes known as NETs that serve to ensnare pathogens (Brinkmann et al., 2004; Yipp et al., 2012).

1.5.8 Mast Cells

Mast cells are resident, granular leukocytes of tissues and organs throughout the body and are important players in type I hypersensitivity reactions and in responses against parasites. Mast cells contain surface receptors (FcεR1) with a high affinity for IgE antibodies. Upon first exposure to an antigen, specific IgE produced by B cells bind to FcεR1 and upon subsequent challenge, the binding of antigen to mast cell mounted IgE simulates the degranulation of the mast cell and the release of granular contents such as histamine, bradykinin, and seratonin (Benoist and Mathis, 2002). This in turn increases vascular permeability and prompts the efflux of leukocytes to the site of challenge via the secretion of a variety of cytokines, and chemokines (Crivellato et al., 2004; Woodbury et al., 1984).

1.5.9 $CD4^+$ T cells

T cells are members of the adaptive immune system and originate from the primary lymphoid organs and play a central role in adaptive immune responses. T cells contain receptors (TCRs) on their surface that uniquely recognize antigens presented by APCs such as dendritic cells or macrophages in secondary lymphoid organs via MHCII receptors. Antigen presentation in the presence of a specific cytokines results in activation via differentiation into specific subsets of $CD4^+$ T cells (Röcken et al., 1992). Once t cells have been activated, they undergo clonal expansion and migrate from secondary lymphoid organs to the sites of infection where they serve as effectors that can directly influence the immune response against the foreign antigen via the production and secretion of cytokines. Initially, differentiated $CD4^+$ T cells were believed to belong to one of two subsets: type 1 or type 2 helper T cells (Th1, Th2) (Mosmann et al., 1986). Since then, the number of $CD4^+$ T cells have expanded to include two additional subsets, Th17 and regulatory T cells (Tregs). Each subset has its own repertoire of cytokines that they secrete along with characteristic cytokines that are specific to the subset (Aggarwal et al., 2003; Chen et al., 2003). Th1 cells are characterized by their production of IFNγ, IL-2, and TNF-α and are involved in immune responses directed primarily against intracellular bacteria and protozoa. The IFN-γ produced by these cells activate and increase the phagocytic activity of macrophages, induces the production of pathogen-killing nitric oxide free radicals (NOx) via inducible nitric oxide synthase (iNOS), induces IgG isotype switching of B cells, and suppresses the induction of Th2 responses (Desmedt et al., 1998).

Th2 cells are characterized by the production of IL-4, IL-5, and IL-13 and are essential in humoral responses against metazoan parasites such as helminthes. Th1 and Th2 share mutual antagonism and Th2 responses also help to suppress the induction of Th1 responses.

Additionally, the cytokines produced by these cells help maintain the Th2 response by activating more Th2 $CD4^+$ T cells via positive feedback loop, induce B cell Ab isotype switching to IgA and IgE, promote mast cell chemotaxis and degranulation, and activate eosinophils (Mosmann and Coffman, 1989). The IL-4 from memory Th2 T cells have also been shown to protect against challenge infections of GIN in the mouse model (Anthony et al., 2006).

1.5.10 B cells

B cells are characterized by the presence of B cell receptors (BCR) on their surface that recognize specific antigen moieties, and are an essential component of humoral immunity. They originate in the bone marrow as lymphoid progenitor cells that begin development via signaling by stromal cells. B cell maturation occurs in a series of steps during which the heavy and light chains of the BCR undergo rearrangement after which, the mature naïve B cell travels through the blood stream to secondary lymphoid organs where they become exposed to antigen via their BCRs (Carrasco and Batista, 2007; von Andrian and Mempel, 2003). If a B cell is unsuccessful in completing any stage of development it is targeted for death via apoptosis. Antigens bound on BCRs are internalized, degraded, and mounted onto MHCII molecules and presented on the surface of the B cell. B cells can undergo T cell dependent activation via B cell/T cell interaction called an immunological synapse. Cytokines released by Th cells induce isotype class switching of B cells to produce IgA, IgG, or IgE antibodies (Gray et al., 1996). B cells that have become activated multiply rapidly into mature plasma cells and memory B cells, the former of which is short-lived (1-2 weeks), while the latter persist for an extended period of time and are primed for rapid replication and antibody production upon challenge exposures with the same antigen (MacLennan et al., 1992).

1.5.11 Early Host-Nematode Interactions

Early host-nematode interactions are important in determining the immunological course of action that will be taken by the host during infection. In sheep, these initial interactions are not as clear as in murine models of GIN infection. As such, the research that has been done in murine models may shed some light on what is occurring in GIN of sheep as well.

Gastrointestinal epithelial cells serve as both physical barriers to invading pathogens and as one of the first responders in GIN invasion. It has been shown that epithelial cells recognize both allergens and nematode products which can lead to Th2 activation (Eisenbarth et al., 2002; Zaph et al., 2007). This is accomplished via the release of cytokines IL-25 andIL-33, and thymic stromal lymphopoetin (TSLP). In the case of allergic responses using a lung model, exposure of lung epithelial cells to allergens promoted the production of TSLP mRNA via recognition of proteases via PAR-2, a seven transmembrane G-protein coupled receptor. It has been shown that when PAR-2 is knocked down, TSLP was significantly reduced compared to control cells when exposed to allergens (Kouzaki et al., 2009). TSLP has been shown to be essential in protection against *Trichuris* spp. infection in murine models (Zaph et al., 2007). *Trichuris* spp. infections in mice with a deficiency in IκB kinase-β-dependent gene expression ($Ikkb^{\Delta IEC}$) in intestinal epithelial cells resulted in a decrease in TSLP expression the inability to establish a Th2 response against the parasite. Indeed, $Ikkb^{\Delta IEC}$ mice also displayed a dysfunction in intestinal epithelial cell/dendritic cell interactions characterized by increased proinflammatory – anti T_H2 cytokines (Zaph et al., 2007). In addition to activation of dendritic cells, basophils can both be activated by TSLP and produce TSLP themselves which when acting as antigen presenting cells have an affinity for priming T helper cells towards Th2 via the suppression of IL-12 secretion. It is important to note however, that while TSLP is able to promote an immunological shift

towards a Th2 response, TSLP is not essential for immunity against all GIN. Using TSLP receptor knockout mice, both *Heligmosomoides polygyrus* and *Nippostrongylus braziliensis* (both murine GIN) were able to bypass TSLP via direct suppression of IL-12p40 production by dendritic cells leading to similar responses against both helminthes in C57BL/6 mice with normal TSLP receptor functions (Massacand et al., 2009; Perrigoue et al., 2009).

In addition to TSLP, epithelial cells also secrete the cytokines IL33 and IL25 as a result of cellular damage from infection or injury. These damage-associated molecular patterns (DAMPs) act on both hematopoietic stem and progenitor cells as well as immune cells in both a regulatory and effector driven manner in response to helminth infection.

IL33 was originally discovered to be expressed in endothelial venules and since has been shown to be expressed in a wide variety of cell types as well as serve a multitude of regulatory functions (Baekkevold et al., 2003). In addition to the wide gamut of immunologically significant cells such as, epithelial cells, dendritic cells, macrophages, and mast cells, IL33 is also expressed in smooth muscle cells, osteoblasts, adipocytes, and fibroblasts (Schmitz et al., 2005). IL-33 activates NFκB as well as the MAP kinases p38, ERK and JNK via the ST2-IL-1 receptor accessory protein complex (Mirchandani et al., 2012; Schmitz et al., 2005). IL-33 is found within the nucleus of cells and is released upon apoptosis in a manner similar to the alarmin IL-1α (Lüthi et al., 2009). Perhaps the most important role played by IL-33 towards GIN infections is the stimulation of lymphocyte effector functions. IL-33 promotes the release of the archetypical antihelminth cytokines IL-4, IL-5, and IL-13 by Th2 Helper T cells as well as induces the release of IL-5 and IL-13 by B-1 B cells (Komai-Koma et al., 2011). These cytokines are known to activate several types of innate immune cells. Additionally, IL-33 is able to prime $CD4^+$ T cells towards Th2 in an IL-4 independent fashion. This may prove to be an

essential component to the rapid early expulsion of incoming larvae seen during GIN infections seen in many different hosts (Kurowska-Stolarska et al., 2008). This early priming of $CD4^+$ T cells to release IL-5 may be a critical factor in the eosinophilia seen in GIN infections. Some innate lymphoid cell populations are also known to respond to IL-33. These four recently discovered cell types are distinct from all others (lymphocytes, neutrophils, dendritic cells, etc.) in that they lack hematopoetic cell associated surface markers. Of the four types of ILCs, three have been reported to be induced during helminth infection (Neill et al., 2010; Price et al., 2010). Innate type 2 helper cells and nuocytes have both been identified in mesenteric lymph nodes and spleen, with innate type 2 helper cells also reported to reside in the liver. Both cell types are Lin-, c-kit+, CD44+ and Sca1+. Both cell types expand in response to IL-25 in addition to IL-33 and secrete IL-5 and IL-13. Using YetCre13-Rosa-DTA mice in which transcription of IL-13 in cells results in the production of diphtheria toxin and subsequent cell death, it was found that administration of exogenous IL-25 resulted in a decrease in eosinophilic infiltration of several organs including spleen peritoneum, liver and mesenteric lymph nodes (Price et al., 2010). Subsequent examination of innate type 2 cells in antihelminth immunity using *N. brasiliensis* indicated the integral role of this cell type, as adoptive transfer of innate type 2 cells rescued worm expulsion and eosinophil responses in mice lacking both adaptive immunity and innate type 2 cells which solidified prior studies that implicated the role of IL-25 in worm expulsion (Fallon et al., 2006; Price et al., 2010). Neill et al. (2010) were able to show, through similar experiments using IL13-eGFP mice, that nuocytes are also an important source of IL-13 and are present in the spleen, mesenteric lymph nodes and bone marrow. Additionally, *N. brasiliensis* infected mice lacking IL-25 or IL-33 receptors (*Il17br*$^{-/-}$ and *Il1rl1*$^{-/-}$ respectively) successfully expelled worms. However, mice with a combined deficiency in IL-25 and IL-33 receptors failed

to expel worms and held a prolonged persistent infection with a marked absence of nuocytes and a reduction in eosinophils that was successfully restored upon adoptive transfer (Neill et al., 2010).

While studies in mouse models have been essential in elucidating the immune responses involved during GIN infections, murine models may not fully reflect what is occurring in other animals due to differences in the life-cycle and host niche of the parasites. This dissertation work focused on the immune responses during GIN infections in ruminants, specifically sheep. As such, the following section will focus on the host response to GIN in ruminants with particular attention to immune responses against *H. contortus*.

1.6 Immune Responses Against GIN in Sheep

Infections with GIN, when broken down into its most simplest form involves the infection of the host, followed by the host immunological response resulting in either the clearance of the infection or the establishment of a patent infection by the adult worms.

1.6.1 Primary Infection

Primary infections with *H. contortus* are characterized by an increase in eosinophilia within the tissues of the abomasum during early infection with numbers of eosinophils declining as the infection period persists(Balic et al., 2000a). This is accompanied by a similar increase in B cells and $CD4^+$ T-cells during the early course of infection, and a subsequent decrease around the time of adult establishment within the abomasum (Balic et al., 2000a). As larvae mature to adults, mucosal mast cells increase within the abomasum (Salman and Duncan, 1984).

These observations indicate that the majority of immune responses seen during primary infection are largely directed against the larval stages of the parasite as evidenced by the peaks in leukocyte levels first seen at the time of larval presence in the mucosa and again surrounding the

time of adult maturation (Stevenson et al., 1994). The overall result of the primary infection is the inability to clear the incoming larval burden due to an insufficiently activated immune response. From an immunological standpoint, primary infections serve to prime the immune response against future infections. Furthermore, the presence of antigens specific to either larvae or adults (Bowles et al., 1995), and the responses seen during primary infections indicate that the host immune system recognizes larval and adult parasites as separate infections (Meeusen et al., 2005).

1.6.2 Secondary Infections

Secondary GIN infections generally have a higher magnitude of response that occurs more rapidly than the responses of the host during the first encounter with the parasites. This is due to the priming of the immune system during the primary infection.

1.6.2.1 Innate Immune Responses

Complement is one of the first innate immune components that GIN larvae encounter (Leid, 1988). The production of the complement peptides C3a and C5a from the alternative complement pathway serve as chemo-attractants for leukocytes to the mucosa. Additionally, the active migration of L3 into the mucosa damages the tissue and induces the production of IL-33 which helps activate $CD4^+$ T cells (Bowdridge, 2009). GIN can also produce products (excretory/secretory molecules) that can directly influence the migration of leukocytes such as eosinophils and neutrophils. Using in vitro cell migration assays, Wildblood et al. (2005) demonstrated that the excretory/secretory products of *H. contortus* and *T. circumcincta* L3 act as chemo-attractants to eosinophils and illicit an eosinophil migratory response as potent as IL-5 and eotaxin, The secretory/excretory molecules of the parasite also act as chemo-attractant agents for neutrophils, and mast cells (Reinhardt et al., 2011; Wildblood et al., 2005).

The role of eosinophils during GIN infection has been the subject of several studies. Rainbird et al. (1998) examined the direct effect of eosinophils on L3 *H. contortus in vitro* by incubating L3 in the presence of mammary wash obtained eosinophils and serum from an infected or uninfected animal. Primed eosinophils caused a reduction in larval motility within 6 hours of incubation while unprimed eosinophils did not adhere to larvae and did not reduce motility until 24 hours. However, the addition of IL-5 with antibodies and complement to unprimed eosinophils caused a decrease in larval motility in a manner similar to primed eosinophils with positive serum. Transmission electron microscopy of immobile larvae showed attached, degranulated eosinophils and cuticular swelling and destruction of the musculature below the cuticle at the site of eosinophilic attachment (Rainbird et al., 1998). The results of the study suggested that eosinophils play a role in the expulsion of larvae by directly damaging the parasites when in the presence of activating factors. This was later supported by Balic et al. (2006) who examined the interplay between eosinophils and *H. contortus* larval stages *in vivo.* In this study, 2-3 year old merino ewes were repeatedly immunized and allowed to rest for either 9 or 22 weeks before challenge infection and subsequently euthanized at 1 or 2 days post challenge. Challenged animals showed elevated levels of eosinophils within the abomasal mucosa and were observed via light and scanning electron microscopy in granulomatous formations enveloping larvae within the mucosa. Transmission electron microscopy of larval cross sections showed similar associations between larvae and eosinophils as described by Rainbird et al. (1998). Additionally, high levels of IL-4 were present in these animals that were not present in controls. However, despite elevated eosinophils in the mucosa, very few were in contact with larvae in sheep challenged 22 weeks after infection. In these animals, levels of IL-4 were undetectable. The results of both studies indicate clear eosinophil mediated killing of

larvae that occurs only when eosinophils are activated (Balic et al., 2006). Activation signals are likely to come from a variety of sources including Th2 $CD4^+$ T cells, B cells, natural helper cells, and alternatively activated macrophages (Balic et al., 2006; Behm and Ovington, 2000; Rainbird et al., 1998)

Studies where the innate effector cells of resistant and susceptible sheep during infection have been compared show mixed results. Eosinophils have been found at increased levels in the peripheral blood and abomasal mucosa of resistant breeds in several studies (Bricarello et al., 2004; Gill, 1991; Muñoz-Guzmán et al., 2006; Saddiqi et al., 2009; Shakya et al., 2009; Shakya et al., 2011; Terefe et al., 2007; Terefe et al., 2009). Alternatively, studies have also shown cases in which no differences in eosinophils are detected between resistant and susceptible breeds despite profound differences in parasitological parameters such as worm burden and FEC (Amarante et al., 2005; Amarante et al., 1999; Bahirathan et al., 1996b; Zajac et al., 1990). Additionally, the *in vitro* ability to kill larvae by eosinophils do not differ between breed type (Terefe et al., 2009).

Mast cells and globule leukocytes have also been shown to play a role during GIN infection in ruminants. Globule leukocytes are a type of intraepithelial mast cell with granules that are larger than traditional epithelial mast cells (Balic et al., 2000b). Mast cells are resident cells within the abomasal mucosa and as such are one of the first cells encountered during GIN infection. However, like eosinophils, the expression of effector functions in mast cells during infection are dependent upon activation of the mast cell by various parasite specific and non-specific stimuli including complement, surface pattern recognition receptors, receptors that bind to inflammatory products that are present during infection, and worm specific IgG and IgE antibodies via FcR receptors (Abraham and St. John, 2010). One of the results of activation is

degranulation and release of effector molecules including leukotrienes which have been shown to promote neutrophil recruitment in humans (McIntyre et al., 1986), as well as IL-4, and IL5 which are important in eosinophil activation and recruitment (Galli et al., 2005). Additionally, mast cells can increase mucus production within mucosal compartments such as the GI tract and increase peristalsis via the release of histamine (Metcalfe, 1984). In ruminant GIN infection, mast cells are most commonly associated with the rapid rejection response that occurs within the first 24 hours of infection (Miller, 1984). Huntley et al (1984) found elevated levels of a mast cell proteinase (SMCP) present in abomasal mucosal tissue and lymph nodes of sheep immunized and subsequently challenged with GIN compared to unimmunized sheep. Similarly, by incubating GI mast cells with *T, colubriformis* and *H. contortus* antigen, Bendixen et al (1995) found that the release of SCMP and histamine occurred within 30 minutes of exposure while leukotriene secretion was found after 3 hours. They also noted that SCMP release was higher in mast cells taken within the first two weeks of immunization and levels decreased without further challenge. While the exact mechanism by which this occurs is largely unknown, it is believed that the products of mast cell and GL degranulation create an inhospitable environment for the larvae and prevent larval establishment (Huntley et al., 1992).

Studies comparing resistant and susceptible sheep breeds have shown mixed results in terms of the role that mast cells and GLs play in breed resistance. Gamble et al. (1992) found higher numbers of GLs within the abomasal mucosa of St. Croix lambs compared to Dorset lambs and were negatively correlated with worm burden. Similarly, Bricarello et al. (2004) found elevated GLs in resistant Spanish Crioula Lanadada lambs compared to Corriedale lambs and in resistant Native compared to Suffolk lambs (Shakya et al., 2009). Studies using Native and Suffolk lambs have found elevated mast cells in all regions of the abomasal mucosa (cardiac,

fundic, and pyloric) in Native lambs during both early and late infection compared to Suffolk lambs (Shakya et al., 2009; Shakya et al., 2011). Elevated mast cells that are negatively correlated with FEC have also been shown during challenge infection in Lohi sheep compared to Kachi and Thalli sheep (Saddiqi et al., 2009). Alternatively, Zajac et al. (1999) found no differences in mast cell and GL numbers within the abomasal mucosa of St. Croix, Florida Native, and Dorset/Rambouillet crossed sheep. Similar results were observed by Amarante et al. (2005) when comparing Santa Ines, Suffolk and Ile de France sheep, Munoz-Guzman et al (2006) when comparing Barbados Black Belly and Columbia and Terefe et al (2007) when comparing INRA 401 and Barbados Black Belly sheep (GLs only). This suggests that multiple mechanisms are responsible for resistance and worm expulsion in sheep.

1.6.2.2 Antibody

Infections with GIN illicit the production of parasite specific antibody isotypes IgG, IgA, and IgE by plasma cells and occur sooner and in greater magnitude during secondary infections compared to primary infections (Canals and Gasbarre, 1990; Schallig et al., 1994). These increases have been shown to occur within 14-21 days post challenge (Canals and Gasbarre, 1990; Lacroux et al., 2006). This coincides with the establishment of patency in GIN infection and suggests that antibodies may play a greater role in immunity towards the adult stages of GIN rather than larval stages. In one study, elevated levels of serum IgA were found in Barbados Black Belly sheep compared to Columbia sheep (Muñoz-Guzmán et al., 2006); however, conflicting results were found by Amarante et al. (2005) who found no differences in IgA in three different sheep breeds. Similar results in IgA were observed by Shakya et al. (2009) using Native and Suffolk lambs, however, the serum IgE levels of Native lambs increased significantly from days 14-42 post challenge compared to Suffolk lambs.

1.6.2.3 Cytokines and Th2 Helper T cells

Whereas eosinophils, mast cells, and GLs are end-point effector cells that can directly affect GIN infection, cytokines play an important role as end-point mediators that coordinate and modulate immune responses directed against parasite challenge. Cytokines are essential for the development of naïve $CD4^+$ T cells to shift to the Th2 lineage that is necessary for protective responses during GIN infection (Lacroux et al., 2006; Meeusen et al., 2005). Dexamethasone is a glucocorticosteroid with immunosuppressive properties that has been shown to prevent T cell growth and proliferation (Migliorati et al., 1997). In a study by Presson et al. (1988) , administration of dexamethasone to genetically resistant sheep abrogated resistance and resulted in parasitological parameters similar to genetically susceptible sheep of the same breed. This was further supported by Pena et al. (2004), who showed a reduction in serum antibody titers to *H. contortus* antigen and a decrease in lymphocyte percentage in circulation in Native lambs that had received dexamethasone treatment. Pena et al. (2006) then demonstrated the requirement of $CD4^+$ T cells in the involvement of breed resistance to *H. contortus* in Native lambs. In this study, depletion of $CD4^+$ T cells via blocking antibody abrogated resistance shown as an increase in FEC and worm burden, compared to non-depleted Native lambs (Peña et al., 2006). These studies show the importance of $CD4^+$ T cells in breed resistance. Th2 cytokines are produced by $CD4^+$ T cells and various other cells. These cytokines are necessary for the activation, proliferation and recruitment of effector cells to the site of infection during GIN infection (Lacroux et al., 2006; Meeusen et al., 2005). Th2 cytokines include IL-4, IL-5, IL-13, IL-25 and IL-33. Various studies have shown that secondary infections with GIN result in elevated levels of IL-4, IL-5, and IL-13 both in the mucosa at the site of infection (Balic et al., 2006; Terefe et al., 2009).

1.6.3 Self-Cure

The self-cure reaction is a phenomenon first observed by Stoll (1929) in which the adult *H. contortus* burden in sheep is expelled following the intake of new L3 and is associated with the molting of L3 to L4 within the abomasal mucosa (Soulsby and Stewart, 1960). Since this observation was made, studies have shown that self-cure occurs naturally on pasture and can be induced artificially through inoculation with L3 (Dargie and Allonby, 1975; Stewart, 1953). Additionally, Stewart (1953) showed that histamine levels were elevated in the peripheral blood of sheep that had undergone self-cure while the same response was absent in those that had not. In order for self-cure to occur, certain parameters must be met. First, an animal must be currently infected with GIN. Second, an animal must go through a period of very low larval intake. An extended latent period increases the likelihood of self-cure (Soulsby, 1982). Third, the animal must experience a large challenge dose of L3. It should be noted however, that host genetic factors play a role in the outcome of self-cure, as evidenced by stronger and more frequent self-cure responses in genetically resistant merino sheep (Allonby, 1976). Indeed, self-cure results in one of four possible outcomes (Dargie and Allonby, 1975):

1. The existing worm population and the challenge larval population are both expelled.
2. The existing worm population is expelled but the larvae remain.
3. Adult worms are not expelled and larvae also establish an infection.
4. Adult worms are not expelled and larvae do not establish an infection.

To date, whether or not the self-cure response is immune mediated is unknown, as no studies have been performed that examine the immunological profiles of sheep undergoing self-cure. Furthermore, evidence shows that genetic resistance influences the outcome of a self-cure event.

However, the studies that have been performed have only examined genetic resistance within a breed and not between breeds known to be either resistant or susceptible to infection.

1.7 Hypothesis

There are differences in the ability of Suffolk and Native lambs to produce a self-cure response that are a result of immunological differences in magnitude and response time.

1.8 Research Objectives

The objectives of this research were to: 1. Assess the ability of GIN resistant Native sheep and GIN susceptible Suffolk sheep to produce a self-cure response; 2. Define cellular recruitment within the abomasal mucosa and peripheral blood; 3. Quantify the mRNA expression of select Th1, Th2, and Treg cytokines; and 4. Establish serum antibody isotype responses.

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Chapter 2: The Self-Cure Phenomenon in Gastrointestinal Nematode Resistant Gulf Coast Native and Susceptible Suffolk Sheep: Comparison of Parasitological Parameters and Cellular Profiles

2.1 Introduction

Gastrointestinal nematode parasitism in small ruminant production systems is responsible for millions of dollars in economic losses worldwide due to decreased gains, cost of treatment and mortality. The GINs of concern are members of the Family Trichostrongylidae and are commonly referred to as trichostrongyles. In tropical and sub-tropical areas such as the southeastern United States, *Haemonchus contortus* is the trichostrongyle of greatest importance. *H. contortus* is hematophagous and parasitizes the abomasum. A single female can account for 50µl of blood loss per day and heavy infections of several thousand worms, can result in acute anemia and death of the animal (Clark et al., 1962).

H. contortus has developed resistance to most commonly used anthelmintics which has further increased the severity the problem and has necessitated the search for control alternatives. Some such alternatives include copper oxide wire particles (Soli et al., 2010), condensed tannin containing forages (Terrill et al., 2009), and nematophagous fungi (Pena et al., 2002). Vaccines are also another potential alternative; however, a better understanding of the mechanism involving parasite expulsion could provide information that is essential for the production of viable vaccines. The absence of a commercial *H. contortus* vaccine indicates that further elucidation regarding the host responses during GIN infection is needed.

Classically, GIN infection illicits a Th2 type of immune response characterized by the production of Th2 cytokines and the recruitment of granulocytes such as eosinophils, mast cells and globule leukocytes to the site of infection during challenge infections (Lacroux et al., 2006; Shakya et al., 2009). However, studies have shown a disparity in levels of these cell types

during challenge infection. For example, Huntley et al. (1992) found that a large challenge infection resulted in increased numbers of mast cells in the mucosa and the clearance of all incoming larvae within 24 hours post challenge. Alternatively, Balic et al. (1999) found elevated levels of eosinophils in close association with larvae that appeared to be degraded. This suggests that multiple mechanisms for worm expulsion may exist.

Self-cure is a phenomenon that occurs in sheep that are infected with GIN and acquire a heavy challenge infection following a period of little to no larval intake (Soulsby and Stewart, 1960; Stoll, 1929). Within the first 7 days of this infection the existing infection is cleared and in some cases the challenge larval infection is also cleared (Dargie and Allonby, 1975).

Several studies have demonstrated that certain sheep and goat breeds display higher levels of resistance against GIN than others, as indicated by decreased FEC and worm burden when compared to breeds that are more susceptible (Baker et al., 1999; Burke and Miller, 2002; Gamble and Zajac, 1992; Saddiqi et al., 2009). Gulf Coast Native (Native) sheep of the southeastern United States has been shown to be more resistant to natural GIN infection than Suffolk sheep under natural pasture challenge conditions (Bahirathan et al., 1996; Miller et al., 1998). Additionally, Pena et al. (2004 and 2006) found that this resistance had an immunological component. Shakya et al. (2009) further found that Native lambs had higher levels of peripheral eosinophils compared to Suffolk lambs at day 7 post infection and elevated levels of eosinophils and neutrophils within the abomasal mucosa at days 7 and 14 post infection.

No studies have been done to examine the possible differences in the self-cure response between resistant and susceptible sheep. The objective of this experiment was to determine the level of "self-cure" achieved in Native and Suffolk sheep and compare and characterize the

cellular immune responses that may be involved in any difference in response during early infection.

2.2 Materials and Methods

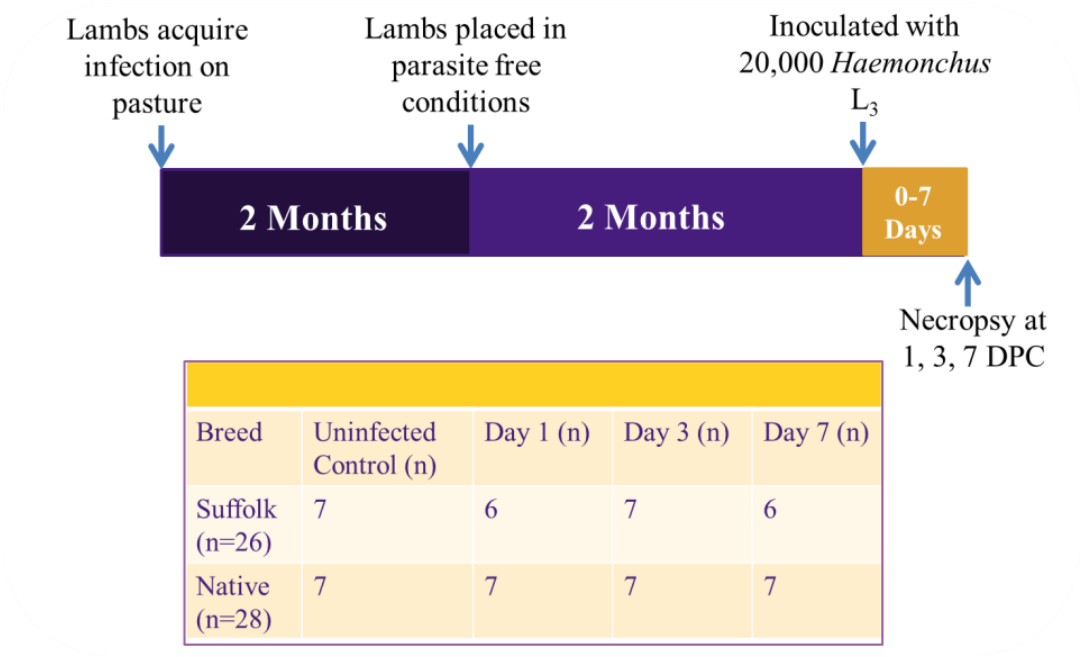
2.2.1 Location and Animals

The study was conducted (July to October 2010) at the School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA. Fifty four 8 month old (26 Suffolk and 28 Native) lambs were maintained in concrete floor pens. Lambs were fed a lamb growing ration and water was provided ad libitum. The protocol was approved by the Louisiana State University Institutional Animal Care and Use Committee.

2.2.2 Experimental Design and Sampling Scheme

Lambs were allowed to initially graze on pasture to acquire a natural GIN infection and then were moved to the study pens. Feces, for FEC, were collected directly from the rectum, at weekly intervals for 2 months, to confirm infection status. Within breed, lambs were randomly assigned to 4 groups (3 infected and 1 control) with 6-8 animals each (Figure 2.1).

For infected groups, lambs were given a challenge infection dose of 20,000 freshly cultured *H. contortus* L3 on day 0 to induce a self-cure response. L3 were administered in 6 ml of water in plastic syringes with a metal extender. Control group lambs were given sham inoculations of 6 ml of water. An equivalent number of lambs in each group were euthanized and necropsied on days 1, 3 and 7 post inoculation. Prior to euthanasia, feces were collected for FEC and culture. Blood samples, for PCV and differential leukocyte counts, were also collected.



Breed	Uninfected Control (n)	Day 1 (n)	Day 3 (n)	Day 7 (n)
Suffolk (n=26)	7	6	7	6
Native (n=28)	7	7	7	7

Figure 2.1 Experimental design and sampling scheme

At necropsy, abomasa, small and large intestines were removed, washed individually in separate buckets and an aliquot of each organ was collected for worm recovery, identification and enumeration (Miller et al., 1987). Abomasa were then soaked overnight and aliquots of the soak samples were collected for recovery of larvae. In addition, abomasal mucosa samples, abomasal lymph nodes and prescapular lymph nodes were taken for histopathology.

2.2.3 Fecal Egg Count Determination

2.2.3.1 McMaster method

Determination of FEC was performed using a modified McMaster method (Whitlock, 1948). Two g of feces were weighed in plastic cups and crushed using a wooden tongue blade. Twenty-eight ml of a saturated salt solution (specific gravity 1.21) was added and mixed thoroughly. The fecal solution was then briefly mixed using an electric mixer and pipetted into the chambers of a McMasters slide (Chalex Corp. Ketchum, ID). Trichostrongyle type eggs on and within the grids of each of the two chambers on each slide were counted on a compound

microscope at 100x. The total number of eggs counted was multiplied by a factor of 50 to convert to eggs per gram (EPG). Fecal samples with a recorded count of 0 were subjected to sucrose double centrifugation.

2.2.3.2 Double Centrifugation Technique

Two g of feces was weighed in plastic cups and crushed using a wooden tongue blade. Fifteen ml of water was added and the contents were mixed thoroughly. The resulting slurry was then strained through a wire mesh tea strainer into 15ml centrifuge tubes. The samples were centrifuged at 2000g for 10 minutes and the supernatant was discarded. The tubes were then filled with approximately 8 ml of sucrose solution (specific gravity 1.25) and thoroughly mixed using 2 wooden applicator sticks ensuring that the sediment was broken up completely. Tubes were then filled to a positive meniscus with sucrose solution, covered with a coverslip and centrifuged at 1500g for 10 minutes. Coverslips where then removed and placed onto individual glass slides and all trichostrongyle-type eggs found on the entire coverslip were counted. The total number of eggs counted on each slide was divided by a factor of 2 to convert to EPG.

2.2.3.3 Fecal Egg Count Reduction Test

The percent reduction in FEC between time points within each breed was determined using the equation: $\frac{Mean\ Cont\ FEC - Mean\ Trt\ FEC}{Mean\ Cont\ FEC}\ x\ 100$, where *Trt* and *Cont* represent treated (challenge inoculation) and control, respectively.

2.2.4 Fecal culture

Feces from each group were pooled, weighed and then mixed with vermiculite in a large plastic bin. The bin was covered with aluminum foil and approximately 30 small holes were punched into the foil to provide aeration of the cultures. Cultures were incubated at 23°C for 14 days. Cultures were checked periodically and water was added as needed to prevent desiccation.

The Baermann technique was used to extract larvae from the fecal cultures. Cultures were wrapped in a sheet of cheese cloth and placed on wire mesh within large funnels with a 15 ml centrifuge tube attached to the spout via a short piece of rubber tubing. Lukewarm water was then used to fill the funnel until the cultures were submerged and then allowed to sit overnight. The 15 ml tubes containing larvae were then removed and 1 ml of the volume was replaced by 10% formalin for preservation.

2.2.5 Larval Identification and Enumeration

A 100 µl aliquot was taken from each tube, placed on a glass slide and stained with lugol's iodine. The first 100 larvae were counted and identified to genus to determine population distribution. If 100 larvae were not counted in the first aliquot, additional 100 µl aliquots were taken until 100 were identified. After 100 larvae were identified, any remaining larvae on the slide were counted and the total number of larvae per g of feces was determined via extrapolation.

2.2.6 Necropsy and Sample Collection

Abomasa, small and large intestines were removed from the animals at necropsy and placed into separate containers. The abomasum was opened and before washing, approximately 1 by 0.5 inch long pieces of mucosa were clipped from 3 different regions (cardiac, fundic and pyloric) and preserved in 10% formalin for histopathology. Then, the abomasum and other GI tract organs were washed with water and contents were collected in a 10 l bucket and raised to 5 l. The contents were mixed and a 500 ml aliquot was taken in a 500 ml HDPE container (Fisher Scientific). After settling for 2-3 hrs, 50 ml was poured off and replaced with 10% formalin for preservation. Abomasa were placed in bins and soaked overnight to stimulate the release of larvae (L3/L4) from within the mucosa and processed in the same manner described above.

2.2.7 Helminth Enumeration

A 100 ml aliquot from each bottle was taken and passed through a 150 µm wire mesh sieve to recover any worms present. Contents were backwashed from the sieve into a beaker and stained with iodine. Subsamples were examined under a dissecting microscope and the first 100 worms found were placed on glass slides and identified to genus, gender, and life stage. If 100 worms were not counted in the aliquot then a subsequent 100 ml aliquot was taken and the procedure repeated until 100 worms were recovered. All worms in the aliquots were counted and the total population of worms was determined via extrapolation.

2.2.8 Packed Cell Volume

Blood was collected via jugular venipuncture into 7 ml EDTA tubes (Becton Dickinson & Co.). Capillary tubes were filled to three quarters full and sealed with clay and spun for 10 m in an Autocrit Ultra 3 hematocrit centrifuge (Becton, Dickson, & Co.). PCV was determined using the PCV scale.

2.2.9 Leukocyte Differentials

EDTA blood was used to prepare thin blood smears on clean glass slides (labeled with animal number) and stained using Diff-Quick. Complete blood counts were performed on the stained blood smears using a compound microscope at 1000x magnification. The first 100 leukocytes encountered were identified to determine the percentage of monocytes, neutrophils, basophils and eosinophils in circulation.

2.2.10 Histopathology

Abomasal tissue samples, abomasal lymph nodes, and prescapular lymph nodes were fixed in 10% phosphate buffered formalin, trimmed, placed in histological cassettes and embedded in paraffin wax. Thick (3 mm) sections were cut from each tissue block using a

microtome (Leica) and adhered to glass slides. Two slides were prepared for each tissue sample. Slides were stained with either Hematoxylin-eosin or Tryptan blue and the average number of white blood cells per 400x field was determined from 20 fields per slide for numbers of eosinophils, neutrophils, globule leukocytes and mast cells, respectively, and recorded as mean number per mm^2.

2.2.11 Statistical Analysis

Data was analyzed using the SAS statistical package version 9.1.6 (SAS Institute Inc.). FEC was log transformed. Transformed data and leukocyte differentials were analyzed with an analysis of variance test (ANOVA) to examine differences within breed across time points and student's t-test to examine differences between breeds at specific time points. P value of ≤ 0.05 was considered significant.

2.3 Results

2.3.1 FEC

After natural infections were established and allowed to stabilize, FEC was higher in Suffolk (14,041 EPG) than Native (5,412 EPG) lambs at the start of the study (day 0) and remained higher than Native lambs throughout the experiment (Table 2.1). Figure 2.2 represents the percent reduction in FEC on day 1, 3 and 7 post-challenge compared to the control group. At day 1 post challenge, FEC increased slightly in both groups. At day 3 post-challenge, FEC reduction for Native and Suffolk lambs was 86% 38%, respectively, and at day 7 98% and 90%, respectively. The difference between Native and Suffolk at days 3 and 7 was significant ($p<0.05$).

Table 2.1 Mean fecal egg count (eggs per gram) and standard error (SE) in non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group.

	Native		**Suffolk**	
DPC	Mean	SE (+/-)	Mean	SE (+/-)
Control	5412.5	2158.2	14041.7	2307.9
1	5950.0	1745.9	15441.7	4186.1
3	542.9	190.4	8702.3	2847.9
7	164.3	132.6	2708.3	1498.9

2.3.2 PCV

Blood PCV remained consistent and at normal levels for both breeds (30% and 31 % for Native and Suffolk, respectively) during the study (data not shown).

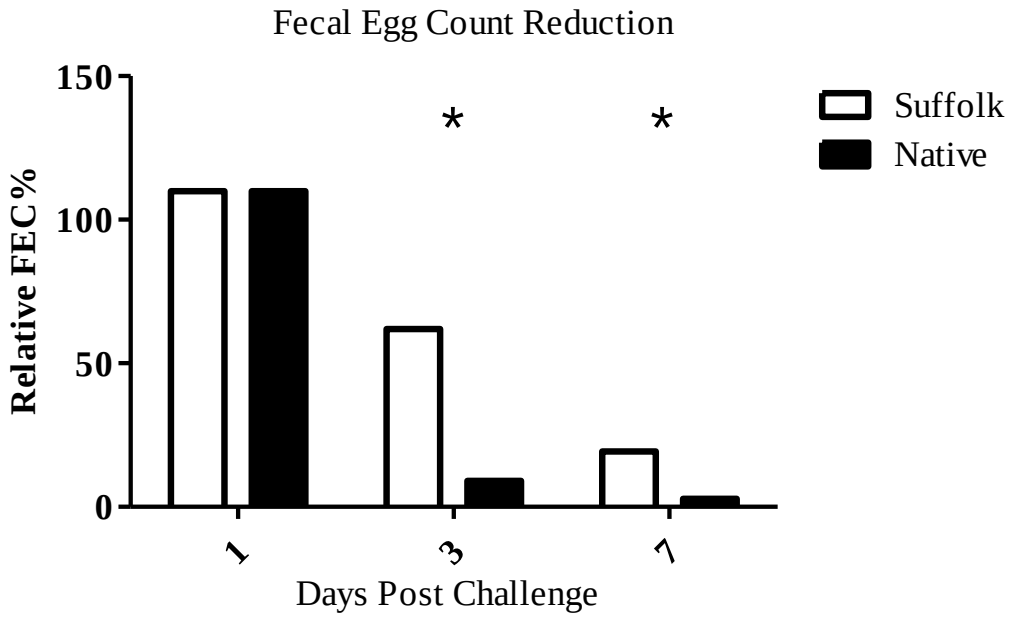


Figure 2.2 Fecal egg count reduction in non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Asterisk (*) indicate significance between breeds. $P<0.05$

2.3.3 Abomasal Worm Burdens

Throughout the study, Suffolk lambs maintained a 4 fold higher adult worm burden than Native lambs (Table 2.2). Worm burden was similar between control and both Native and Suffolk challenged groups on day 1 and 3, post infection. At day 7, adult worm burden decreased significantly in both breeds

No larvae were found in either Suffolk or Native control lambs. Larvae appeared in both Suffolk and Native challenged lambs on day 1 (Table 2.2). For Suffolk challenged lambs, L3 remained at the same level on days 1 and 3, and were undetectable at day 7. For Native challenged lambs, L3 burden was similar to Suffolk challenged lambs on day 1 and then decreased to significantly ($p<0.05$) lower levels on day 3. On day 7, L4 were present in both breeds. Between breed differences were significant ($p<0.05$) on days 3 and 7.

Table 2.2 Mean adult, L4, and L3 recovery from the abomasum of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Different superscript letters indicate significance ($p<0.05$) within breed. Asterisk indicates significance ($p<0.05$) between breeds.

	Native						**Suffolk**					
Groups	Adult		L4		L3		Adult		L4		L3	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	430.0^{a*}	241.8	0.0^{a}	0.0	0.0^{a}	0.0	2007.1^{a*}	595.8	0.0^{a}	0.0	0.0^{a}	0.0
1 DPC	392.8^{a*}	167.9	0.0^{a}	0.0	448.5^{b}	141.4	1941.3^{a*}	651.7	0.0^{a}	0.0	336.6^{b}	114.2
3 DPC	254.2^{a*}	57.4	0.0^{a}	0.0	98.5^{c*}	32.0	1862.0^{a*}	575.3	0.0^{a}	0.0	416.6^{c*}	210.6
7 DPC	30.0^{b}	30.0	724.2^{b*}	554.3	0.0^{a}	0.0	266.6^{b}	212.3	4440.0^{b*}	620.6	0.0^{a}	0.0

2.3.4 Leukocyte Differentials

2.3.4.1 Lymphocytes

Lymphocyte numbers in Suffolk challenged lambs were similar throughout the study, with no significant ($p>0.05$) difference at any time point (Figure 2.3). In contrast, lymphocyte

numbers in Native lambs increased on days 3 and 7 compared to control day 1. Differences were not significant ($p>0.05$) between Suffolk and Native lambs compared to control lambs on day 1, but were significantly ($p<0.05$) higher in Native lambs compared to Suffolk lambs on days 3 and 7.

2.3.4.2 Eosinophils

Peripheral eosinophils increased in both Suffolk and Native challenged lambs at each time point (Figure 2.4). Eosinophils on day 7 were significantly ($p<0.05$) higher than control lambs for both breeds. No significant ($p>0.05$) differences were observed between breeds at any time point.

2.3.4.3 Neutrophils

Neutrophils were significantly ($p<0.05$) higher in Suffolk than Native lambs (challenged or control) on days 1, and 3 but not at day 7 (Figure 2.5).

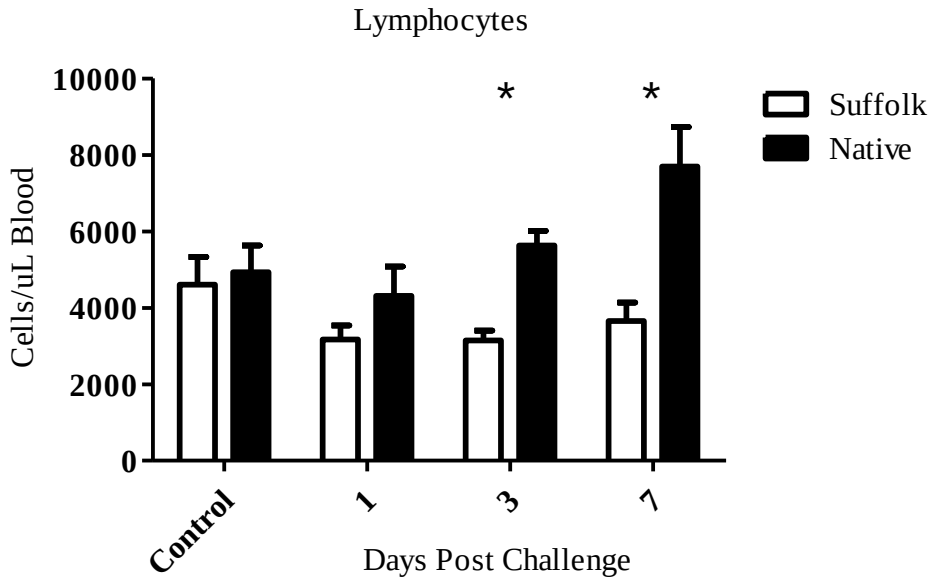


Figure 2.3 Mean peripheral lymphocyte counts of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Asterisk (*) denote significant differences between breeds

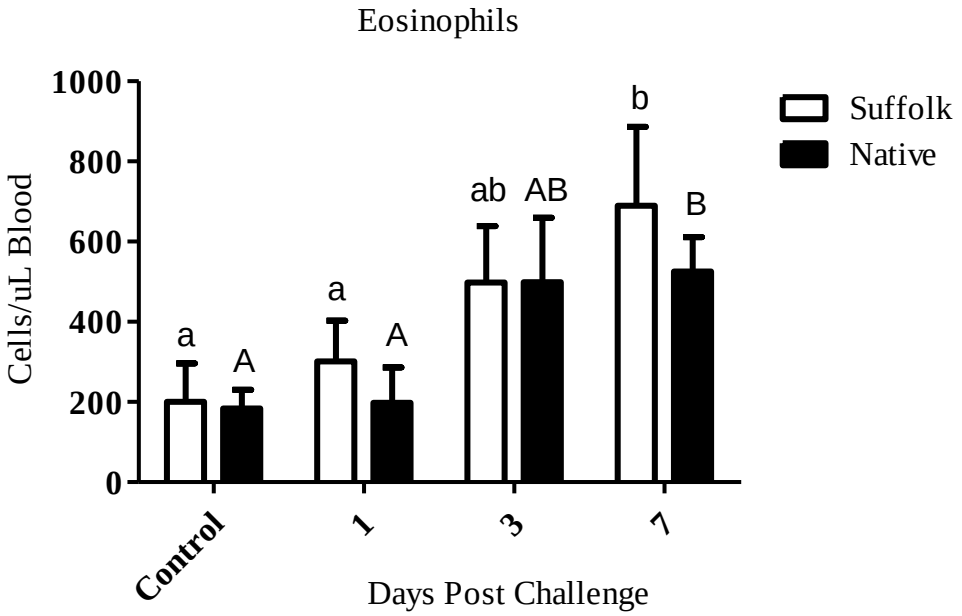


Figure 2.4 Mean peripheral eosinophil counts of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Different letters indicate significance within breed. $p<0.05$

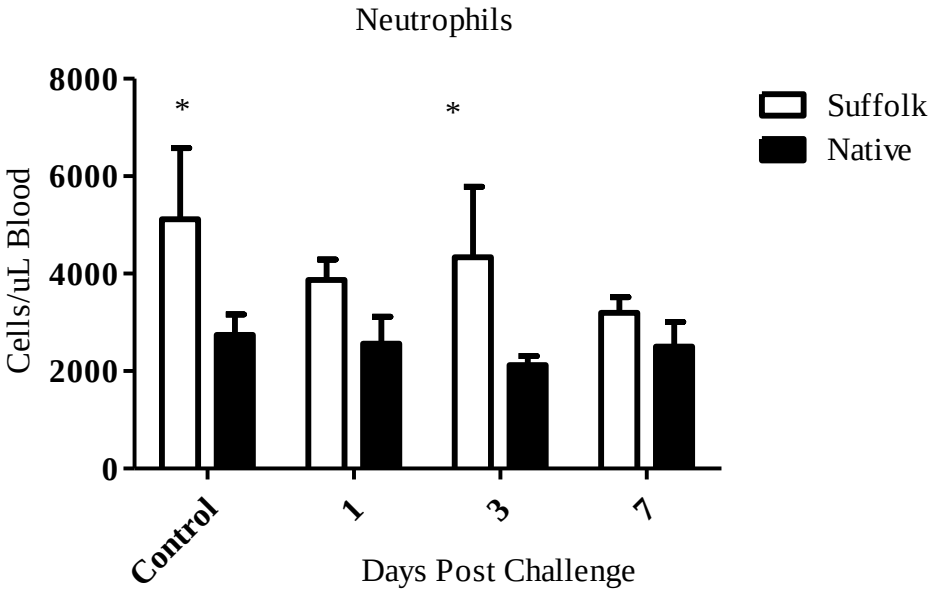


Figure 2.5 Mean peripheral neutrophil counts of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Asterisk (*) denote significance between breeds. $p<0.05$

2.3.4.4 Monocytes

Monocytes were similar ($p<0.05$) for both breeds throughout the study (Figure 2.6).

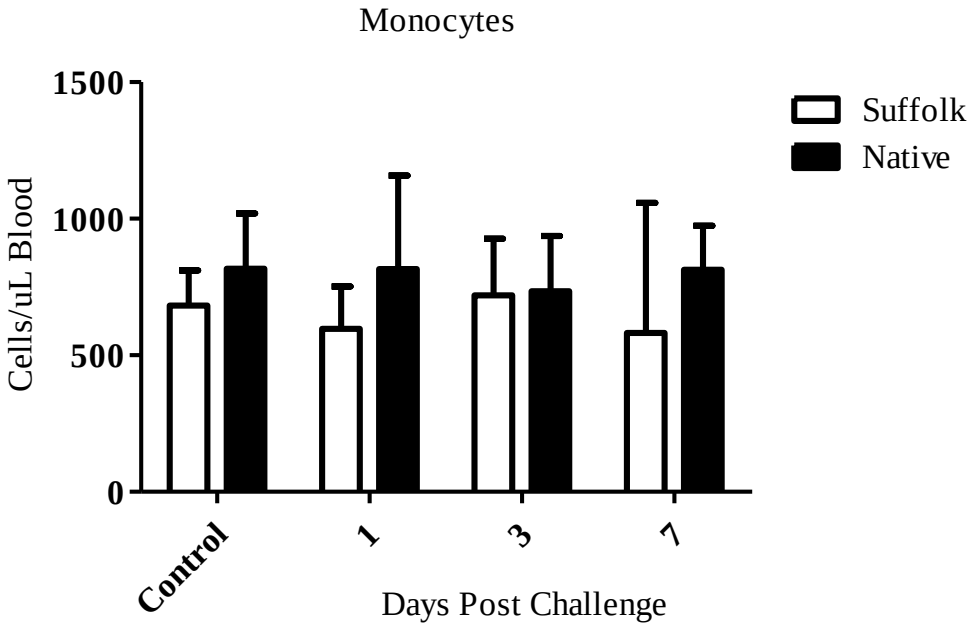


Figure 2.6 Mean peripheral monocyte counts of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group.

2.3.5 Abomasal Cell Numbers

2.3.5.1 Eosinophils

Eosinophils were similar ($p<0.05$) in all sections (cardiac, fundic, pyloric) of the abomasal mucosa for Suffolk and Native control lambs (Figure 2.7).

On day 1, there was no difference ($P>0.05$) in eosinophils in any abomasal section of either breed; however, numbers were higher in Native than Suffolk lambs.

On day 3, eosinophils were significantly ($p<0.05$) higher in all abomasal sections in Native than Suffolk challenged. Of note, numbers in both fundic and pyloric sections were six-

fold higher, while cardiac sections were three-fold higher in Native lambs compared to Suffolk lambs.

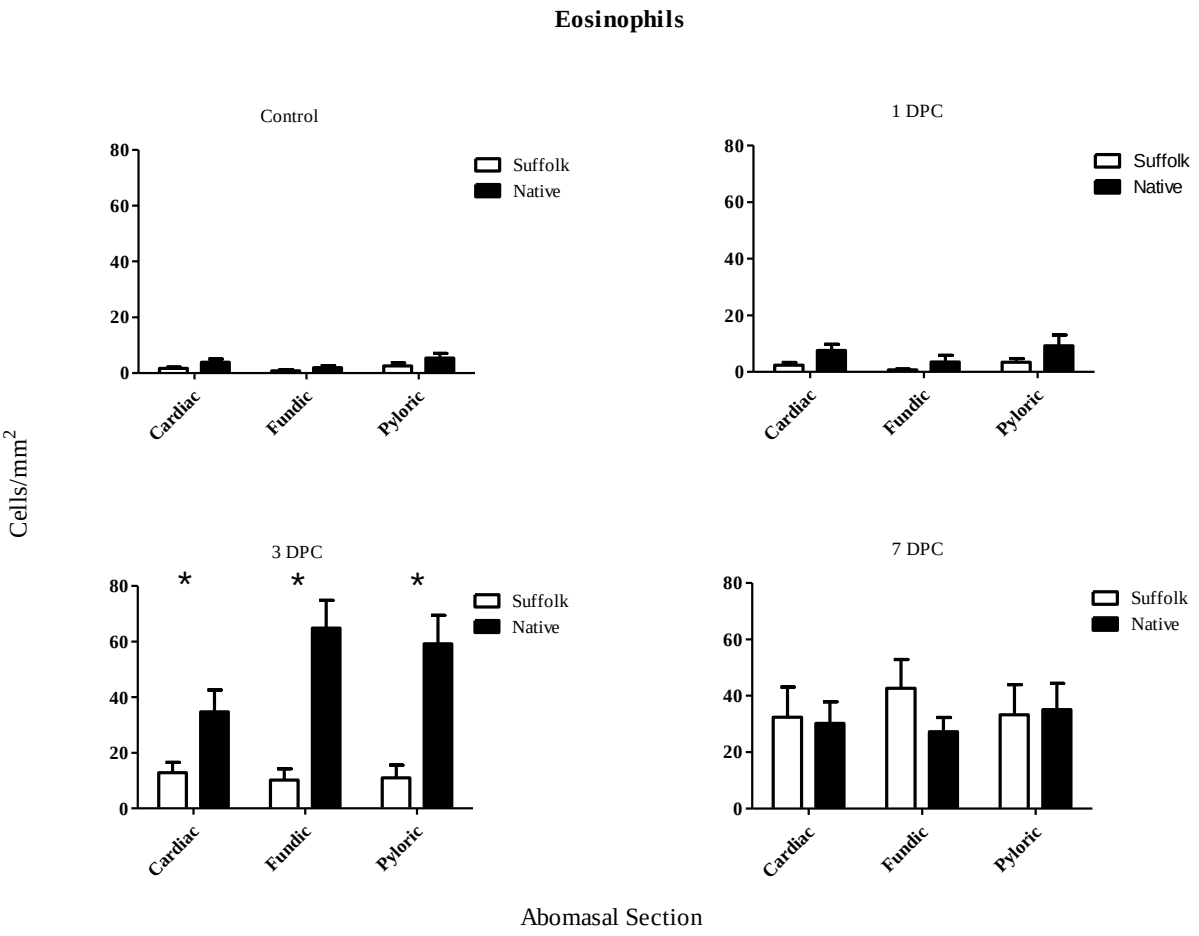


Figure 2.7 Mean eosinophil counts in within the cardiac, fundic and pyloric regions of the abomasum of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Asterisk (*) denote significant differences between breeds. P>0.05

On day 7, there was no difference ($p>0.05$) in eosinophils of any section or between breeds. For Suffolk lambs, eosinophils in each section were significantly ($p<0.05$) higher on day 7 than in days 1, 3 and control. For Native lambs, eosinophils in each section were significantly ($p<0.05$) higher on day 3 than on days 1, 7 and control, and significantly ($p<0.05$) higher on day 7 than on day 1 and control.

2.3.5.2 Neutrophils

Neutrophils were similar ($p>0.05$) and present in very low numbers in both control and challenged lambs of both breeds during the study with the exception of the pyloric region of Native lambs on day 7, which was significantly ($p<0.05$) higher than Suffolk lambs (Figure 2.8).

2.3.5.3 Mast Cells

Mast cells were similar ($p>0.05$) and present in very low numbers in both control and challenged lambs of both breeds on days 1 and 3 (Figure 2.9).

On day 7, mast cells were significantly ($p<0.05$) higher in the pyloric regions of both breeds compared to control on days 1 and 3. Additionally, mast cells were significantly ($p<0.05$) higher in Native lambs compared to Suffolk lambs in all regions.

2.4 Discussion

A successful self-cure event is evidenced by a decrease in FEC followed by expulsion of the established adult worm burden in sheep (Dargie and Allonby, 1975). On order for this to occur, the established worm burden must experience a period in which no or very few new larvae are being ingested (Dargie and Allonby, 1975) (Soulsby and Stewart, 1960).

To ensure that a successful self-cure event would occur, we optimized the parameters necessary as outlined in Soulsby et al. (1960). The animals in this study acquired a natural infection on pasture for two months to best simulate on farm conditions and were subsequently removed from pasture and placed in parasite free conditions for an additional 2 months before being challenged with *H. contortus* L3. While self-cure has been shown to occur on pasture (Soulsby, 1957), artificial challenge was chosen to ensure that a consistent inoculation dosage was delivered to each animal.

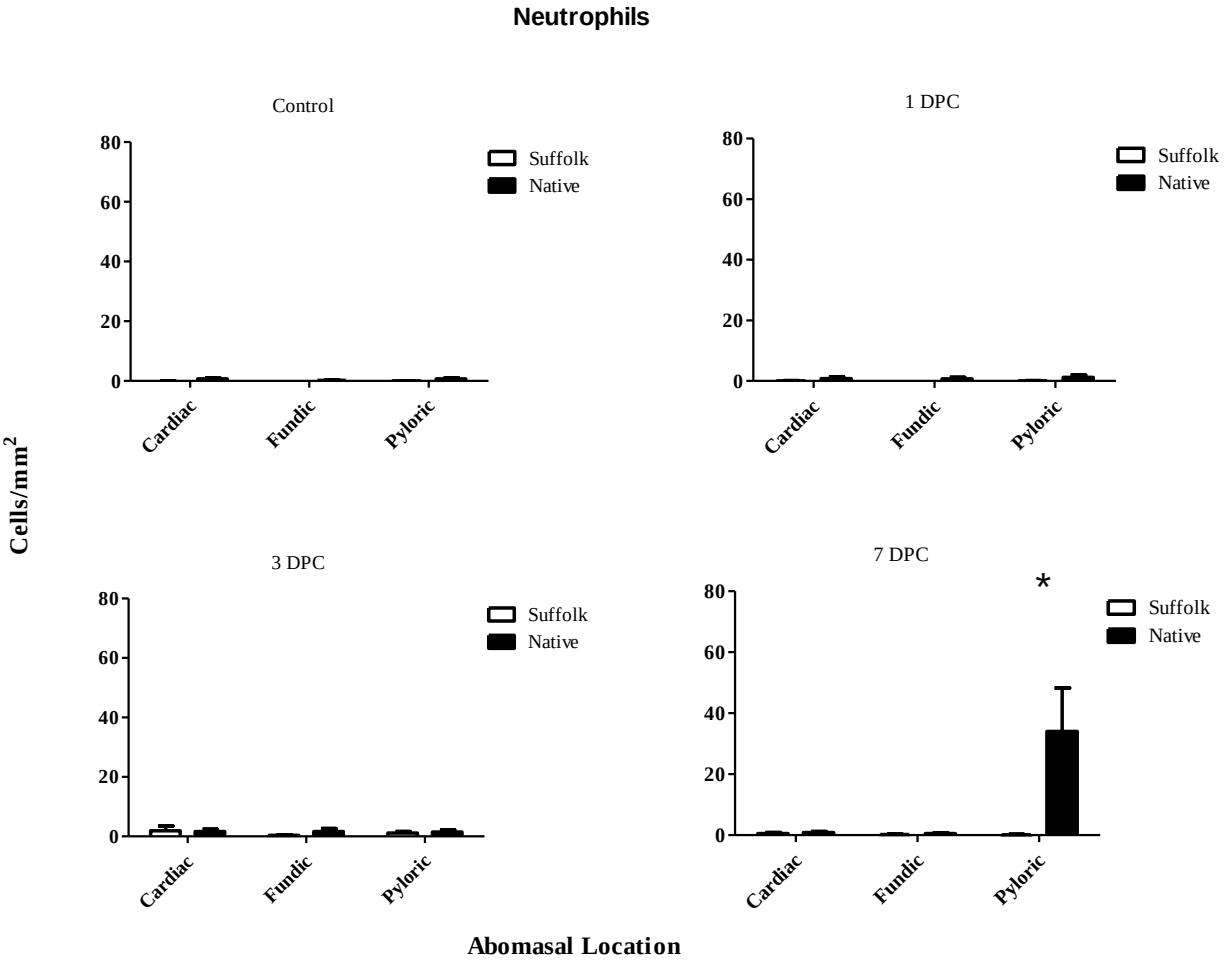


Figure 2.8 Mean neutrophil counts in the cardiac, fundic, and pyloric regions of the abomasa of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Asterisk (*) denote significant differences between breeds. $P<0.05$

At the start of the study, FEC was higher in Suffolk lambs than in Native lambs. This was expected, as it is known that Suffolk lambs normally carry a higher GIN burden than Native lambs (Miller et al., 1998). As expected for a self-cure event, reductions in FEC were seen in both breeds and by day 7, reductions were 98% and 90% in Native and Suffolk lambs, respectively. In addition, on day 3, FEC in Native lambs had already been reduced by 86%, an almost two-fold increase in FEC reduction compared to Suffolk lambs.

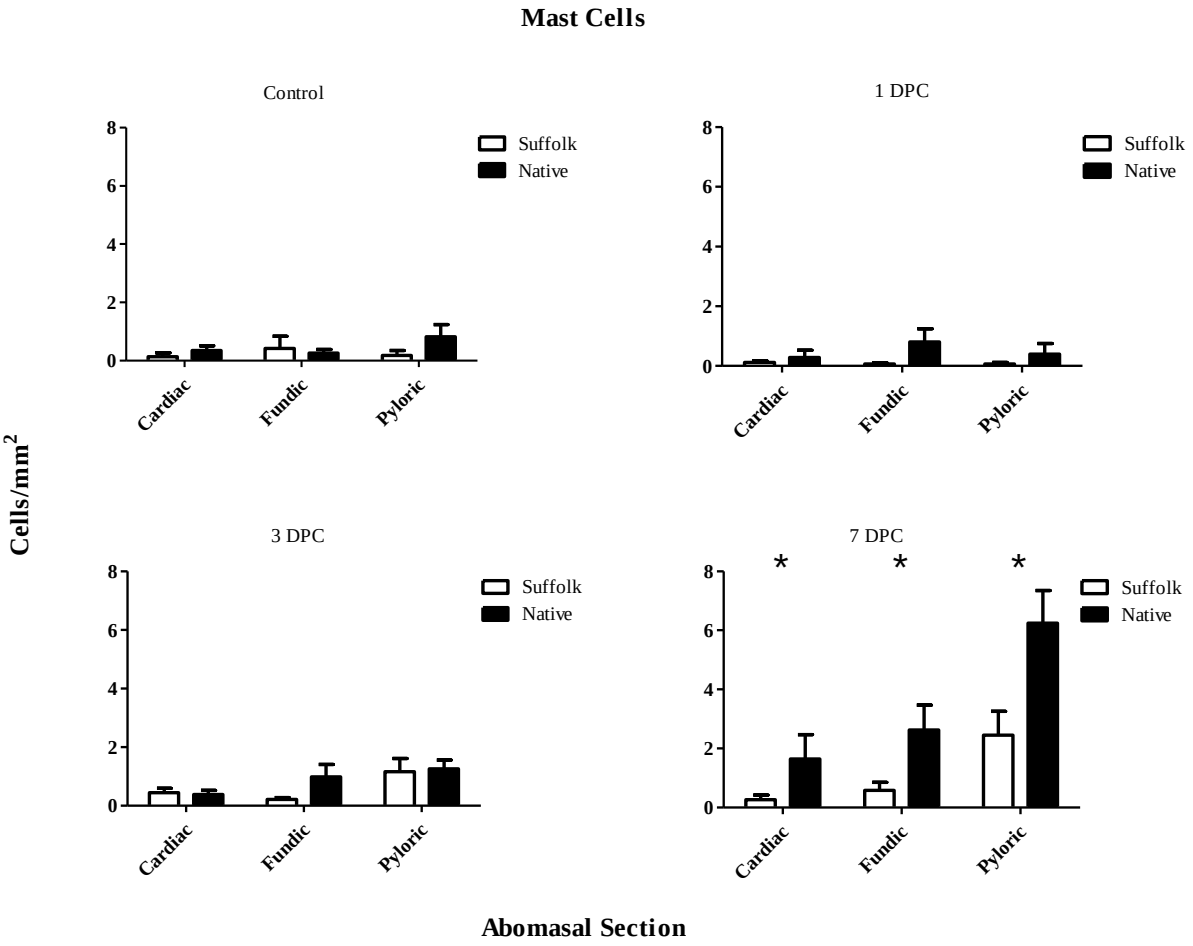


Figure 2.9 Mean mast cell counts in the cardiac, fundic, and pyloric regions of the abomasa of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Asterisk (*) denote significant differences between breeds. $P<0.05$

In order to further substantiate a successful self-cure event, adult and larval burdens were examined. Similar to FEC, more adult worms were present in the abomasa of Suffolk lambs compared to Native lambs and decreases were not detected in either breed until 7 days post challenge. This suggested that both Suffolk and Native breeds were able to effectively clear the established adult worm burden during self-cure. Interestingly, examination of larval burdens suggested that while Native lambs were able to clear larval burdens within the first three days of

challenge, Suffolk lambs did not, as indicated by a larval burden compared to a negligible level in Native lambs. Previous studies have compared self-cure between genetically resistant and susceptible Merino sheep and have found that resistant lambs within the same breed show a much stronger self-cure response in natural infection settings (Allonby, 1976). However, no studies have shown differences in larval expulsion during self-cure between resistant and susceptible breeds of sheep. This study is the first to show differences in parasitological parameters during self-cure between a resistant and a susceptible breed of sheep.

The increase in peripheral eosinophils in both Suffolk and Native lambs is similar to what has been seen in resistant Barbados Black Belly sheep and susceptible Columbia sheep (Muñoz-Guzmán et al., 2006). In this study, the increase in eosinophils in circulation coincides with the decline in FEC in both breeds. Similar results were observed in Romney lambs during challenge infections with *Trichostrongylus* (Buddle et al., 1992). Shakya et al. (2009) observed baseline levels of peripheral eosinophils in both Suffolk and Native lambs during the first 4 days of infection which was followed by an increase in Native lambs beginning at day 7 and continuing to day 42 with peaks which were significantly higher than Suffolk lambs occurring at days 14 and 35 post challenge infection.. This is in contrast to the observations in this study and may be due to differences in infection parameters such as the number of larvae used, and the infection status of the lambs prior to challenge infection. The number of larvae used for infection can affect the magnitude of immune response produced by the animal. If too few larvae are used, then the immune response might be undetectable. Additionally, it is known that the immune response to GIN in sheep varies depending on whether the exposure is primary or secondary in nature, thus the infection status of lambs prior to challenge infection can result in different immune responses (Balic et al., 2000; Salman and Duncan, 1984).

Unlike the levels of peripheral eosinophils, eosinophils in all three abomasal regions were significantly elevated in Native lambs compared to Suffolk lambs on day 3 post-challenge. Additionally, levels in both breeds were higher at day 3 compared to control and day 1. This increase coincides with the reduction in FEC observed on day 3 post challenge and may reflect the differences in magnitude of response between breeds. The increased number of eosinophils on day 3 also corresponds with the expulsion of larvae in Native lambs. The presence of elevated eosinophils within the abomasum during the time of larval expulsion in Native lambs, and the lower levels of eosinophils with accompanying lack of larval expulsion in Suffolk lambs suggested that eosinophils may play a role in larval expulsion during self-cure. The data also suggested a delayed response to the larval challenge in Suffolk lambs as indicated by the significant increase in abomasal eosinophils on day 7 when larvae have already become established within the abomasal mucosa. This is supported by studies that show the larval killing ability of eosinophils occurs before larvae enter the mucosa (Balic et al., 2006; Rainbird et al., 1998; Rotman et al., 1996).

Mucosal mast cells have been shown to play an important role in GIN infection. In this study, mast cells were found significantly elevated in all abomasal regions of both breeds on day 7 and, in addition the level in Native was significantly higher than Suffolk. This elevated level of mast cells in both breeds coincided with the expulsion of adult worm burdens. While elevated mast cells in the Suffolk lambs were relatively low compared to Native lambs, numbers alone do not necessarily indicate functionality (Rothwell, 1989). It is possible that mast cells help to expel the adult worm burden via the release of inflammatory mediators such as histamine and mast cell protease which can cause increased permeability and an inhospitable environment leading to expulsion (Huntley et al., 1992; Stevenson et al., 1994).

Neutrophils were significantly elevated in the pyloric region of the abomasum of Native lambs on day 7. Bowdridge et al (2009) similarly observed increased levels of neutrophils at day 7 post-challenge with *H. contortus* in resistant hair sheep compared to more susceptible wool sheep. Neutrophils have been shown to be involved in the formation of granulomas during infections with *Heligmosomoides polygyrus* in mice (Anthony et al., 2007). However, the role that neutrophils play during GIN in ruminants is largely unknown.

The results of this study indicated that both resistant Native and susceptible Suffolk sheep can undergo a self-cure event, and expulsion of larvae and adult worm burdens occurred sooner in resistant sheep. This might be associated with a greater local eosinophil and mast cell response in the abomasal mucosa on day 3 and day 7, respectively. The delayed response observed in Suffolk sheep to incoming larvae may allow for larval establishment and development within the mucosa before the expulsion response has been expressed. Multiple mechanisms appear to be involved during the self-cure event and any role that neutrophils may play a still undiscovered.

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Chapter 3: Comparison of Cytokine Gene Expression and Antibody Profiles During Self-Cure in Gulf Coast Native and Suffolk Lambs

3.1 Introduction

Gastrointestinal nematode parasitism causes extensive economic losses worldwide. These losses are due to cost of treatment, mortality, and decreased production in the form of decreased milk, wool, and carcass size. In the southeastern United States and other tropical and subtropical areas, *Haemonchus contortus* is the GIN of primary concern. *H. contortus* is hematophagous and parasitizes the abomasum of various ruminants including cattle, sheep, goat, and deer (McGhee et al., 1981). Cases of heavy infection, *H. contortus* can cause anemia, unthriftiness, anorexia, and death.

Infection with GIN in sheep illicits a classic Th2 response characterized by the production of the Th2 cytokines IL-4, IL-5, and IL-13 early during secondary challenge at the site of infection (Craig et al., 2007; Lacroux et al., 2006). Shakya et al (2009) found elevated levels of IL-4 expression at 10 days post infection and elevated expression levels of IL-10 from 7 to 21 days post infection in circulation. Similarly, Bowdridge et al (2009) saw elevated expression levels of IL-13 and IL-33 in abomasal tissue of hair sheep compared to wool sheep at 3 days post infection.

Levels of antibodies have been shown to become elevated during GIN in ruminants. In particular, serum IgA levels increased in response to larval stages, while levels of IgG1 increased in response to adults (Schallig et al., 1994). Additionally, resistant bred sheep have been shown to produce higher levels of parasite specific serum IgG1 and IgA between 10 and 31 days post challenge (Gill et al., 1993). In another study, Gill et al. (2000) found higher levels of IgG1 and IgE produced at 28 days post infection in resistant bred sheep. Shakya et al. (2009) also found

elevated levels of serum IgE in Gulf Coast Native lambs at 14 days post infection while levels remained at baseline in Suffolk lambs.

Self-cure is a phenomenon that occurs in sheep that are infected by GIN and subsequently ingest a new challenge infection following a period of little to no larval intake (Stoll, 1929). This process occurs early during challenge and typically results in the expulsion of the already established infection as well as the incoming larval burden (Dargie and Allonby, 1975).

The purpose of this study was to evaluate the cytokine gene expression profiles at the site of infection and in tissues of immunological importance, and to measure antibody production during self-cure in Gulf Coast Native and Suffolk lambs.

3.2 Materials and Methods

3.2.1 Animals, Experimental Design, and Sample Collection

Details regarding the procedures and parasitological parameters are discussed in detail in Chapter 2. Briefly, Lambs were allowed to graze on pasture to acquire a natural infection of trichostrongyle nematodes for 2 months and subsequently removed from pasture and placed in pens with concrete floors under parasite-free conditions for an additional 2 months. Within breed, lambs were randomly assigned to 4 different groups (3 infected and 1 control) with 6-8 animals each. For infected groups of each breed, lambs were given an infection dose of 20,000 freshly cultured *H. contortus* L3 on day 0. At necropsy, adult *H. contortus* were removed from abomasal contents and washed repeatedly in phosphate buffered saline (PBS) and stored at -20°C until processing for whole worm antigen preparation. Blood samples for serum separation and immunoglobulin isotypes were collected each week prior to infection and at necropsy in red top 10 ml plain vacutainer tubes (No Additive, Becton, Dickinson, & Co.). Whole blood was allowed to sit at room temperature for an hour then centrifuged at 1500x g for ten min. Serum

was then removed from the tubes and stored at -20°C until used. Abomasal mucosa samples were obtained by scraping with a glass slide and placed in RNA Later (Qiagen) for cytokine gene expression analysis via quantitative polymerase chain reaction (qPCR). Abomasal lymph nodes were also collected and placed in RNA Later for qPCR. Abomasal and prescapular lymph nodes were also collected and weighed.

3.2.2 Whole Worm Antigen (WWA) Preparation

Approximately 50 mg of adult *H. contortus* in 1 ml of ice cold PBS were placed into two 2 ml Precellys tissue homogenizing tubes containing 2.8 mm zirconium oxide beads (Bertin Technologies). The tubes were placed into a Tissuelyzer II tissue homogonizer (Qiagen Corp) and homogenized at 125Hz for 30 sec, removed, and immediately placed on ice for 1 min. The process was repeated 7 times. Tubes were then centrifuged at 15,000 rpm for 2 min in a refrigerated microcentrifuge (Eppendorf) to clear the supernatant. The supernatant of each tube was removed carefully using a micropipette to ensure that the sediment plug at the bottom of the tube was undisturbed and transferred into 1.5 ml microcentrifuge tubes and then put on ice. The concentration of soluble protein from the supernatant in each tube was determined using a bicinchoninic acid assay adapted for a microtiter plate (Pierce, Rockford, IL) and stored at -80°C until used.

3.2.3 Enzyme Linked Immunosorbent Assay (ELISA)

Fifty µl of *H. contortus* WWA in 0.5M carbonate buffer at a pH of 9.6 at a concentration of 2 µg/ml was put in each well of high binding polyestrine 96 well microtiter plates (Corning, Inc).. Plates were sealed using a microplate sealing sheet (Fisher Scientific) and incubated at 4°C overnight. Plates were then washed 5 times using PBS with 0.05% Tween 20 and blotted dry. Wells on each plate were blocked with 100 ul of blocking buffer consisting of PBS and 3% fish

gelatin (Sigma-Aldrich), and incubated for one h at 37°C. Wells were then washed 5 times in washing buffer and blotted dry. Serum samples were diluted in dilution buffer (PBS with 0.5% Tween 80 and 0.5M NaCl) for each isotype at the following concentrations: 1:160 for IgG, and 1:80 for IgE and IgA. Fifty µl of diluted serum was added into each well, incubated for 1 h at 37°C and subsequently washed 5 times using wash buffer. Secondary antibodies at dilutions of 1:2500 for IgG, 1:1000 for IgA, and 1:100 for IgE in dilution buffer were added at a volume of 50 µl/well and incubated at 37°C for 1 h. Following another 5 times wash step, wells were incubated with rabbit anti-mouse Ig-HRP diluted 1:1000 in dilution buffer at a volume of 50 µl/well for 1 h at 37°C and subsequently washed 5 times using wash buffer and blotted dry. Plates were then incubated with OPD substrate (Sigma-Aldrich) at a volume of 100 µl/well for 6 min in darkness at room temperature. The reaction was stopped using 25 µl of 2.5M H_2SO_4 and the absorbance in each well was read using an ELISA plate reader at an optical density of 492 and presented as optical density (OD).

3.2.4 RNA Extraction and Synthesis of cDNA

Tissue samples stored in RNA Later were weighed and 20 mg of each sample was placed into 2 ml eppendorf tubes containing 1 ml RLT buffer with 1% β-mercaptoethanol and two 5 mm stainless steel beads. Samples were homogenized using a TissueLyser II for 2 min at 125 Hz and then centrifuged for 3 min at 4°C using a microcentrifuge.at 11,000 x g. Supernatants were removed and RNA was extracted using the RNeasy RNA Extraction kit (Qiagen), including the on-column gDNA clean-up step according to the manufacturers protocol. A Nano-Drop 1000 spectrophotometer (Thermo Scientific) and purity of all samples were evaluated by measurement of the A260:280 absorbance ratio. All samples had a ratios above 1.9, indicating purity of samples.

First strand cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories) as per the manufacturer specified protocol and the concentration of cDNA was measured using the Nano-Drop 1000 spectrophotometer and stored at -20°C until used.

3.2.5 Real-time PCR Quantification of Cytokine Gene Expression

Real-time PCR was used to determine the relative quantification of cytokine gene expression of IL-4, IL-5, IL13, IFN-γ, IL-10 and Foxp3 in cDNA derived from tissues using the standard curve method on an Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems) and the corresponding sets of primers. 25 µl reactions were performed using 1 µl of cDNA, 12.5 µl of SYBR Green qPCR Super Mix (Invitrogen), and 0.2 µl of both forward and reverse primers. Negative controls were included in each assay and reactions were performed in duplicate. Standard curves were generated via ten-fold dilutions ranging from 10^2 to 10^8 using plasmids containing the genes of interest previously constructed (Tom McNeilly, Moredun Research Institute, Edinburgh, Scotland), performed in triplicate on each plate and generated using the Applied Biosystems 7000 system software package.

The thermal cycling protocol followed a 10 min pre-incubation time at 95°C, followed by 40 denaturation cycles at 95°C, primer annealing at 57°C, and primer extension at 72°C all for 30 sec each, respectively. Acquisition of data was performed after each extension step. This was followed by a dissociation cycle to produce a dissociation curve to ensure that the desired amplicon was detected. Following data collection, the number of copies per microliter of cDNA was determined and normalized to the ribosomal protein 26 reference gene.

3.2.6 Statistical Analysis

Lymph node weights, qPCR and ELISA data was analyzed using the ProcGLM (Generalized linear model) procedure of SAS version 9.1.6 (SAS Institute Inc). The effects of

breed, day and breed by day interactions were analyzed. P values less than or equal to 0.05 were considered significant.

3.3 Results

3.3.1 Lymph Node Hypertrophy

No differences ($p>0.05$) in weight were found in prescapular lymph nodes between breeds at any time point or with the Controls (Figure 3.1). Similarly, no difference ($p>0.05$) in weight was found within Suffolk and Native lambs across time points or from the Controls.

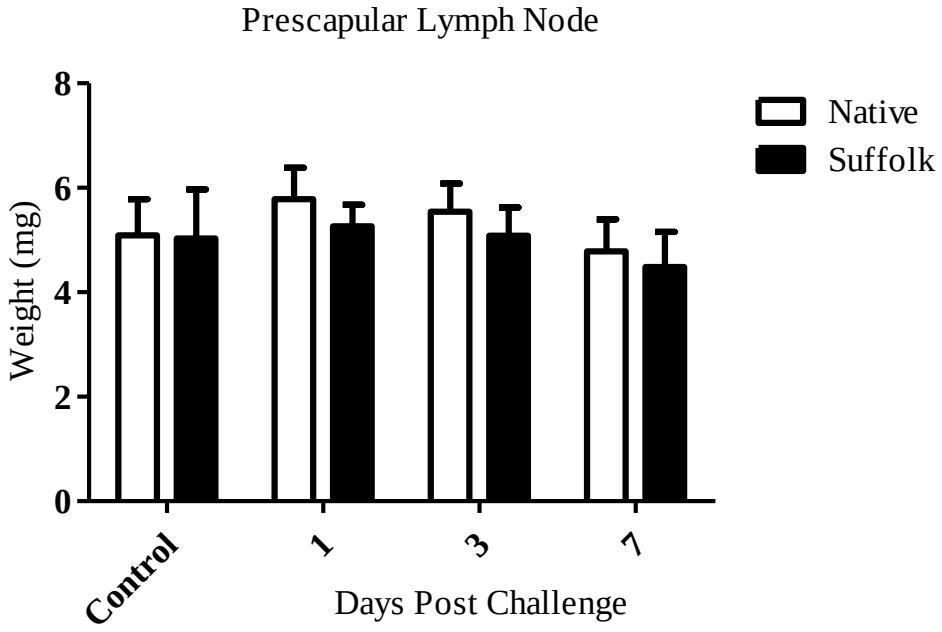

Figure 3.1 Prescapular lymph node weights of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group.

No differences ($p>0.05$) in abomasal lymph node weights were found between breeds at any time point and in the Controls (Figure 3.2). In contrast, lymph node weights were significantly ($p<0.05$) greater on days 3 and 7 compared to the control for both breeds.

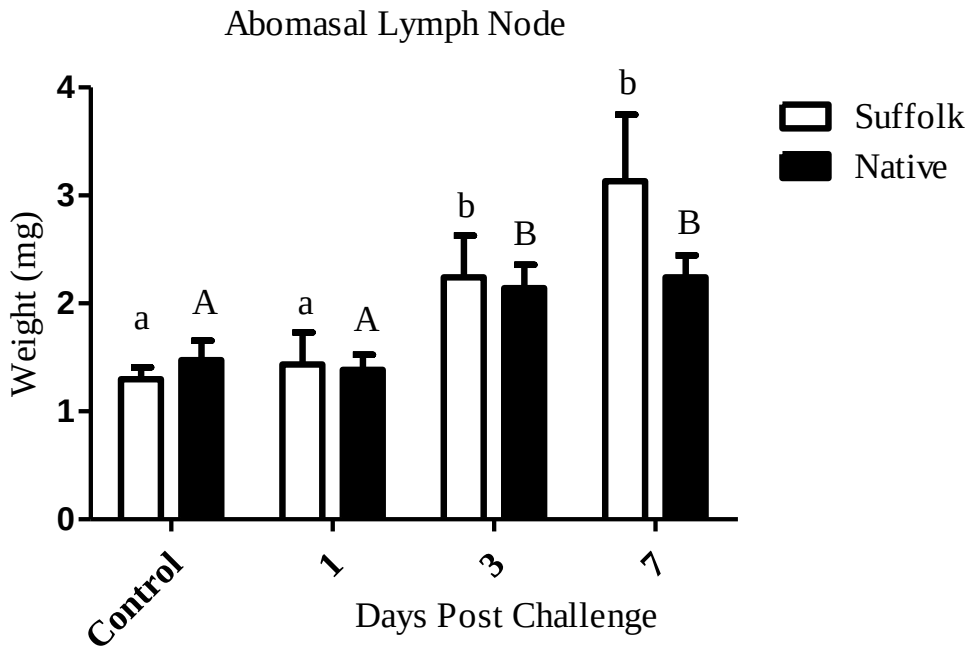

Figure 3.2 Abomasal lymph node weights of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Different letters indicate significance within breed. $p<0.05$

3.3.2 Cytokine Gene Expression

3.3.2.1 Abomasal Lymph Node

No differences ($p>0.05$) were seen in IL-4 levels between Suffolk and Native lambs at any time point or in the controls, but there was significant ($p<0.05$) breed by time interactions for both breeds (Figure 3.3). IL-4 levels increased significantly ($p<0.05$) in both Native and Suffolk lambs across time points. Levels in Native lambs on Day 3 and 7 were significantly ($p<0.05$) higher than Day 1 and Controls, while levels in Suffolk lambs were significantly ($P<0.05$) higher only at day 7 compared to Days 1 and 3. However, levels were not significantly ($p>0.05$) different from the Controls.

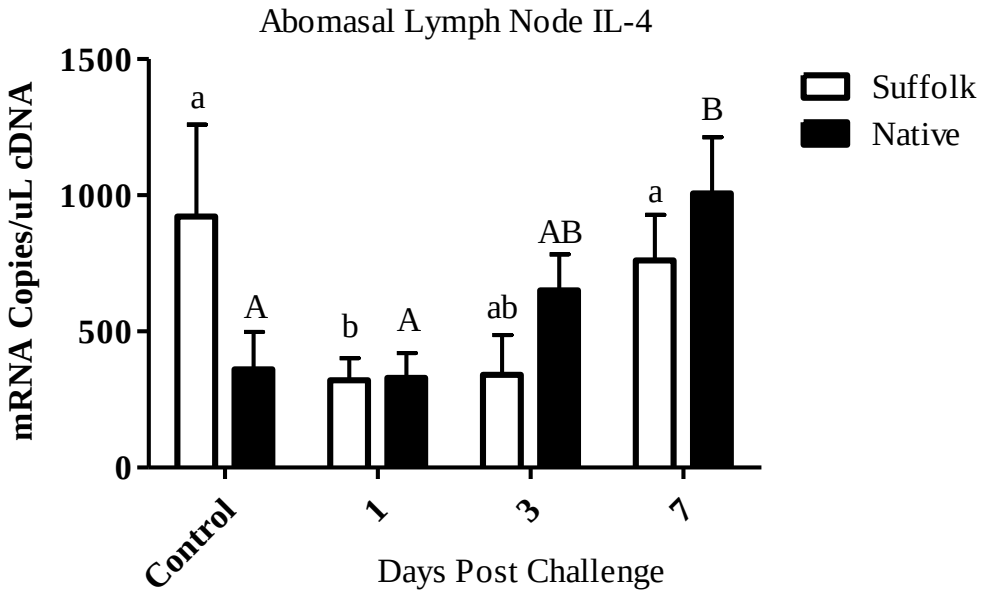

Figure 3.3 IL-4 gene expression in the abomasal lymph nodes of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Different letters indicate significance within breed. $p<0.05$

There was no difference ($p>0.05$) between breeds in IL-5 levels for Controls or at any time point, but there was a significant ($p<0.05$) breed by time interaction for both breeds (Figure 3.4). IL-5 levels were significantly ($p<0.05$) higher on day 7 for Native lambs compared to Days 1, 3 and the Controls. Similarly, mean IL-5 levels were higher at day 7 for the Suffolk lambs, but not significant ($p>0.05$). This suggests an increasing trend in IL-5 over time, similar to IL-4.

IL-13 showed an increasing trend over time in both breeds, however, the increase was not significant ($p>0.05$) (Figure 3.5). Additionally, there was no difference ($p>0.05$) between breeds at any time point or for the controls.

No differences were observed in IFN-γ levels between breeds or across time points within breeds (Figure 3.6).

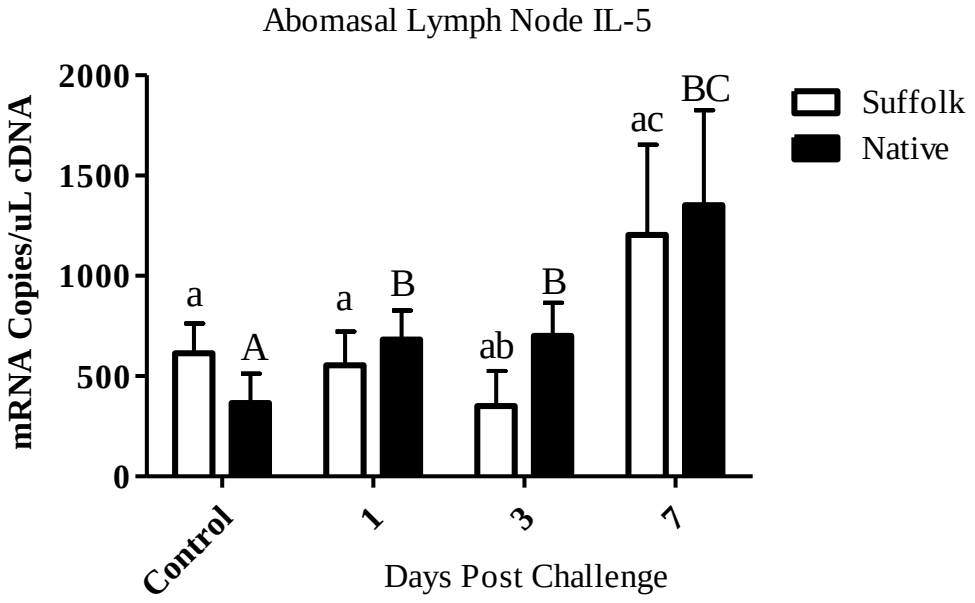

Figure 3.4 IL-5 gene expression in the abomasal lymph nodes of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. $p<0.05$

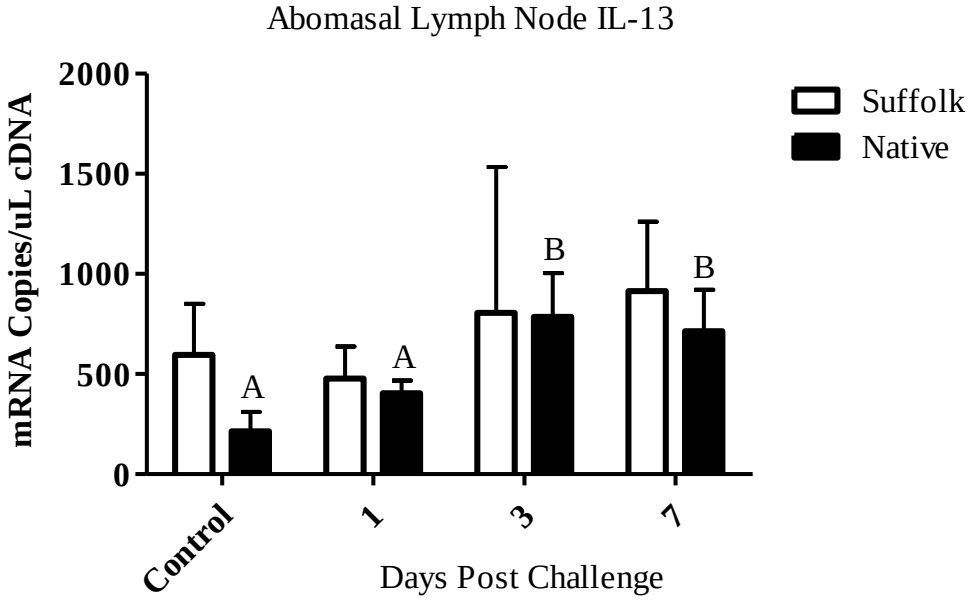

Figure 3.5 IL-13 gene expression in the abomasal lymph nodes of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. $P<0.05$

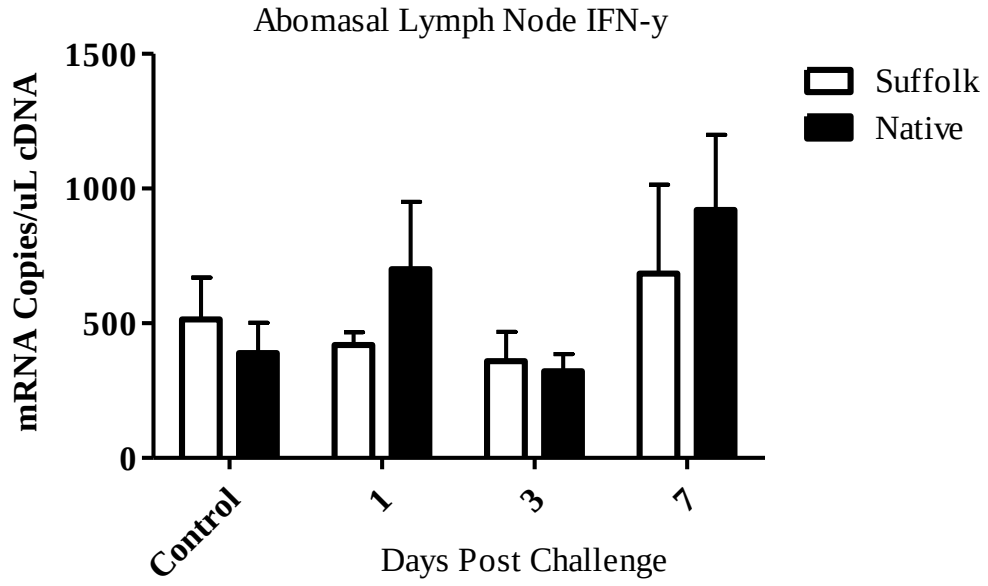

Figure 3.6 IFN-γ gene expression in the abomasal lymph nodes of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group.

There was no significant ($p>0.05$) difference in IL-10 levels between breeds or interaction over time or compared to Controls in Suffolk lambs (Figure 3.7). However, IL-10 levels for Native lambs were significantly ($p<0.05$) higher on day 7 compared to Days 1, and 3 and to Controls.

There were no differences ($p>0.05$) in FOXp3 levels between breeds at any time point or within breeds between any time point or with the Controls (Figure 3.8).

3.3.2.2 Abomasal Mucosa

There was no difference ($p>0.05$) in IL-4 levels between breeds at any time point (Figure 3.9). IL-4 levels increased across time points in both breeds and days 3 and 7 were significantly ($p<0.05$) higher in both breeds compared to the Controls.

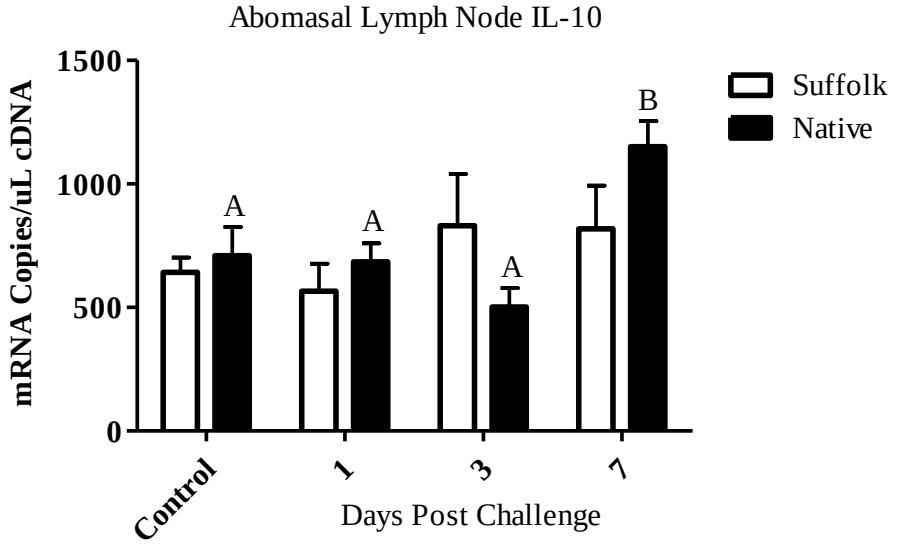

Figure 3.7 IL-10 gene expression in the abomasal lymph nodes of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Different letters indicate significance within breed. $p<0.05$

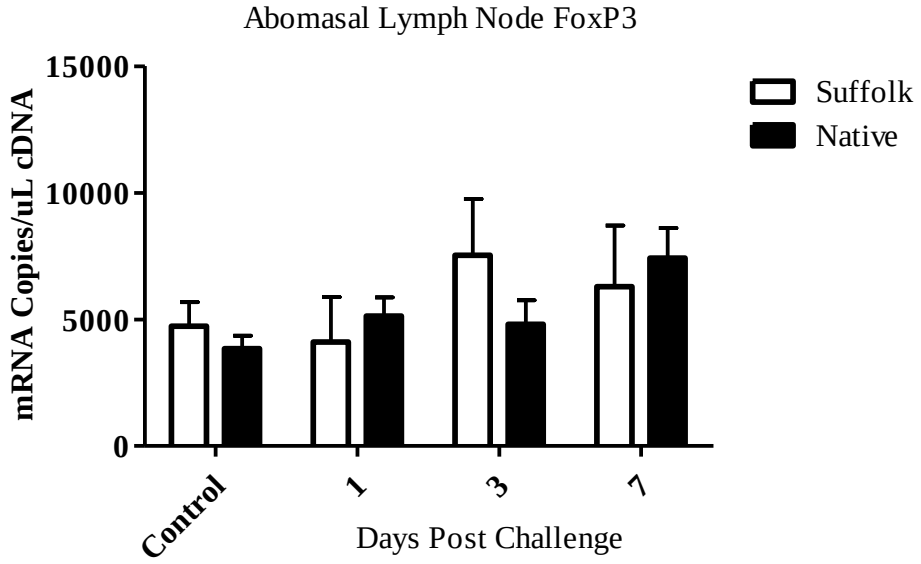

Figure 3.8 FoxP3 gene expression in the abomasal lymph nodes of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group.

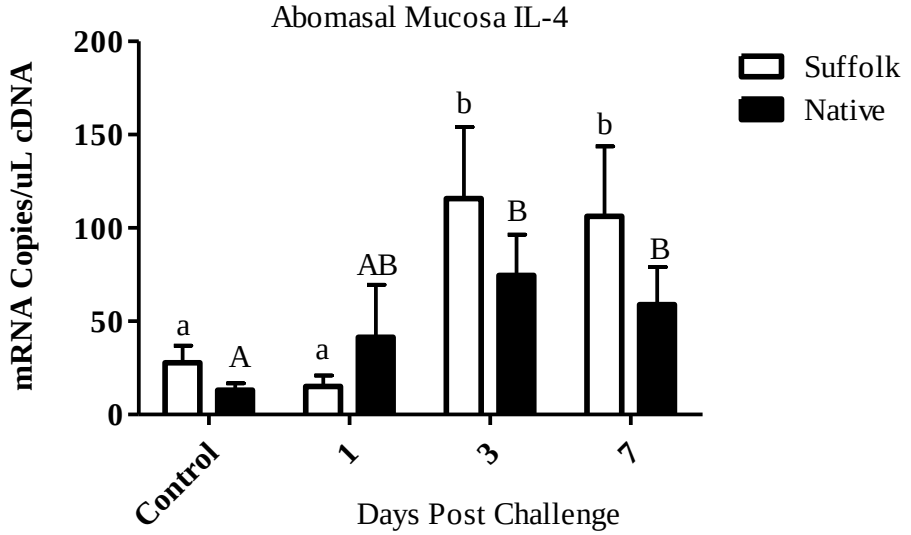

Figure 3.9 IL-4 gene expression from the abomasal mucosa of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Different letters denote significance within breed. $p<0.05$

IL-5 levels were significantly ($p<0.05$) different between breeds on Days 1 and 3 with Native lambs higher on Day 1 and Suffolk lambs higher on Day 3 (Figure 3.10). Within breed, IL-5 levels were significantly ($p<0.05$) higher for Native lambs on Day 1 and for both breeds on Days 3 and 7 compared to Controls.

IL-13 levels were significantly ($p<0.05$) higher in Native lambs compared to Suffolk lambs on Day 7, but were not different ($p>0.05$) between breeds at any other time point (Figure 3.11). Within breed, IL-13 levels increased across time points and were significantly ($p<0.05$) higher on Days 3 and 7 compared to Day 1 and the Controls.

There was no difference ($p>0.05$) in IFN-γ levels between breeds or between time points (Figure 3.12).

IL-10 levels were significantly ($p<0.05$) higher on Day 1 in Native lambs compared to Suffolk lambs (Figure 3.13).There were no other differences ($p>0.05$) between breeds or within breed at any time point or compared to Controls.

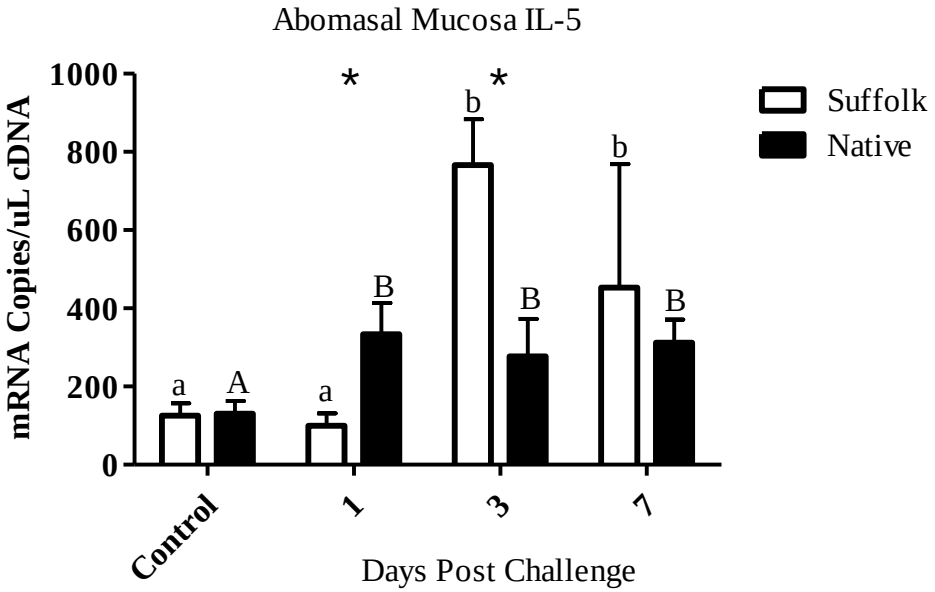

Figure 3.10 IL-5 gene expression in the abomasal mucosa of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Asterisk (*) denotes significant differences between breeds. Different letters indicate significance within breed. $p<0.05$

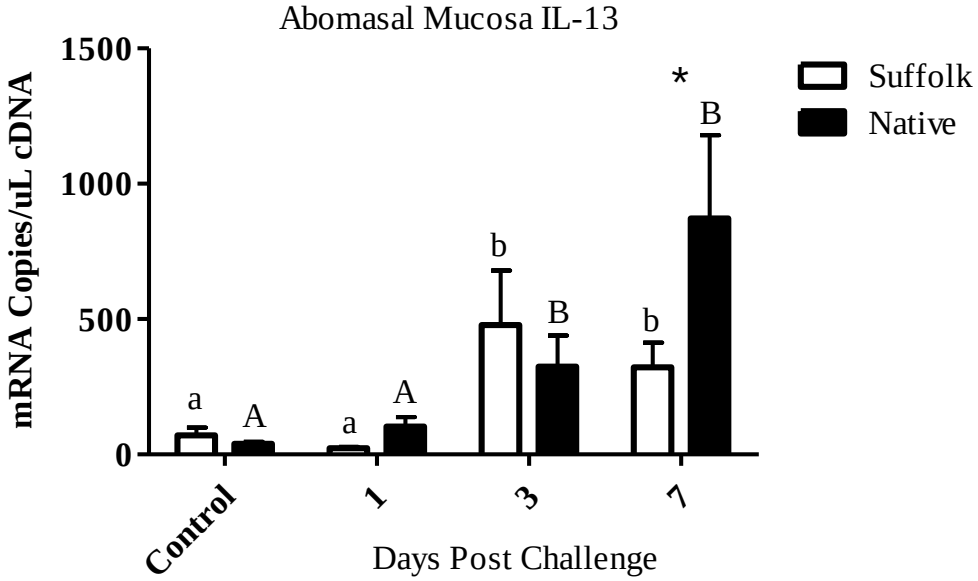

Figure 3.11 IL-13 gene expression in the abomasal mucosa of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Asterisk (*) denotes significant differences between breeds. Different letters indicate significance within breed. $p<0.05$

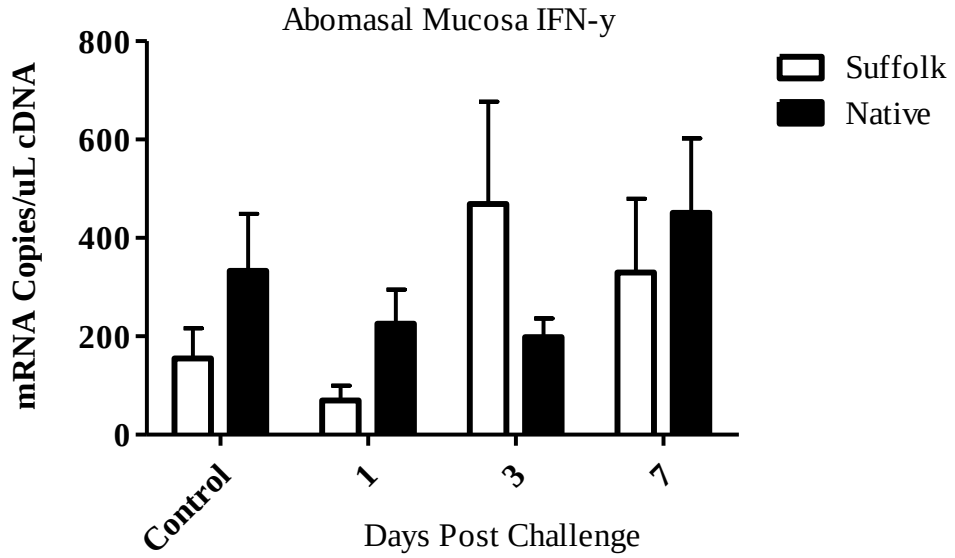

Figure 3.12 IFN-γ gene expression in the abomasal mucosa of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Asterisk (*) denotes significant differences between breeds. Different letters indicate significance within breed. $p<0.05$

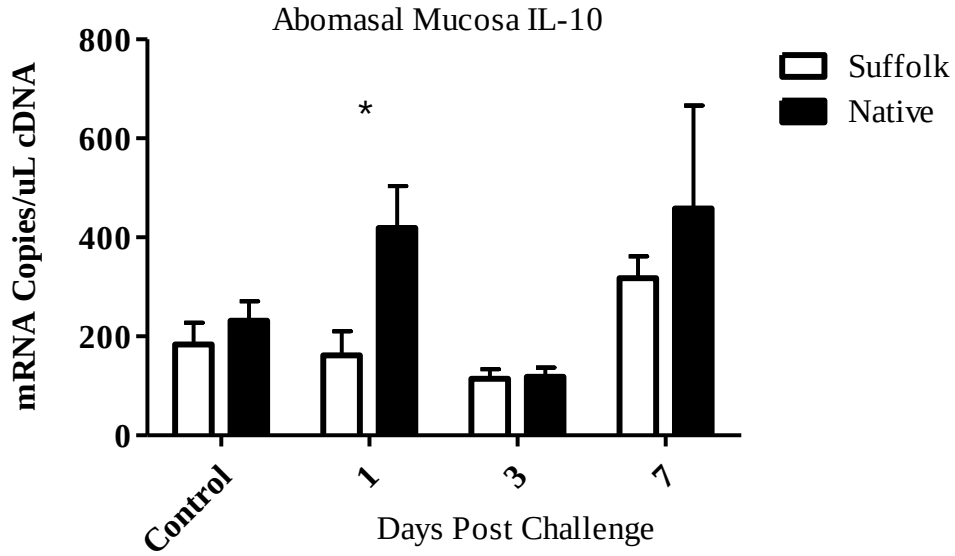

Figure 3.13 IL-10 gene expression in the abomasal mucosa of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Asterisk (*) denotes significant differences between breeds. $P<0.05$

FoxP3 levels were not different ($p>0.05$) between breed at any time point but were significantly higher at day 7 compared to day 3 in both breeds ($p<0.05$) (Figure 3.14).

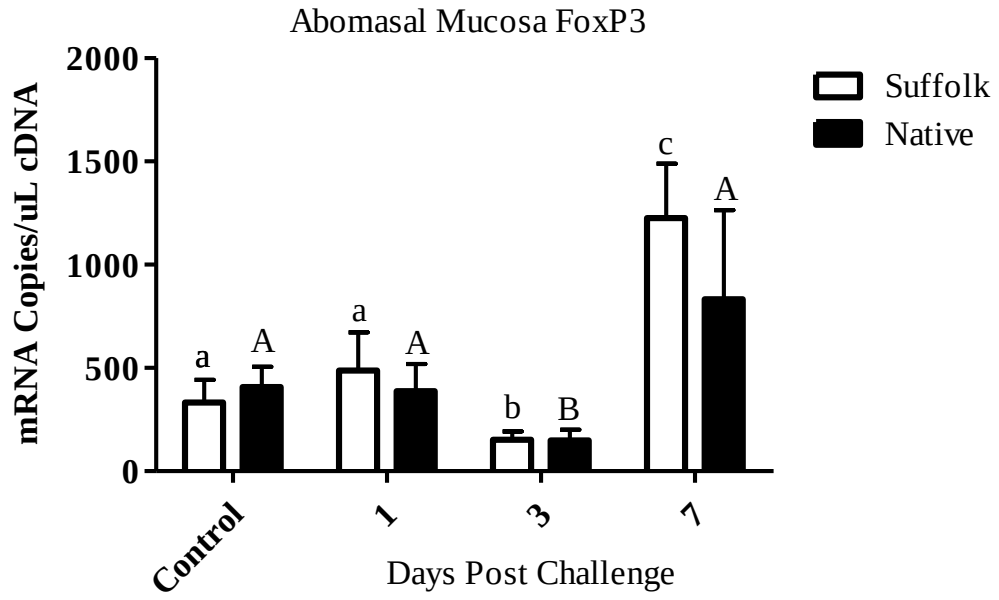

Figure 3.14 FoxP3 gene expression in the abomasal mucosa of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Different letters indicate significance within breed. $p<0.05$

3.3.3 Serum Immunoglobulin

Serum IgA levels increased over time in both breeds with no difference ($p>0.05$) between breeds (Figure 3.15). . Serum IgG levels were similar ($p>0.05$) between breeds for Controls and on Days 3 and 7 (Figure 3.16). On Day 1, the serum IgG level was significantly ($p<0.05$) higher for Native lambs compared to Suffolk lambs.

Serum IgE levels were similar between breeds for Controls and on Days 1 and 7 (Figure 3.17). Though not significant ($p>0.05$), IgE levels in Native lambs were higher than Suffolk lambs on day 3

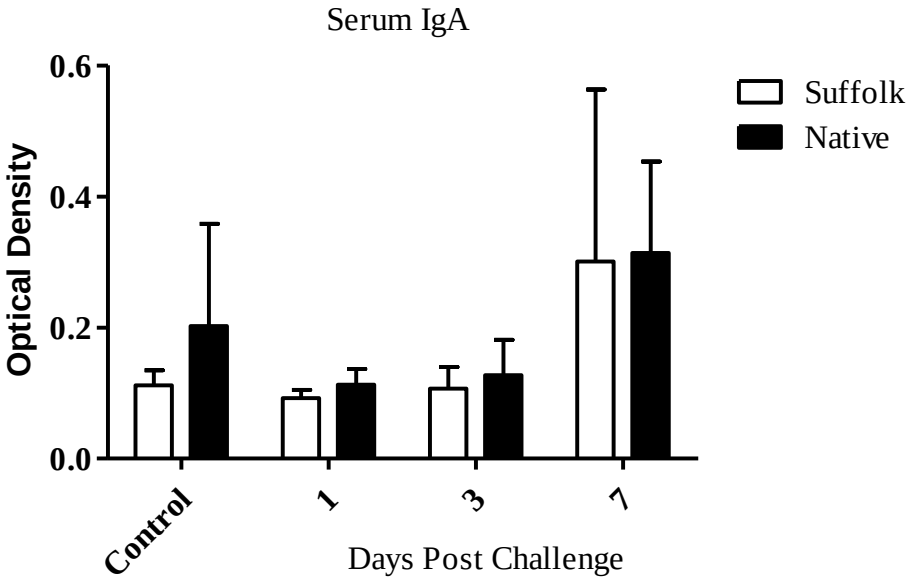

Figure 3.15 Optical densities representing serum IgA levels of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group.

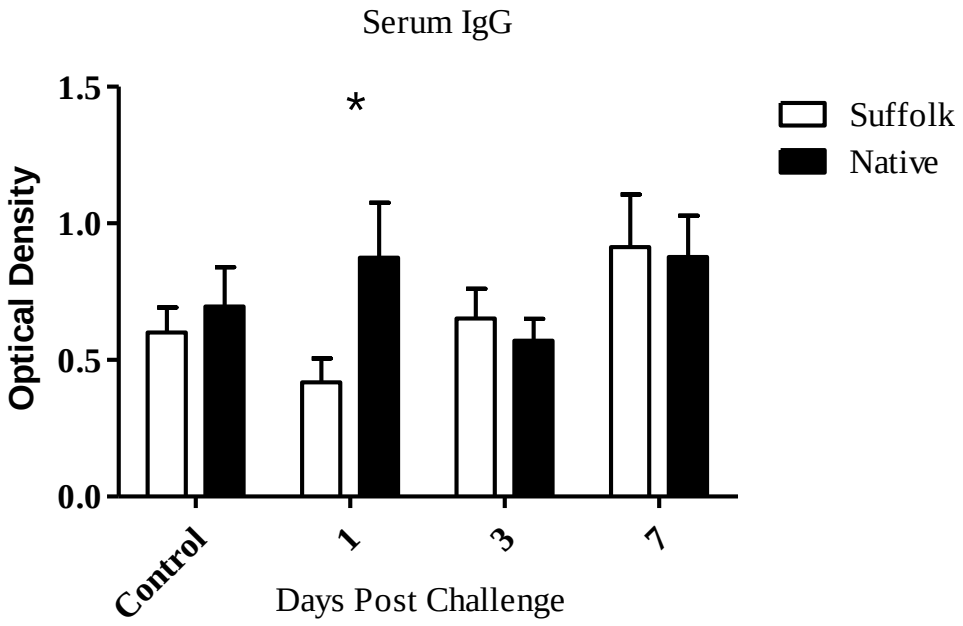

Figure 3.16 Optical densities representing serum IgG levels of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group. Asterisk (*) denotes significance between breeds. $P<0.05$

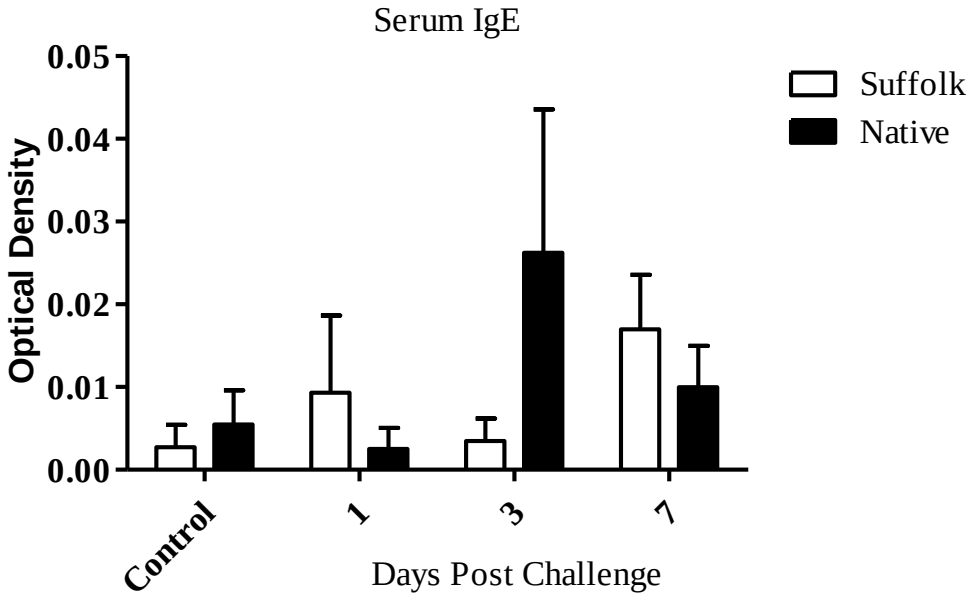

Figure 3.17 Optical densities representing serum IgE levels of non-challenged control and *Haemonchus contortus* challenged Gulf Coast Native and Suffolk lambs necropsied on days 1, 3, and 7 post challenge. n = 6-7 lambs per group.

3.4 Discussion

Prescapular lymph node weights remained consistent throughout the study while abomasal lymph node weights increased in both breeds over time. This finding is similar to abomasal lymph node weights at later time points observed by Shakya et al. (2011) and is in contrast to Bowdridge et al. (2013), who found significantly heavier abomasal lymph nodes in resistant hair breed sheep compared to susceptible wool breed sheep. Lymph nodes are sites of antigen presentation and clonal expansion of plasma cells for the production of antigen specific antibody isotypes. These results suggest that self-cure responses are site specific rather than a systemic type of response.

In abomasal lymph nodes, no differences were observed between breeds with the exception of elevated levels of IL-10 gene expression in Native lambs at day 7 compared to Suffolk lambs. However, IL-4 and IL-5 gene expression increased over time in both breeds.

Alternatively, differences in Th2 cytokine gene expression were observed between Suffolk and Native lambs. Interestingly, IL-5 gene expression first increased in Native lambs at day 1 post inoculation while at day 3, levels of IL-5 cytokine gene expression was increased in Suffolk lambs compared to Native lambs. IL-5 is a known to be chemotactic for eosinophils (Coffman et al., 1989; Gleich and Adolphson, 1986). It is possible that the increases in eosinophils within the abomasal mucosa discussed in the previous chapter are due to the elevated levels of IL-5 cytokine gene expression observed in this study.

IL-13 gene expression within the abomasal mucosa was higher in Native lambs at day 7 compared to Suffolk lambs. This is similar to the findings of Bowdridge et al. (2013) in which IL-13 gene expression was higher in hair sheep. However, these differences occurred earlier than what was observed in the present study. IL-13 has been shown to be involved in worm expulsion by stimulating the increased production of mucus and increase smooth muscle contractions (Anthony et al., 2007).

Surprisingly, no differences in IL-4 gene expression between breeds were observed in this study. This was unexpected as other studies have shown elevated levels of this cytokine in resistant breeds of sheep compared to susceptible breeds early during secondary infections (Bowdridge et al., 2013; Terefe et al., 2007).

The increasing levels of Th2 cytokines during this study indicated that self-cure may be a Th2 type of immune response. This was expected as immune responses directed against *H. contortus* are Th2 in nature (Lacroux et al., 2006; Peña et al., 2006).

With the exception of elevated serum IgG at day 1 post inoculation in Native lambs compared to Suffolk lambs, no other differences between breeds were observed. These results

are similar to other breed comparison studies (Bowdridge et al., 2013; Shakya et al., 2009). The results suggest that these antibodies may not be important in self-cure responses.

Overall, the data suggested that self-cure caused elevated Th2 cytokine gene expression that increased over time in both susceptible and resistant breeds of sheep. Antibodies are likely to play a negligible role in self-cure worm expulsion.

3.5 References

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Chapter 4 General Discussion and Summary

4.1 Discussion

The objective of this research was to evaluate the ability of GIN resistant and susceptible breeds of sheep to undergo self-cure worm expulsion, and to identify, quantify and compare the parasitological parameters and immune responses involved using the Native (resistant) and Suffolk (susceptible) model system. The design of the study was optimized such that all animals acquired a natural GIN infection on pasture before being housed under parasite free conditions for an extended period of time before inoculation with an *H. contortus* L3 challenge infection. The period of rest before inoculation was essential for self-cure to occur (Soulsby, 1957) (Dargie and Allonby, 1975). It has been shown that self-cure worm expulsion occurs within the first seven days of challenge, as such, this study examined time points within that time frame. This study examined parasitological parameters that included FEC, PCV, and adult and larval worm counts. Immunological parameters included examination of immune cells in peripheral blood, as well as at the site of infection (abomasum), and in sites of immunological importance (lymph nodes). Additionally, serum levels of parasite specific antibody isotypes IgG, IgA, and IgE as well as quantification of cytokine gene expression of select Th1, Th2, and Treg cytokines within the abomasum and abomasal lymph nodes were examined.

Prior to, and at the start of the study, FEC were much lower in Native lambs. This is consistent with results from previous studies using this model system and decreased FEC is a common observation in resistant sheep breeds compared to susceptible breeds (Bahirathan et al., 1996; Miller et al., 1998; Shakya et al., 2009; Shakya et al., 2011; Terefe et al., 2007). Similarly, adult GIN burdens were much lower in Native lambs prior to, and at the start of the study.

The results of this study suggested that the immune response of Native lambs was stronger and faster than Suffolk lambs during self-cure which resulted in the expulsion of both larval and adult GIN burdens and an early reduction in adult fecundity. Based on this, we accept our hypothesis that there are differences in the ability of Suffolk and Native lambs to produce a self-cure response due to immunological differences in magnitude and response time.

The following is a proposed model for self-cure responses in resistant and susceptible breeds of sheep based on the data collected during this study (Figure 4.1).

On day 1 following inoculation with 20,000 *H. contortus* L3, the larvae reached the abomasum and were present in the lumen. At this point, in Native lambs, elevated levels of IL-5 served as an eosinophilic chemoattractant and eosinophils began to migrate to the site of larval challenge. Larvae became opsonized by increased levels of parasite specific IgG in Native lambs. At day 3 post challenge, the larvae were beginning to reach the mucosa where eosinophils were present in very high numbers in Native lambs. Larvae were killed via degranulation of eosinophils attached to the larval cuticle. Degranulation caused degradation of the cuticle and damage to the muscular sheath of the larvae in the presence of complement and antibodies (Balic et al., 2006; Rainbird et al., 1998). Eosinophils began to increase within the abomasum of Suffolk lambs due to very high levels of IL-5 but were unable to effectively kill larvae possibly due to insufficient numbers of eosinophils. At the same time, FEC began to decrease in both breeds at day 3 post challenge, but no decreases in adult worm burden had yet occurred. The decrease in FEC in the absence of adult worm expulsion may have been due to the increasing presence of eosinophils within the abomasum, as the magnitude of eosinophils strongly reflects the reduction in FEC in both Native and Suffolk lambs at day 3 post inoculation. It is possible some eosinophils may have attached to adult worms and degranulated on the cuticle

in a method similar to larvae and caused slight damage resulting in a decrease in fecundity. By day 7 post inoculation, mast cells had been recruited to the site of infection in both breeds. Mast cell degranulation occurred and resulted in increased mucous production by goblet cells and increased smooth muscle contractions, creating an inhospitable environment for the adult GIN population resulting in their expulsion in both breeds (Huntley et al., 1987; McDermott et al., 2003).

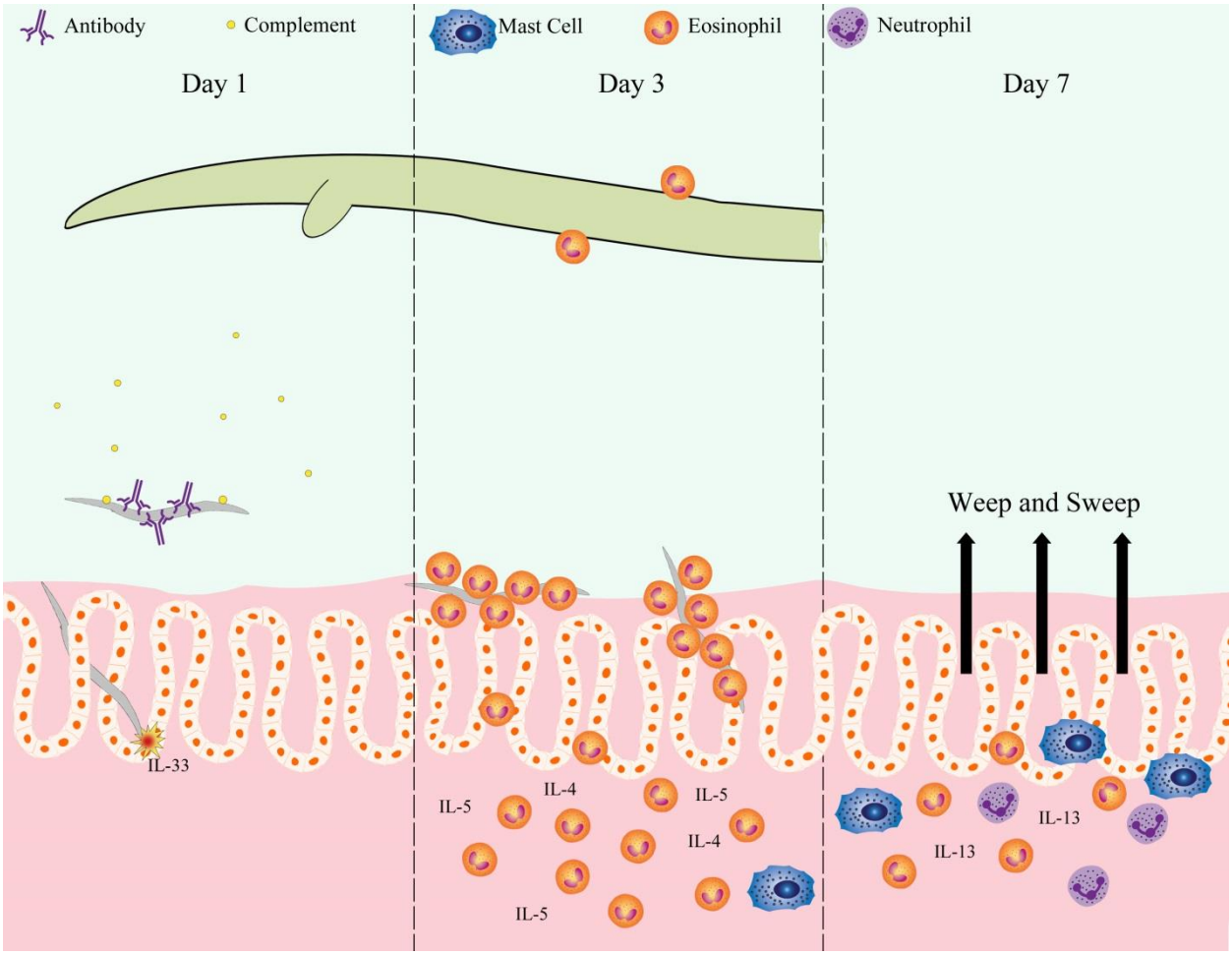

Figure 4.1 Proposed model of self-cure GIN expulsion.

IL-13 may play a role in this process as well, as studies have shown involvement of IL-13 in smooth muscle functions and is essential for worm expulsion in mouse models (Anthony et al., 2007). In Suffolk lambs, eosinophils reached levels similar to those in Native lambs at day 7

post inoculation. However, by day 7, larvae are imbedded within the abomasal mucosa and are protected from eosinophil mediated killing. This results in the eventual establishment of patency by this larval population.

The overall result of self-cure that was observed in Native lambs was the successful expulsion of both the pre-existing adult worm population and the challenge larval infection. In Suffolk lambs, self-cure also resulted in the expulsion of the pre-existing adult infection; however, there was a delayed recruitment of eosinophils to the site of infection that allowed the larvae to establish in the abomasal mucosa and avoid eosinophil mediated killing.

The delayed recruitment of eosinophils in response to larvae may be due to differences in antigen recognition (Scott Bowdridge, personal communication). Impairment in antigen recognition would likely be evident in responses to larval challenge, where the larva is susceptible to expulsion by the host immune responses for a short period of time before maturation (1-2 days). Impairment in antigen recognition in Suffolk sheep could also account for the higher GIN burdens that they acquire during grazing on infected pastures.

Differences in eotaxin levels within the abomasums of Suffolk and Native sheep may also be responsible for delayed eosinophil recruitment. This study examined the gene expression of IL-5 within the abomasal mucosa during self-cure and found that levels increased in both breeds after larval challenge. IL-5 is a known eosinophil chemo-attractant that is important for recruitment and activation of eosinophils into tissue from the periphery while eotaxin is important for recruitment of eosinophils within tissue (Dent et al., 1990; Garcia-Zepeda et al., 1996; Knott et al., 2009).

4.2 Future Direction

Examination of additional time points during self-cure would provide a clearer picture of which components of the host immune response may be responsible for worm expulsion in resistant sheep. Additionally examination and quantification of cell types using flow cytometry would allow for the identification of additional cell types including alternatively activated macrophages, and γδ T cells that have shown to be important in worm expulsion in murine models. Analysis of additional cytokines such as IL-33 could aid in more effectively characterizing the Th2 responses seen during self-cure. Blocking of specific cell types and cytokines via the development of blocking antibodies would definitively confirm their roles during self-cure worm expulsion. Repeating the study using additional breeds with known resistance or susceptibility to GIN would provide evidence for determining whether the responses observed occur in all breeds that are resistant or susceptible to GIN, or are limited to Suffolk and Native sheep.

This study suggests the importance of eosinophils in early larval expulsion in sheep. However, studies can be performed to further reinforce these results. A study using Native lambs in which self-cure is induced and eosinophils are ablated would confirm the importance of this cell type in larval expulsion. Similarly, a study in which eosinophils from *H. contortus* challenged Native lambs are injected into Suffolk lambs prior to self-cure could further strengthen results of this study.

4.3 Conclusions

This study showed that resistant Native and susceptible Suffolk sheep were able to successfully undergo self-cure worm expulsion. Additionally, there were differences in magnitude and timing in immune responses between breeds. These findings have contributed to

the knowledge base of immune responses to GIN infection in Native and Suffolk sheep and further validated this model. The results of this study provide some previously unknown insights on the immune responses during self-cure worm expulsion and further confirms the presence of multiple mechanism for GIN expulsion in sheep. This knowledge may prove beneficial in the production of vaccines for GIN in sheep by providing insight on the specific immune responses necessary for parasite clearance. This study shows the importance of the timing and magnitude of an eosinophilic response for the prevention of parasite establishment via the expulsion of L3. A vaccine focus on priming an eosinophilic response could yield a vaccine that can both clear an existing infection and prevent future infections as well. Additionally, if a product can be created that can replicate the responses involved during adult GIN expulsion in both breeds, then such a product could serve as an anthelmintic alternative. Furthermore, since eosinophils appear to be important for larval clearance, selection of animals with a faster eosinophilic response for breeding may yield progeny that are more resistant to GIN infection.

4.4 References

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Vita

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