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# Evaluation of selected immune response to *Haemonchus contortus* in Gulf Coast native compared to Suffolk lambs

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**EVALUATION OF SELECTED IMMUNE RESPONSE TO  
*HAEMONCHUS CONTORTUS* IN GULF COAST NATIVE COMPARED TO  
SUFFOLK LAMBS**

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 Interdepartmental Program in  
Veterinary Medical Sciences through the  
Department of Pathobiological Sciences

by  
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## ABSTRACT

*Haemonchus contortus* is one of the major nematode parasites causing substantial economic losses in small ruminant farming worldwide. Recently, effect of anthelmintic treatment has decreased due to an increasing problem of nematode populations that have developed resistance to anthelmintics. Efforts to develop effective vaccines are at various stages of success. There are certain breeds of sheep that are identified as being relatively resistant to the parasite including Gulf Coast Native (Native) sheep. Understanding the mode of immune response that helps these breeds of sheep control infection could help design vaccines and enhance control programs. This experiment was designed to evaluate the immunological responses of Native, compared with susceptible Suffolk sheep that might be responsible for this resistance.

In Experiment 1, groups (n = 5) of 6 month old Native and Suffolk lambs were given infective larvae as one time (bolus) or trickle experimental infections. Fecal, blood, and serum samples were collected on day 0, 2, 7, 14 and 21 post-infection. Abomasal mucosa and regional lymph node samples were collected at the time of necropsy on day 14 and day 21. There was no significant difference in number of worms recovered at necropsy but the ratio of adult vs larvae was significantly greater in bolus infected Suffolk than Native. Native lambs had significantly greater numbers of mast cells and eosinophils in the abomasal mucosa and serum IgG production was significantly greater compared to Suffolk lambs. Native lambs also showed a trend of increased level of serum IgA and IgE compared to Suffolk lambs.

In Experiment 2, immune responses were evaluated in naturally infected Native and Suffolk lambs that grazed pasture contaminated predominantly with *H. contortus*. Ten lambs of each breed grazed together for 42 days. Fecal, blood and serum samples were collected on 0, 2, 4, 7, 10, 14, 21, 28, 35 and 42 days of exposure. Five lambs of each breed were necropsied on day 35 and five on day 42 for nematode recovery and abomasal tissue sample collection.

Throughout the course of infection, Native lambs had significantly lower FEC, significantly lower PCV reduction percent, and significantly higher serum IgE after day 14 and increased expression of Il-4 on day 10 post exposure compared to Suffolk lambs. At both necropsy time points, Native lambs had significantly greater numbers of mucosal mast cells, eosinophils and globule leukocytes in abomasal mucosa than Suffolk lambs.

Results indicated that Native lambs had a more pronounced immune response to infection with *H. contortus* than Suffolk lambs which may be responsible for the observed resistance to infection.

# CHAPTER 1

## GENERAL BACKGROUND

### 1.1. Introduction

Gastrointestinal (GI) parasitism is a very common and economically important condition affecting domestic livestock species worldwide (Krecek and Waller, 2006; Miller et al., 1998). The major parasites of concern differ by the prevailing host animal species and climatic conditions in a particular geographic location and no farm animal species in general is free from GI parasitism. Small ruminants are specifically vulnerable to infection with *Haemonchus contortus* which is most prevalent in regions with warm and humid tropical and subtropical climates. In the US, such conditions occur along the coastal area of the Gulf of Mexico, and southeastern states are quite favorable for this parasite. Hence small ruminant farming is affected to a great extent (Miller et al., 1998). However, this parasite is also present in temperate regions with focal areas of similar climatic conditions (Waller et al., 2004; Waller et al., 2006).

*Haemonchus contortus* is a trichostrongylate nematode (also known as the barber pole worm) which resides in the abomasum of sheep, goats and other wild ruminants (Davidson et al., 1980). The parasite is a highly pathogenic hematophagous nematode. Clinical signs in affected animals include anemia, unthriftiness, hypoproteinemia resulting in bottle jaw, diarrhea (not a consistent feature) and eventual death if untreated.

Economic losses are incurred through morbidity and mortality and increased investment due to cost of preventative as well as curative treatments (Miller and Horohov, 2006). It is very difficult to assess the exact economic impact of this parasite in small ruminant farming due to the complicated nature of sub-clinical infection by multiple species of parasites. However, the economic losses in various countries due to helminthiasis including haemonchosis are high and therefore control and prevention need attention. As reviewed by Miller and Horohov (2006), GI

parasitism has been a problem of moderate to high concern for US farmers. Gasbarre (1997) estimated that economic losses incurred in the US alone to be over \$2 billion per year due to ostertagiosis in cattle and haemonchosis in small ruminants. Total losses in Australia due to all nematodes combined, of which *H. contortus* is a major contributor, was estimated to be US \$500 million (Emery, 1991). One third of total sheep production, equivalent to \$946 million was attributed to nematode infection in New Zealand (Vlassoff and McKenna, 1994). The estimated treatment cost alone for *H. contortus* per year in Kenya, South Africa and India was estimated at US \$26, \$46 and \$103 million, respectively (Peter and Chandrawathani, 2005). In a Kenyan study conducted to determine the contribution of parasitic diseases in small ruminant mortality, 32% of total deaths in sheep were due to parasitism and about 63% were due to helminthiasis of which haemonchosis accounted for 40% (Kagira and Kanyaria, 2001). Similarly 26% of goats were found to have died due to parasitism of which helminthiasis occurred in 55% of the cases and haemonchosis accounted for 27%. In another study conducted to determine the losses attributable to different conditions and infections, it was found that weight gain cost due to *H. contortus* infections in sheep and goat was 48.7% and 32.2%, respectively (Beriajaya and Copeman, 2006). These are just a few examples of estimated economic losses especially in underdeveloped countries where the situation may be much more severe.

## **1.2. Life Cycle of the Parasite and Pathogenesis**

*Haemonchus contortus* is a nematode parasite belonging to Phylum Nematelminthes, Class Nematoda, Order Strongylida, Suborder Strongylina, Superfamily Trichostrongyloidea, Family Trichostrongylidae and Subfamily Haemonchinae (Urquhart et al., 1996a). This hematophagous nematode is a parasite of sheep and goats but also is reported in wild ruminants like white tailed deer, *Odocoileus virginianus* (Davidson et al., 1980). *Haemonchus placei*, another parasite belonging to the same genus, inhabits the abomasum of cattle and other

ruminants (Urquhart et al., 1996a). As described by Soulsby (1982), the life cycle of parasites belonging to this genus is direct and has pre-parasitic as well as parasitic stages. The adult worms live in the abomasum where the male and the female worms mate and produce ova that are excreted in the feces. Under favorable environmental conditions, especially in warm temperature and higher humidity, the eggs hatch into 1<sup>st</sup> stage larvae (L1) which molts twice to become the 3<sup>rd</sup> stage infective larvae (L3). The L3 is attained in about 4 to 6 days after hatching. L3 are very active and motile moving up grass blades in the pasture where they are ingested by grazing animals. Following ingestion, the L3 exsheath in the rumen and move to the abomasum, where they penetrate the gastric epithelium into the gastric glands where they molt and emerge back into the lumen as 4<sup>th</sup> stage larvae (L4) (Soulsby, 1982; Miller, 1984). They develop into immature adults (L5) for a short period of time and become mature adults soon after. The pre-patent period (L3 ingestion to mature adult) in sheep is 15-18 days (Soulsby, 1982).

*Haemonchus contortus* is the most pathogenic species of all the GI nematode parasites in small ruminants. Transmission can occur year round in favorable warm and humid climates. L3 can survive in the pasture up to one year under these conditions. However in cooler climates, survival is usually weeks to a few months. The major clinical sign of infection with *H. contortus* is anemia. The L4, L5 and adult worms feed on blood and an average worm consumes about 0.05 ml of blood in a day (Urquhart et al., 1996a). Therefore, degree of anemia depends on the number of worms present in the abomasum. The severity of blood loss is increased additionally by bleeding of raw ulcers created by the worms even after they leave the abomasal wall. Clinically, haemonchosis can be classified into three types; hyper acute, acute and chronic. Hyper acute cases result when the animal ingests a massive number of L3 leading to heavy blood loss causing severe anemia. The color of the feces from these animals usually becomes dark due to digested blood and sudden death may take place due to massive blood loss. Acute cases

usually occur in young lambs that get heavily infected, but expansion of erythropoiesis compensates for the loss of blood to a certain extent. The animals may or may not have diarrhea. Anemia is also accompanied by hypoproteinemia and edema which may contribute to death. A common observation in these cases is sub-mandibular edema termed “bottle-jaw” (Taylor, 1990). Chronic haemonchosis is seen when the animals are infected with relatively few worms, therefore it is characterized by high morbidity and low mortality. Infected animals are unthrifty, weak and emaciated, however anemia may or may not be appreciable depending upon the erythropoietic status of the animal. Pregnant ewes and does around the time of parturition are found to have increased fecal egg counts (FEC) known as the periparturient rise. Immunity against parasites shortly before and after parturition appears to be reduced due to reasons yet to be confirmed (Houdijk et al., 2003) which results in increased fecundity of female worms and subsequent increased infection level for both the dam and their offspring.

### **1.3. Prevention and Control Measures**

There are several methods for controlling nematode infection, some of them being more successful than others. Methods and strategies continue to be developed and are at different stages of evaluation.

#### **1.3.1. Anthelmintic Treatment**

The most widely used method for control of nematode parasites has been the use of chemical anthelmintics both as a preventative or curative treatment. The benzimidazoles and avermectin anthelmintics are the most common; however, the evolution of resistance in helminth parasites including *H. contortus*, against these anthelmintics has posed a great threat to successful use of these chemical agents in farm animals (Waller, 1987; Prichard, 1990; Craig, 1993; Rahman, 1994; Sangster, 1999; Miller and Horohov, 2006). There are reports of very high degree of resistance in parasites against multiple drug agents including complete failure to

eliminate the parasites (Kaplan et al., 2005; Burke and Miller, 2006). Apart from *H. contortus*, *Ostertagia* spp. and *Trichostrongylus* spp. are other nematode parasites that have developed resistance to common anthelmintics (Eddi et al., 1996). Newly introduced anthelmintics of the same chemical class have been shown to have reduced efficacy after a short time in use due to side resistance (Wooster, 2001).

Anthelmintic resistance in *H. contortus* has been reported to be very high from locations where haemonchosis is endemic and farmers practice frequent anthelmintic treatment. Hence, development of alternative strategies for parasite control is essential for modern livestock farming. Additionally, increased consumer demand for “clean and green” animal products free of residual chemicals and growth promoters, is a powerful driving force towards the investigation, development and adaptation of alternative control methods (Waller, 2003). There are several other methods of control utilized in the animal production industry which are yet to achieve success rates delivered by various chemotherapeutic agents.

### **1.3.2. Biological Control**

Nematophagus or parasite larvae trapping fungi species like *Duddingtonia flagrans* have been investigated for GI nematode parasite control and have shown promising results in field trials with sheep and goats (Chandrawathani et al., 2002; Chandrawathani et al., 2004). Spores fed to animals pass through the GI tract and sporulate in the feces. The resulting hyphal loops trap and kill the developing larvae. It was demonstrated that *D. flagrans* treated grazing animals resulted in reduced L3 pasture contamination (Wolstrup et al., 1994; Nansen, 1995; Larsen, 1998; Fernandez, 1999). Therefore, utilization of nematophagus fungi may be a useful alternative method of controlling nematode parasites. However, there are issues concerning the delivery system and the use of certain anthelmintics (i.e. benzimidazoles) that are antifungal which may interfere with efficiency (Pena, 2001). *Duddingtonia flagrans* has also been shown to

reduce residual fecal L3 that survived treatment with copper oxide wire particles (COWP) which suggests another use of biological control for haemonchosis (Burke et al., 2005).

### **1.3.3. Immunization**

Successful vaccination against nematodes may be the most effective strategy for prevention and control. Hence a substantial amount of effort has been put into research and development of vaccines against helminth parasites including *H. contortus*. The early approaches in the development of vaccine were to attenuate the L3 through irradiation. Vaccination with irradiated L3 was reported to confer a very high level of protection (Smith and Christie, 1979). Helminth excretory and secretory (ES) products from *H. contortus* with molecular weights of 15 and 24 kDa have also been used as vaccine candidates and are reported to induce an immunity resulting in 70% reduction of FEC and worm burden (Schallig and Van Leeuwen, 1997). H-11, the best known *H. contortus* hidden gut antigen, is a gut membrane glycoprotein which is a naturally extracted antigen from adult worms which can induce a very high level of protective immunity with a reduction of over 90% in FEC and over 75% in worm burden (Newton and Meeusen, 2003). Another hidden gut antigen that extends a high level of protection against *H. contortus* is H-gal-GP which has been reported to reduce FEC by 80% and worm burden by 60% (Newton and Meeusen, 2003). Studies in the US have shown that both of these antigens, H-11 and H-gal-GP, induced significant protective immunity in lambs and kids as measured in terms of IgG production, PCV, FEC and worm burden (Kabagambe et al., 2000; Olcott, 2006). Among other natural antigens, Hc-sL3 from the L3 surface has also been reported to induce a protective level of immunity in terms of both reductions in FEC and worm burden by 64-69% and 45-55%, respectively (Jacobs et al., 1999). Despite all these efforts, there are no commercial vaccines presently on the market. This may be attributed to the lack of complete understanding of the protective immune responses to the helminth parasites and the inability to produce recombinant

antigens equivalent to the natural antigens. Extraction of natural antigens from the worms would be very expensive hence they are not feasible (Dr. Miller, personal communication).

#### **1.3.4. Breeding for Resistance**

There are various breeds of sheep that are known to be relatively resistant to *H. contortus* which include Scottish Blackface (Altaif and Dargie, 1978; Abbott et al., 1985a, 1985b), Red Massai (Preston and Allonby, 1978, 1979), Barbados Blackbelly, Saint Croix (Bradley et al., 1973; Yazwinski et al., 1980; Courtney et al., 1985; Gamble and Zajac, 1992) and Gulf Coast Native (Bradley et al., 1973; Bahirathan et al., 1996; Miller et al., 1998; Amarante et al., 1999b). There is variability in the magnitude of resistance among individuals within a breed that can be attributed to their genetic make up. Some of the mechanisms responsible for resistance have been identified while others are under investigation (Pena, 2001). Some lines within breeds have also been identified as resistant to *H. contortus* infection such as within the Australian Merino (Gray et al., 1992) and Scottish Blackface against *Teledorsagia circumcincta* (Stear and Murray, 1994). Crossbreeding Gulf Coast Native with Suffolk (Barras, 1997) and Rambouillet (Amarante et al., 1999b) has been shown to improve the breed characteristics with regards to parasite resistance. The desired characteristics in the crossbred offspring may differ by local needs however, and breeding for “resistance to effects of infection” rather than breeding for “resistance to infection” may be another strategy that should be given serious thought while planning such breeding programs (Bisset and Morris, 1996).

#### **1.3.5. Rotational Grazing**

The concept of rotational grazing to reduce infection is based on the assumption that substantial larval mortality occurs during the period between grazing. Unfortunately, the time between rotations to make the best use of available and nutritious forage (usually about 28-30 days for most forages) co-insides with the time period that results in high levels of L3 becoming

available for re-infection. In a study conducted in the US (Illinois), it was reported that lambs that grazed under such optimal rotational grazing acquired more nematodes, of which most were *H. contortus* and gained less weight compared to non rotated control lambs, hence rotational grazing for sheep has not been recommended (Levine et al., 1975). Extended periods of time between rotations (60-90 days) in some cases may be sufficient to substantially reduce pasture infectivity. Rotating more resistant mature animals with susceptible younger animals may also prove beneficial. However, this strategy may not be sufficient because of practical reasons (van Wyk et al., 2006).

### **1.3.6. Selective Treatment of Infected Animals**

Selective treatment of individual animals instead of treating all animals is another economic strategy for control of *H. contortus*. The FAMACHA system, which involves comparison of conjunctival mucous membrane color with an eye color chart to determine the severity of anemia, is used to decide whether an animal needs treatment (Kaplan et al., 2004). This method has facilitated quick identification of *H. contortus* infected sheep and goats without the aid of any laboratory procedures and delivers the treatment only to those who require it (Vatta et al., 2002; Kaplan et al., 2004; van Wyk et al., 2006). This system has enabled farmers to limit the expense of anthelmintics and at the same time reduce undue exposure of the worm to anthelmintics which will slow the evolution of resistance.

### **1.3.7. Condensed Tannins**

Condensed tannin containing forages may prove to be another alternative means of controlling GI nematodes. Inclusion of condensed tannin equivalent to 5% of dietary dry matter in goats significantly reduced FEC but there was no effect found on worm recovery (Paolini et al., 2003). In another study, *Onobrychis viciifolia* extracts were found to inhibit *H. contortus* L3 in sheep (Barrau et al., 2005). A significant reduction in FEC and a noticeable decrease in worm

burden were reported in goats fed *Acacia karoo* dried leaves mixed in a basal diet compared to the control group given basal diet alone (Kahiya et al., 2003). *Lespedeza cuneata* (sericea lespedeza) fed to sheep and goats as hay significantly reduced FEC and worm burden (Shaik et al., 2006; Lange et al., 2006). In addition, a dose dependant reduction in *Haemonchus* egg hatching in animals given condensed tannins has also been reported (Iqbal et al., 2007). The effects of condensed tannin in reducing the FEC and egg hatching percentage may be beneficial by reducing the level of pasture larvae contamination leading to reduced infection level thereby decreasing overall economic losses.

### **1.3.8. Manipulating Supplementation of Nutrients**

Manipulation of nutrients, especially protein supplement, and reducing physiological requirement of protein in the host around the time of parturition has been reported to improve immunity and reduce *T. circumcincta* FEC thereby decreasing pasture larval contamination leading to lower infection level (Houdijk et al., 2003, 2005; Houdijk et al., 2006). Effects of higher levels of protein in the diet were observed in lambs resilient and resistant to *H. contortus* (Strain and Stear, 2001; Bricarello et al., 2005). Similarly, supplementation of soybean and sorghum meal to grazing kids increased resilience against helminth parasites (Torres-Acosta et al., 2004).

## **1.4. Genetic Immune Status of Gulf Coast Native and Suffolk Breeds of Sheep**

### **1.4.1. Gulf Coast Native Sheep**

Gulf Coast Native (Native) sheep have been derived from Spanish sheep imported in the Gulf Coast region of the US by the Spanish and French settlers in the 1500s and were bred within or with other breeds that were also imported into the region (Pena, 2001). These animals are very well acclimatized to the warm, humid climate of the Gulf Coast and are well known for their hardiness and mothering instinct. They are known to require minimum anthelmintic

treatment and found to be relatively resistant to *H. contortus* as substantiated by extensive epidemiological studies (Bahirathan et al., 1996; Miller et al., 1998). Native sheep have a small body size and open face with clean legs and underline and can survive under least care and management systems (Bahirathan et al., 1996).

#### **1.4.2. Suffolk Sheep**

Suffolk sheep originated in England and gained popularity among sheep farmers in the US. They are known to be highly prolific and produce high quality wool and meat. Black head, ears and legs are characteristic of this breed and head and legs do not have any wool. They do not have horns and they are the largest sheep breed in the US (Ensminger, 2002). Suffolk sheep compared to Native sheep are highly susceptible to *H. contortus* infection (Bahirathan et al., 1996; Miller et al., 1998).

### **1.5. Immune Response to Gastrointestinal Nematode Parasites**

Both humoral and cellular arms of the mammalian adaptive immune system are actively involved in response to nematode infection. Generally, T-lymphocytes, soluble cytokines, B-lymphocytes, plasma cells, various immunoglobulin isotypes, mast cells, eosinophils and globule leukocytes are known to actively take part in immunological reactions, although variability in their production and magnitude of action in different species of parasite and host has been observed. The ultimate result of parasitic invasion of a host animal is either establishment of infection or expulsion of the invading parasite. The latter being the consequence of protective immune response of the host.

#### **1.5.1. Self Cure Phenomenon**

In endemic areas, sometimes FEC in *H. contortus* infected sheep drops sharply following heavy spells of rain due to expulsion of the adult worm population from the abomasum. This phenomenon is described as the self cure phenomenon which is due to superinfection by large

numbers of L3 that are released from feces following the rain which are ingested over a very short period of time. Immunologically, this phenomenon is attributed to the development of immediate or type-I hypersensitivity to the larval antigens mediated by IgE (Mitchell et al., 1983; Smith et al., 1984; Yakoob et al., 1983). The sensitization to worm antigens has already occurred with the current established population of worms and upon subsequent exposure to incoming infection the immediate type of hypersensitivity takes place that leads to expulsion of adult worms (Miller, 1984; Urquhart et al., 1996a). However, a series of consequences may be observed apart from the self sure phenomenon first of which is rapid expulsion, where the incoming larvae are expelled very fast, before their establishment. This phenomenon takes place within 24 to 48 hours of their entry. A second consequence is as a result of action against the larvae that have established and are in the developing phase of their life cycle. And thirdly the fecundity of adult female worms is reduced which results in less pasture contamination (Miller, 1984). Female worm length is reported to have a positive relation with fecundity (Terefe et al., 2005).

After being ingested, L3 start the process of growth and development which includes exsheathing, molting from one stage to another, and shedding ES products. During this process antigens are shed in the GI tract and are presented by epithelial cells to underlying gut associated lymphoid tissue. Presentation of parasitic antigens are transported by M cells to the antigen specific T and or B cells in the Peyer's patches which is followed by a cascade of cellular and subcellular activities such as activation of antigen specific T and or B cells, production of a variety of cytokines that bring about activation of various cells like eosinophils, mast cells, macrophages and globule leukocytes. In addition, production of different immunoglobulin isotypes brings about immune responses leading to expulsion of worms and protection against re-infection.

### **1.5.2. Cytokines and Cellular Interaction**

T helper cells have membrane bound receptors for antigens; however the receptors do not recognize the free antigen. Antigens presented with the major histocompatibility complexes (MHC) on antigen presenting cells are recognized and further development takes place. The T helper cells of interest in this study are those which present the membrane glycoprotein molecule CD4 which recognize the antigen presented with MHC-II (Goldsby et al., 2000b). After recognizing the antigen, these T cells start secretion of cytokines. The pattern of cytokine gene expression following infection and antigen presentation to the antigen specific T lymphocytes is very important for the course of infection to be determined (Sher et al., 1992). Cytokines are messenger proteins that are produced by a variety of cells especially the cells of the immune system and are able to modulate the immune response which includes lymphocyte activation, proliferation, differentiation, survival and apoptosis (Goldsby et al., 2000a; Budhia et al., 2006). Two distinct types of cytokine patterns are produced by CD4+ T helper cells that orchestrate the cascade of cellular interplay after the antigen is presented. The first subset of these helper cells are commonly known as Th1 cells and produce interleukin (IL)-2, interferon-gamma (IFN- $\gamma$ ) and tumor necrosis factor-alpha (TNF- $\alpha$ ). This subset is involved in cell mediated immune response which is mediated by macrophages and is mainly targeted against intracellular invaders. The second subset is Th2 type cells which produce IL-4, IL-5, IL-9, IL-10 and IL-13. These are involved in immunoglobulin production, mastocytosis, and eosinophil activation and function. Immune response to nematode parasites is generally associated with this subset of T cells (Finkelman and Urban, 1992; Janeway et al., 2004).

Immunoglobulins are produced by lymphocytes known as B cells which mature in bone marrow and carry membrane bound immunoglobulin molecules that act as receptors for antigens. Interaction between antigen, macrophages and T cells activate and differentiate B cell clones of

particular specificity. They multiply by dividing repeatedly thereby generating a plasma cell population and memory cells. Plasma cells lack membrane bound immunoglobulin and synthesize any one of the several isotypes specific to the antigen presented. Plasma cells are terminally differentiated B cells and have a life of one to two weeks. Memory cells live for a long time and become activated during subsequent exposure to the antigen (Goldsby et al., 2000c).

Mast cells have acidic proteoglycan granules on their surface which take up basic dye. Mast cells are highly specialized cells that originate from myeloid lineage and are distributed in mucosal and epithelial tissues (Haig et al., 1984; Janeway et al., 2004).

Eosinophils are granulocytic leukocytes that originate from bone marrow. They have eosinophilic granules on their surface which contain arginine-rich basic proteins. Normally very few eosinophils are present in peripheral circulation (Haig et al., 1984).

Globule leukocytes are mononucleate cells that contain cytoplasmic acidophil hyaline bodies and are often associated with erythropoietic and macrophage functions. They are usually found close to the mucosal surface (Dobson, 1966) and are first (Stankiewicz et al., 1993) to be associated with helminth infections. Worm free abomasal mucosae are without globule leukocytes (Dobson, 1966).

Neutrophils are short lived with a multilobulated nucleus and originate from bone marrow. They are recruited at the beginning of an immune response. They are involved mainly in innate immunity and can recognize the pathogens by means of cell surface receptors without prior exposure. They are found to be surrounding the parasitic larvae in the first few hours after infection (Janeway et al., 2004).

Understanding the protective immune responses against nematode parasites is a prerequisite for development of efficient vaccines and designing effective strategies for their

control. There are various parasite-host models which are used to delineate the various components of immune responses and their kinetics. Due to inherent differences between the species of parasite as well as host, these model systems do not totally replicate the responses of all nematodes hence they can not be regarded as the representative of natural course of host parasite interaction. However, they greatly facilitate the understanding of different immunological responses in a convenient way which may not be always possible with the nematode and host species of concern in their natural settings. The following is a brief review of different parasite-host model systems.

### **1.5.3. Interaction between Nematodes and Host Animals**

The initiation of immune responses occurs in Payer's patches and regional lymph nodes and the spleen both play important roles in immunogenesis. Their importance was demonstrated in an experiment using rats in which these organs were surgically removed and infected with *Nippostrongylus brasiliensis* (Heatley et al., 1982). There was no effect of infection observed on cellular composition of whole blood, plasma histamine level, peripheral leukocytosis or antibody levels, mast cell recruitment or histamine content, nor on FEC. As demonstrated by Haig et al (1984), activated lymphocytes produce mucosal mast cell (MMC)-growth factors from day 10 onward after *N. brasiliensis* infection. In another study, *N. brasiliensis* infection was found to induce a 25-40 fold increase in MMC count compared to uninfected control mice (Madden et al., 1991). This increase was suppressed by about half with either anti-IL-3 or anti-IL-4 treatment alone and up to 90% with combined treatment of both. This experiment showed that IL-3 and IL-4 play an important role in MMC growth and recruitment. On the other hand degranulation of mast cells was found to be dependant on the quantity of IgE bound to their surface. Both IgE receptor density and occupancy on mast cells appeared to be T cell dependent and long lasting as demonstrated by diminished response in athymic compared to euthymic mice and rats to a *N.*

*brasiliensis* infection (Chen and Enerback, 1995; Chen et al., 1995). Ishizaka et al. (1975) found increased serum IgE in *N. brasiliensis* infected Sprague-Dawley rats and degranulation of MMC, however, no significant difference was seen in MMC between control and infected rats with respect to histamine content. Anti-IL-4 mAb treatment was found to completely abrogate *N. brasiliensis* induced IgE but not the peripheral and pulmonary eosinophilia which were inhibited by administration of anti-IL-5 mAb (Coffman et al., 1989; Chai et al., 1999). In another study, *N. brasiliensis* infected rat bone marrow cells showed higher IgE-bearing cells when cultured with serum. Similar effects were induced by cell-free supernatants (CFS) from MLN cell culture as well. This was a specific action for generation of IgE bearing cells because there was no change in the proportion of IgM bearing cells. Neutralization of IgE did not affect the generation of IgE bearing cells (Urban et al., 1977). Both T-cells and eosinophils are known to produce IL-5 which has been implicated in their differentiation and maturation. In resistant BALB/c mice infected with *N. brasiliensis*, higher IL-5 mRNA level was expressed in lamina propria and crypts in the small intestine. The difference in IL-5 mRNA level correlated with changes in blood and tissue eosinophilia, mastocytosis, IgE production, FEC and worm expulsion. However, no difference in intestinal IgA+ cells, except on day 11, was observed when they were significantly higher in BALB/c mice indicating IL-5 is an important regulatory cytokine in determining the host immunity to parasites (Madden et al., 1991). In another study, the number of adult *N. brasiliensis* recovered was significantly lower in IL-5 transgenic mice with increased total leukocytosis as well as eosinophilia which decreased on day 21 compared to normal C3H/HeN mice. However, IgE level was remarkably higher in normal lines than IL-5 transgenic mice (Chai et al., 1999; Shin et al., 2001). In another experiment, IL-5 transgenic mice resisted primary *N. brasiliensis* infection that resulted in damage to the adults in the intestine as well as inhibition of development, migration and viability in other tissues (Daly et al., 1999). The majority of the

worms were trapped at the site of inoculation for 24 hours where as in normal mice, only less than 20% were found at the site after two hours of inoculation. These findings indicate that IL-5 is necessary for resistance against *N. brasiliensis* infection (Chai et al., 1999). In an *in-vitro* culture study, eosinophils adhered to the worm surface within few minutes, flattened and degranulated; leaving cytoplasmic remnants only after 24 hr in culture (Mackenzie et al., 1981). Whereas mast cells adhered for a short time (4-6 hrs), and were neither flattened nor degranulated. On the contrary, only a small area of the cytoplasmic membrane of neutrophils flattened on the surface of the worms and adherence ceased after 2-24 hours. Macrophages adhered permanently to the surface of *Trichinella spiralis* and *N. brasiliensis* larvae and did not flatten and retained their integrity. Cytoplasmic inclusions appeared to decrease in size during culture with fewer granules present and an increased number of vacuoles. Giacomini et al., (2005) reported the adherence of complement 3 and eosinophil rich leukocytes on migrating L3 but not lung stage L4 of *N. brasiliensis*, however this worm overcame the complement mediated attack within 24 hours. These larvae were damaged by the electron dense materials released by eosinophils, and this effect was abrogated by heat treatment of serum, but IgG or IgM depletion did not have any effect (Shin et al., 2001). Similarly *Ancylostoma caninum* infection in Swiss albino mice was also associated with eosinophilic recruitment and degranulation (Vardhani, 2003). Uber et al (1980) observed the “self cure” effector mechanism in expulsion of *N. brasiliensis* in rats. It was also noted that the absence of mast cell did not change the natural course of this parasite and goblet cell proliferation in the small intestine may have a role in expulsion. Likewise, epithelial mastocytosis in intestine coincided roughly with expulsion of *T. spiralis* in BALB/C mice but not with the site of attachment or expulsion. Hence, Brown et al (1981) concluded that mastocytosis and degranulation are independent phenomenon from worm expulsion. Also mast cell deficient WWv mice were found to have expelled *N. brasiliensis*

normally like their litter mates during the second or third exposure although they were slower in rejection during the primary infection. IgE level was high but no mastocytosis was observed indicating MMC are not essential for *N. brasiliensis* rejection (Crowle and Reed, 1981). However, Ogilvie et al (1977) has the opinion that antibody damaged *N. brasiliensis* are later expelled by non-immunoglobulin bearing cells.

*Trichinella spiralis* is also a nematode parasite of many mammals including canine, porcine and feline (Urquhart et al., 1996b). Adult worms live in the small intestine and larvae parasitize the skeletal muscles. Wild type mice upon primary exposure to *T. spiralis*, respond with a production of IgE and splenic mastocytosis compared to knockout counterparts. Although, no differences in peripheral or tissue eosinophilia and jejunal mastocytosis was reported in these two types of mice, serum MMC protease-1 level in IgE deficient mice was significantly low and worms were eliminated slowly from the small intestine (Gurish et al., 2004). Infected muscle fibers in normal mice were covered with IgE which was absent in null mice. Another experiment demonstrated that increased IL-5 gene expression was accompanied by eosinophilia and wild type mice had increased contraction of intestinal muscle compared to IL-5 knockout mice in which worm expulsion was delayed (Vallance et al., 1999). Eosinophils from intestines of infected mice are also reported to resist apoptosis and lived longer (Gon et al., 1997), indicating that changes in the microenvironment that activated the eosinophils was also helpful in survival and recruitment. It was also noted that resistance to gastrointestinal parasites is accompanied by increased mucosal permeability as a result of degradation of occludin that seals the intercellular junction in the epithelium (McDermott et al., 2003). IL-9 transgenic mice exhibiting mastocytosis were able to induce increased epithelial permeability leading to expulsion of the worms but when given mast cell specific protease they failed to generate both increase in permeability and expulsion of worms. This indicated that mast cell products are directly

associated with worm expulsion. Furthermore, the mast cell knockout mice were also found to expel *T. spiralis* but slower than normal littermates, and bone marrow grafting in these mice regained the rate of worm expulsion (Ha et al., 1983). Similarly, in another experiment, degranulated mast cell granules in *T. spiralis* infected mice were observed bound to the subcutaneous injected *T. spiralis* larval antigens and engulfed by phagocytic cells. Such activity was demonstrated *in-vitro* with peritoneal mast cells that were found to take up fluoresceine labeled antigen which were detected by perigranular fluorescence which supported a role in parasitic infections (Justus and Morakote, 1981). These studies support the important role that mast cells play in expulsion of this worm.

Apart from the requirement of cytokines, presence or absence of receptors on target cells play an important role in determination of the immune status of a host. For example, IL-4R $\alpha$  expression is required for elimination of *T. spiralis* and *N. brasiliensis*, additional recruitment of T cells and mast cells are a prerequisite for IL-4-induced expulsion of *T. spiralis* but not *N. brasiliensis* (Urban et al., 2001). Although *T. spiralis* expulsion required IL-4R $\alpha$  expression by both bone-marrow and non-bone marrow-derived cells, IL-4R $\alpha$  expression by bone marrow derived cells can be bypassed by IL-4 administration. Thus, direct IL-4R $\alpha$  signaling of non-immune gastrointestinal cells may be generally required to induce worm expulsion, even when mast cell and T cell responses are also required.

Likewise, IL-10 contributes to regulation of worm expulsion. IL-10 abrogated mice are susceptible to *T. spiralis* infection. Infected mice demonstrate elevated Th1 and Th2 cytokine expression with significantly lower MMC in the jejunum and higher production of IFN- $\gamma$  resulting in increased resistance to larval stages (Helmbly and Grecnis, 2003). Hence, IL-10 has a negative regulatory effect on the tissue dwelling larval of *T. spiralis* but plays a role in MMC response. However, with *Strongyloides ratti* infection in rats, mast cells were induced in IL-4

deficient mice and parasites were expelled, therefore IL-4 is not a must for protection against *S. ratti* infection (Watanabe et al., 2001).

Like other helminth parasites, *Heligmosomoides polygyrus* also induces typical components of immediate-type hypersensitivity, including elevated serum IgE, eosinophilia, and mucosal mast cells which are T-cell-dependent and associated with rapid expulsion of parasitic worms from a sensitized host (Urban et al., 1991). In mice infected with *H. polygyrus*, the level of parasite specific serum total IgG, IgG1, IgG2b, and IgG3 isotypes of immunoglobulin correspond to the level of protection as determined in terms of infectivity, fecundity, and adult worm length (Zhong and Dobson, 1996). IgE response to *H. polygyrus* infected mice was diminished by anti IL-4 or anti IL-4R treatment compromising immunity while anti-IL-5 antibody treatment prevented eosinophilia but not protection. Long acting IL-4 (IL-4/anti-IL-4 mAb complexes) treatment resulted in decreased egg production and termination of *H. polygyrus* and *N. brasiliensis* infection in normal, severe combined immunodeficient mice and anti-CD4 mAb-treated BALB/c mice (Urban et al., 1995). Larval *H. polygyrus* were more resistant to IL-4 than the adults. Therefore, it was inferred that IL-4 alone was able to limit the survival and egg production of GI nematode parasites. Mice doubly infected with *T. spiralis* (day 0) and *H. polygyrus* (day-14), rejected *T. spiralis* at a significantly slower rate and was associated with a reduced mastocytosis and lower serum MMC protease levels (Behnke et al., 1993). Therefore, adult *H. polygyrus* was able to selectively modulate the Th2 response including depression of mucosal inflammation and promoted survival. Similarly, concurrent infection of *H. polygyrus* was found to modulate host immunity against other pathogens such as malaria by induction of high level of IL-10, TGF- $\beta$  and diminishing IFN- $\gamma$  production (Su et al., 2005). In another study, *N. dubius* (*H. polygyrus*) infected naive mice failed to express MMC response, which is prominent feature in other nematode infections, regardless of host strain and sex (Dehlawi et al.,

1987). The MMC response elicited by *N. brasiliensis* or *T. spiralis* in these mice was delayed or depressed when *N. dubius* was established earlier or concurrently with these two species, as was expulsion of the worms. However, *N. dubius* had no effect on already established mastocytosis due to *T. spiralis* infection. Furthermore, the Th2 related cytokines IL-4, IL-5 and IL-13 were not detected in *H. polygyrus* sensitized mice mesenteric lymph node lymphocytes until 48 hour culture but were seen after ConA stimulation (Doligalska et al., 2006). Therefore, it was concluded that *H. polygyrus* was able to modulate the immune responses by inducing IL-10, however, it was also reported that IL-10 may be able to regulate MMC in an autocrine manner that may be very useful in a regulatory function during their activation (Lin and Befus, 1997).

*Trichuris muris* is a nematode parasite of mice that lives in the cecum. This worm has been used as a model for *T. trichiura*, the whip worm of humans. The anterior end burrows into the mucosa which results in close contact with the host. Chronic infection with *T. muris* is associated induction of a Th1 type immune response characterized by elevated level of IFN- $\gamma$ , whereas Th2 response with increased secretion of IL-4, IL-5, IL-9 and IL-13 symbolize resistance (Artis et al., 1999). Mice that were IL-4, IL-10, and IL-10/IL-4 deficient were not able to expel *T. muris*, but those deficient in IL-10 suffered morbidity and mortality. In contrast, double IL-10/IL-12-deficient mice were completely resistant and mounted a highly polarized Th2 response, demonstrating that the increased susceptibility of IL-10-deficient mice was dependent on IL-12. Marked Th1 response was observed in IL-10 and IL-10/IL-4 deficient mice which corresponded to severe inflammation, loss of Paneth cells, and lack of mucus in the cecum (Schopf et al., 2002). It was concluded that IL-10 plays a major role in polarization of Th2 responses and development of protective immunity in *T. muris* infection. However, recently it was discovered that expulsion of *T. muris* is brought about by increased epithelial cell turnover induced by increased production of the Th2 cytokine IL-13 and chemokine CXCL10 and it was

argued that antibodies, eosinophils, natural killer cells,  $\gamma\delta$  T cells and mast cells are not required in this process (Cliffe et al., 2005). Increased epithelial turnover acts as an “epithelial escalator” removing the worms.

### **1.6. *Haemonchus contortus* Interaction in Sheep**

As in other nematode host models, the consequences of *H. contortus* infection in small ruminants may be either development of protective immunity leading to rejection of the worms or establishment of patent infection resulting in a disease condition. Compared to adult sheep; young weaned lambs are highly susceptible to *H. contortus* infection. Adult Romney sheep raised worm free until the age of 28 and 16 months and pasture infected for four weeks demonstrated lower FEC compared to four month old worm free lambs although there was no significant difference in worm burden (Douch and Morum, 1993). Similarly Barbados Blackbelly and INRA401 crossbred F1 lambs were found to be more resistant at 7 month of age compared to 3 months (Gruner et al., 2003). However, there was no such increased resistance detected in INRA401 lambs. The degree of susceptibility may also be variable among the breeds based on their resistant characteristics such as found in Native and Suffolk breeds by Bahirathan et al., (1996). Likewise, susceptibility was found to differ among the Barbados Blackbelly, Dorset, Suffolk and their crossbred lambs (Yazwinski et al., 1980). In another comparative study, Amarante et al., (1999a) demonstrated a difference in susceptibility among Florida Native, Rambouillet and their crossbred lambs. Based on the number of treatment required, the Suffolk and Ile de France sheep were highly susceptible to a mixed infection of *H. contortus*, *Trichostrongylus colubriformis*, and *Oesophagostomum columbianum* infections compared with Santa Ines sheep (Amarante et al., 2004). Similarly, Gamble and Zajac (1992) demonstrated a significantly higher resistance in St. Croix lambs compared with Dorset lambs in both artificial as well as natural infection of *H. contortus*. Gulf Coast Native lambs were found relatively more

resistant to *H. contortus* compared to Suffolk lambs based on the number of treatment required, FEC, PCV and worm burden in an epidemiological study (Miller et al., 1998). In another breed comparison study, Red Maasai sheep were found to be resistant to *H. contortus* in both natural as well as artificial infections compared to Blackheaded Somali, Dorper and Romney Marsh breeds (Mugambi et al., 1997).

Various immunological components such as changes in systemic as well as local cellular composition, production of immunoglobulins and cytokines after infection of *H. contortus* may be responsible for the resistance or susceptibility demonstrated by individuals, lines or breeds of sheep. Cytokines may also be responsible for regulation of cellular composition. Therefore, cellular and other immunological components have been evaluated. Histological examination of abomasal and small intestinal mucosa from *H. contortus* and *Trichostrongylus* spp. infected Romney sheep revealed significantly greater numbers of globule leukocytes in adults than in lambs but not eosinophil count compared to uninfected control adults and lambs that demonstrated similar cell count (Douch and Morum, 1993). Gamble and Zajac (1992) demonstrated increased *H. contortus* specific antibodies in the abomasal mucus and significantly greater number of globule leucocytes in resistant St. Croix lambs compared with susceptible Dorset lambs. *H. contortus* L3 challenged goats demonstrated presence of eosinophils, mast cells, CD3+ T lymphocytes, CD79 $\alpha$ +B cells and IgG+ plasma cells in the abomasal mucosa as early as 10 days post infection (Perez et al., 2003). They also noted that during the later part of infection infiltration of these cells were found in significantly greater numbers in previously exposed animals compared to primary infected animals. In resistant Romney sheep, the peripheral eosinophil count started increasing 1 week earlier than in random bred susceptible sheep which was not found to be correlated with FEC in resistant lambs but showed correlation at some points in susceptible lambs (Pernthaner et al., 1995). In another study, abomasal

recruitment of eosinophils, mast cells, globule leukocytes, IgA+ cell counts and histamine concentration were inversely related with *H. contortus* worm burden and FEC indicating a role in inhibition of development and or fecundity of the parasites in resistant Santa Ines breed of sheep (Amarante et al., 2005). However, upon enumeration, these cells were found not significantly different between in resistant Santa Ines and susceptible Suffolk or Ile de France breeds. Therefore, it was concluded that the number of cells does not represent the physiological status and therefore resistance may be variable among individuals and breeds with similar cell count. Furthermore, cellular recruitment is not a constant feature and varies depending on the time point at which they were counted.

Gill (1991) reported resistance status of the host positively correlated with mast cell hyperplasia and anti-*Haemonchus* antibodies. Similarly, another study revealed FEC in pasture infected lambs negatively correlated with globule leukocytes and MMC (Douch and Morum, 1993). Likewise, *H. contortus* infection in lambs of different resistance status resulted in a prominent peripheral eosinophilia in artificially challenged Florida Native and their F2 lambs that peaked at 21 days and then declined (Amarante et al., 1999a). Although the *H. contortus* burden was significantly higher in Rambouillet and F1 lambs than in Florida Native and F2 lambs, the abomasal cell counts were not different. FEC and worm burden had a negative correlation with PCV, plasma protein and mast cell counts in the abomasal mucosa. However, eosinophilia was found as a common feature in Romney (Buddle et al., 1992) and Australian lines of Merino (Hohenhaus and Outteridge, 1995) that were resistant to *T. colubriformis* and *H. contortus* based of low FEC. Resistant lambs consistently demonstrated higher levels of vasoactive amines and leukatreines in their gut mucous which suggested that these mediators were involved in resistance against *T. colubriformis* and *H. contortus*. Globule leukocytes were reported to be in abundance just posterior to the site of adult worm attachment (Stankiewicz et

al., 1993). In contrast to the earlier reports, they reported that globule leukocytes migrate to the intestinal lumen indicating the mode of effector mechanism. They also observed that multi-nematode species immunized sheep had a significantly greater globule leukocyte count and no parasites after challenge compared to non-immunized sheep that had few globule leukocytes and high worm count. This trend was also observed in resistant and susceptible animals (Stankiewicz et al., 1993).

The mucus layer over the mucosa acts as a physical barrier in addition to the presence of larval migration inhibitory compounds produced by MMC and globule leukocytes (Lee and Ogilvie, 1982; Douch et al., 1984; Stankiewicz et al., 1993). Resistant animals demonstrated a high degree of larval migration inhibition activity compared to non-immunized and susceptible animals (Stankiewicz et al., 1995). The larval migration inhibition activity was positively correlated with globule leukocytes and eosinophils. Sheep that were given an immunizing infection with *T. colubriformis* and *Teladorsagia (Ostertagia) circumcincta* demonstrated increased levels of globule leukocytes in the tissue as well as in the lumen. There was a positive correlation between protection and anti-larval activity in the mucus (Stankiewicz et al., 1995).

Smith et al (1977a) found slower development of response to repeated infections of *H. contortus* that declined in the absence of larvae with no immunological memory 9 weeks after the first exposure. In contrast, infections of *T. colubriformis* terminated with thiabendazole treatment induced a considerable immunity to homologous L4 and L5 challenge and were equally effective at stimulating immunity (Douch et al., 1986). Furthermore, a strong association between resistance and globule leukocytes was demonstrated but such association was lacking with mast cells or goblet cells. They also found globule leukocyte and eosinophil count correlated with anti-parasitic activity in mucus.

Eosinophils from mammary washes of sheep were found to immobilize and kill *H. contortus* larvae *in vitro* in the presence of anti-larval antibody (Rainbird et al., 1998). Those from primed animals were more efficient and addition of complement increased their efficiency. Naive eosinophils were also able to immobilize *H. contortus* larvae in the presence of antibody, complement and IL-5 which suggested that eosinophil effector function was enhanced by IL-5. Upon ultra structural analysis, eosinophils were degranulated onto the larval surface at 6 hour and by 24 hours larvae showed surface damage and most of the eosinophils were degenerated. These results suggested that eosinophil-mediated killing may be an effector mechanism for the elimination of *H. contortus* L3 in immune sheep.

Effector cell growth and activation are mediated by various cytokines produced in response to infection. Therefore, a clearer understanding of cytokine expression is important to further delineate the immune response. In *H. contortus* infected naive lambs, expression of IL-4 mRNA was induced 7 days after infection, but in previously sensitized lambs, increased expression was observed in abomasal lymph nodes (ALN) and fundic mucosa 3 days post infection whereas it was totally absent in uninfected controls (Gill et al., 2000). IL-5 mRNA expression was also found to be at higher levels in fundic mucosa but only after 28 days post infection in ALN cells. IL-13 mRNA showed a higher trend in production by day 7. However, these cytokine levels did not correlate with worm burden. Draining lymph nodes like ALN and mesenteric lymph node (MLN) cells from *H. contortus* infected lambs expressed lower levels of IFN- $\gamma$  compared to uninfected lambs regardless of resistance status (Gill et al., 2000). IL-5 was expressed by both groups of cells *in-vitro* but it was higher in resistant compared to random bred lambs and IgE and IgG1 levels followed the same trend. In another study, IFN- $\gamma$ , IL-13 and IL-5 increased 3 days after challenge, but each showed a different trend (Meeusen et al., 2005). IL-4 peaked at 5 days post challenge and remained at a high level throughout the challenge period

which indicated its importance in the early or rapid expulsion while IL-5 and IL-13 may be critical in delayed rejection when the larvae reach the mucosal tissue niche. Similarly, a *T. colubriformis* resistant line of sheep also expressed IL-5 and IL-13 genes at a higher level compared to susceptible lines (Pernthaner et al., 2005). However, IFN- $\gamma$ , IL-4 and IL-10 gene expression was not different between the two lines.

All of the immunoglobulin isotypes have been reported to be produced after *H. contortus* infection in sheep with variation in magnitude and time sequence. This variation may be an indication of their importance and role played in different stages of infection. Kooyman et al., (1997) reported increased level of IgE specific to *H. contortus* 2-4 weeks post infection. Increased level of IgG1 has been reported to be followed by IgG2, IgM and IgA (Schallig et al., 1995). Similarly, resistant lines of sheep were found to produce higher level of IgE and IgG1 when compared with random bred lambs (Gill et al., 2000). On the contrary, all the isotypes were reported to have been produced in sheep however they were found not significantly correlated with resistance against *H. contortus* (Gomez-Munoz et al., 1999). Lacroux et al., (2006) reported induction of IgA production in secondarily challenged animals by day 7 and 15 in mucus while serum IgG and IgA were detected on 15 and 28 days in response to *H. contortus* ES products. Significantly higher levels of IgG1 and IgM were also found in *T. colubriformis* resistant lambs (Bisset et al., 1996). The effector function of mast cells can be expressed in the presence of IgE (Gurish et al., 2004). They also demonstrated low levels of mouse mast cell protease-I in deficient mice which resulted in slower elimination of nematode parasites. A trend of antibody production in response to *T. colubriformis* and *H. contortus* primary infection was also suggested as another indication of resistance (Hohenhaus and Outteridge, 1995). Immunization of naive adults with irradiated *H. contortus* larvae generally resulted in production of serum IgG, mucosal IgA, IgG which was not seen in two-month-old lambs (Smith and Angus, 1980). Four doses of

the attenuated larvae induced resistance in 3 of 6 lambs against a challenge. However, lambs vaccinated with larval antigens and two doses of irradiated larvae had much higher levels of circulating and mucosal IgG, but mucosal IgA levels were similar to those in the other immunized groups. Smith (1977a) demonstrated increased levels of IgA and IgG in abomasal mucus after repeated infections of *H. contortus* in lambs which could be of local and systemic origin, respectively. In contrast, lambs immunized with irradiated larvae demonstrated increased level of systemic IgG but not IgA in the abomasal mucus which was not protective (Smith, 1977b).

The treatment of immune sheep with dexamethasone is known to down regulate resistance by reduced numbers of mast cells in the mucosal as well as reduced serum mast cell protease (Douch et al., 1984; Douch et al., 1986; Douch et al., 1996; Huntley et al., 1992). Similarly, fewer tissue mast cells and eosinophils were associated with higher *Nematodirus battus* worm burden and higher fecundity of the worms in lambs treated with dexamethasone compared to untreated lambs (Winter et al., 1997). Rats primed with *N. brasiliensis* when challenged intravenously with homologous whole worm antigen (WWA) had systemic anaphylactic shock with increased mucosal permeability and MMC protease, and rat mast cell protease II (RMCP-II) was present in the gut and circulation (King et al., 1985). However, treatment with corticosteroids prevented this from happening. Diminished response to infection in corticosteroid treated rodents may be due to inhibition of cytokine production by T cells. However, MMC count and mouse intestinal mucosal cell protease (MIMCP) in mouse intestine with established infection of *N. brasiliensis* did not change post treatment, which was in contrast to decreased MMC count as well as RMCP II concentration after steroid treatment in *Nippostrongylus*-infected rats (Newlands et al., 1990). Presson et al., (1988) reported that genetic resistance of Merino sheep was abolished after treatment with dexamethasone which resulted in

equal susceptibility to *H. contortus* infection. Similarly, Native lambs treated with dexamethasone demonstrated high FEC, lower PCV accompanied by high worm count and lower serum level of anti-*Haemonchus* antibody with a depressed lymphocyte count compared to untreated control animals (Pena et al., 2004). Genetic resistance of sheep to the abomasal parasite *H. contortus* appeared to depend on the ability to express an acquired immune response, and mastocytosis, eosinophils and globule leukocytes along with anti-parasite immunoglobulins like IgA, IgE, IgG1 play an important role. Length of the adult worms were affected by degree of resistance an animal acquires as *O. ostertagia* were shortest in resistant, intermediate in truncated infections and normal full length in controls (Claerebout et al., 1996). Sheep doubly infected with *Oestrus ovis* and *H. contortus* had shorter *Haemonchus* females and decreased *in utero* egg count, which was attributed to induction of eosinophils and globule leukocytes by *O. ovis* larvae (Terefe et al., 2005).

Native sheep have demonstrated relatively greater resistance to infection of *H. contortus* (Bahirathan et al., 1996; Miller et al., 1998). In these studies, the characteristic responses in Native lambs were compared with susceptible Suffolk lambs. Dexamethasone treatment resulted in reversion to susceptibility which indicated a genetic component was involved in control (Pena et. al, 2004). In another experiment, CD4+ T cell depletion also was found to abolish the resistant characteristics indicating it to be a adaptive immune response . Determining the immunological components that are responsible for the demonstrated resistance of the Native lambs against *H. contortus* is therefore important and it would help understand the immune response in resistant breeds of sheep. This knowledge may also be helpful in selection of individuals for breeding of animals for resistance against *H. contortus* infection.

## 1.7. Research Objectives

The objectives of this study were to 1) compare infection level between Native and Suffolk lambs, 2) define cellular recruitment in the abomasum, 3) quantify cytokine mRNA expression of IL-4, IL-10 and IFN- $\gamma$ , and 4) establish serum immunoglobulin isotype profiles.

## 1.8. Hypothesis

There is difference in infection level and expression, magnitude and time sequence of the various components of the immune response in Native compared to Suffolk lambs.

## 1.9. References

Abbott, E.M., Parkins, J.J., Holmes, P.H., 1985a, Influence of dietary protein on parasite establishment and pathogenesis in Finn Dorset and Scottish Blackface lambs given a single moderate infection of *Haemonchus contortus*. Res Vet Sci 38, 6-13.

Abbott, E.M., Parkins, J.J., Holmes, P.H., 1985b, Influence of dietary protein on the pathophysiology of ovine haemonchosis in Finn Dorset and Scottish Blackface lambs given a single moderate infection. Res Vet Sci 38, 54-60.

Altaif, K.I., Dargie, J.D., 1978, Genetic resistance to helminths. The influence of breed and haemoglobin type on the response of sheep to primary infections with *Haemonchus contortus*. Parasitology 77, 177-187.

Amarante, A.F., Bricarello, P.A., Huntley, J.F., Mazzolin, L.P., Gomes, J.C., 2005, Relationship of abomasal histology and parasite-specific immunoglobulin A with the resistance to *Haemonchus contortus* infection in three breeds of sheep. Vet Parasitol 128, 99-107.

Amarante, A.F., Bricarello, P.A., Rocha, R.A., Gennari, S.M., 2004, Resistance of Santa Ines, Suffolk and Ile de France sheep to naturally acquired gastrointestinal nematode infections. Vet Parasitol 120, 91-106.

Amarante, A.F., Craig, T.M., Ramsey, W.S., Davis, S.K., Bazer, F.W., 1999a, Nematode burdens and cellular responses in the abomasal mucosa and blood of Florida Native, Rambouillet and crossbred lambs. Vet Parasitol 80, 311-324.

Amarante, A.F., Craig, T.M., Ramsey, W.S., El-Sayed, N.M., Desouki, A.Y., Bazer, F.W., 1999b, Comparison of naturally acquired parasite burdens among Florida native, Rambouillet and crossbred ewes. Vet Parasitol 85, 61-69.

Artis, D., Potten, C.S., Else, K.J., Finkelman, F.D., Grecnis, R.K., 1999, *Trichuris muris*: host intestinal epithelial cell hyperproliferation during chronic infection is regulated by interferon-gamma. Exp Parasitol 92, 144-153.

Bahirathan, M., Miller, J.E., Barras, S.R., Kearney, M.T., 1996, Susceptibility of Suffolk and Gulf Coast Native suckling lambs to naturally acquired strongylate nematode infection. *Vet Parasitol* 65, 259-268.

Barras S. E., 1997, Epidemiology of gastrointestinal nematode parasitism in Suffolk, Native and Crossbred lambs in relation to breed resistance and production. MS Thesis, Louisiana State University, Baton Rouge, LA, USA.

Barrau, E., Fabre, N., Fouraste, I., Hoste, H., 2005, Effect of bioactive compounds from Sainfoin (*Onobrychis viciifolia* Scop.) on the in vitro larval migration of *Haemonchus contortus*: role of tannins and flavonol glycosides. *Parasitology* 131, 531-538.

Behnke, J.M., Wahid, F.N., Grecis, R.K., Else, K.J., Ben-Smith, A.W., Goyal, P.K., 1993, Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): downregulation of specific cytokine secretion (IL-9 and IL-10) correlates with poor mastocytosis and chronic survival of adult worms. *Parasite Immunol* 15, 415-421.

Berijaya, Copeman, D.B., 2006, *Haemonchus contortus* and *Trichostrongylus colubriformis* in pen-trials with Javanese thin tail sheep and Kacang cross Etawah goats. *Vet Parasitol* 135, 315-323.

Bisset, S.A., Morris, C.A., 1996, Feasibility and implications of breeding sheep for resilience to nematode challenge. *Int J Parasitol* 26, 857-868.

Bradley, R.E., Radhakrishnan, C.V., Patil-Kulkarni, C.G., Loggins, P.E., 1973, Responses in Florida Native and Rambouillet lambs exposed to one and two oral doses of *Haemonchus contortus*. *Am J Vet Res* 34, 729-735.

Bricarello, P.A., Amarante, A.F., Rocha, R.A., Cabral Filho, S.L., Huntley, J.F., Houdijk, J.G., Abdalla, A.L., Gennari, S.M., 2005, Influence of dietary protein supply on resistance to experimental infections with *Haemonchus contortus* in Ile de France and Santa Ines lambs. *Vet Parasitol* 134, 99-109.

Brown, P.J., Bruce, R.G., Manson-Smith, D.F., Parrott, D.M., 1981, Intestinal mast cell response in thymectomised and normal mice infected with *Trichinella spiralis*. *Vet Immunol Immunopathol* 2, 189-198.

Buddle, B.M., Jowett, G., Green, R.S., Douch, P.G., Risdon, P.L., 1992, Association of blood eosinophilia with the expression of resistance in Romney lambs to nematodes. *Int J Parasitol* 22, 955-960.

Budhia, S., Haring, L.F., McConnell, I., Blacklaws, B.A., 2006, Quantitation of ovine cytokine mRNA by real-time RT-PCR. *J Immunol Methods* 309, 160-172.

- Burke, J.M., Miller, J.E., 2006, Evaluation of multiple low doses of copper oxide wire particles compared with levamisole for control of *Haemonchus contortus* in lambs. *Vet Parasitol* 139, 145-149.
- Burke, J.M., Miller, J.E., Larsen, M., Terrill, T.H., 2005, Interaction between copper oxide wire particles and *Duddingtonia flagrans* in lambs. *Vet Parasitol* 134, 141-146.
- Chai, J.Y., Shin, E.H., Takatsu, K., Matsumoto, N., Kojima, S., 1999, Eosinophil and IgE responses of IL-5 transgenic mice experimentally infected with *Nippostrongylus brasiliensis*. *Korean J Parasitol* 37, 93-99.
- Chandrawathani, P., Jamnah, O., Adnan, M., Waller, P.J., Larsen, M., Gillespie, A.T., 2004, Field studies on the biological control of nematode parasites of sheep in the tropics, using the microfungus *Duddingtonia flagrans*. *Vet Parasitol* 120, 177-187.
- Chandrawathani, P., Jamnah, O., Waller, P.J., Hoglund, J., Larsen, M., Zahari, W.M., 2002, Nematophagous fungi as a biological control agent for nematode parasites of small ruminants in Malaysia: a special emphasis on *Duddingtonia flagrans*. *Vet Res* 33, 685-696.
- Chen, X.J., Enerback, L., 1995, Surface expression of IgE receptors, IgE occupancy and histamine releasability of mast cells: influence of genetic and T cell factors. *Int Arch Allergy Immunol* 107, 132-134.
- Chen, X.J., Wiedermann, U., Dahlgren, U., Hanson, L.A., Enerback, L., 1995, T-cell-independent and T-cell-dependent IgE responses to the nematode *Nippostrongylus brasiliensis*: comparison of serum IgE and mast-cell-bound IgE. *Immunology* 86, 351-355.
- Claerebout, E., Hilderson, H., Meeus, P., De Marez, T., Behnke, J., Huntley, J., Vercruyse, J., 1996, The effect of truncated infections with *Ostertagia ostertagi* on the development of acquired resistance in calves. *Vet Parasitol* 66, 225-239.
- Cliffe, L.J., Humphreys, N.E., Lane, T.E., Potten, C.S., Booth, C., Grecis, R.K., 2005, Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. *Science* 308, 1463-1465.
- Coffman, R.L., Seymour, B.W., Hudak, S., Jackson, J., Rennick, D., 1989, Antibody to interleukin-5 inhibits helminth-induced eosinophilia in mice. *Science* 245, 308-310.
- Courtney, C.H., Parker, C.F., McClure, K.E., Herd, R.P., 1985, Resistance of exotic and domestic lambs to experimental infection with *Haemonchus contortus*. *Int J Parasitol* 15, 101-109.
- Craig, T.M., 1993, Anthelmintic resistance. *Veterinary Parasitology* 46, 121-131.
- Crowle, P.K., Reed, N.D., 1981, Rejection of the intestinal parasite *Nippostrongylus brasiliensis* by mast cell-deficient W/W<sup>v</sup> anemic mice. *Infect Immun* 33, 54-58.

- Daly, C.M., Mayrhofer, G., Dent, L.A., 1999, Trapping and immobilization of *Nippostrongylus brasiliensis* larvae at the site of inoculation in primary infections of interleukin-5 transgenic mice. *Infect Immun* 67, 5315-5323.
- Davidson, W.R., McGhee, M.B., Nettles, V.F., Chappell, L.C., 1980, Haemonchosis in white-tailed deer in the southeastern United States. *J Wildl Dis* 16, 499-508.
- Dehlawi, M.S., Wakelin, D., Behnke, J.M., 1987, Suppression of mucosal mastocytosis by infection with the intestinal nematode *Nematospiroides dubius*. *Parasite Immunol* 9, 187-194.
- Dobson, C., 1966, Studies on the immunity of sheep to *Oesophagostomum columbianum*: the nature and fate of the globule leukocyte. *Aust. J. Agric. Res* 17, 955-966.
- Doligalska, M., Rzepecka, J., Drela, N., Donskow, K., Gerwel-Wronka, M., 2006, The role of TGF-beta in mice infected with *Heligmosomoides polygyrus*. *Parasite Immunol* 28, 387-395.
- Douch, P.G., Harrison, G.B., Buchanan, L.L., Brunsdon, R.V., 1984, Relationship of histamine in tissues and antiparasitic substances in gastrointestinal mucus to the development of resistance to trichostrongyle infections in young sheep. *Vet Parasitol* 16, 273-288.
- Douch, P.G., Harrison, G.B., Elliott, D.C., Buchanan, L.L., Greer, K.S., 1986, Relationship of gastrointestinal histology and mucus antiparasite activity with the development of resistance to trichostrongyle infections in sheep. *Vet Parasitol* 20, 315-331.
- Douch, P.G., Morum, P.E., 1993, The effect of age on the response of Romney sheep to gastrointestinal nematodes during grazing. *Int J Parasitol* 23, 651-655.
- Douch, P.G., Morum, P.E., Rabel, B., 1996, Secretion of anti-parasite substances and leukotrienes from ovine gastrointestinal tissues and isolated mucosal mast cells. *Int J Parasitol* 26, 205-211.
- Eddi, C., Caracostantogolo, J., Pena, M., Schapiro, J., Marangunich, L., Waller, P.J., Hansen, J.W., 1996, The prevalence of anthelmintic resistance in nematode parasites of sheep in southern Latin America: Argentina. *Vet Parasitol* 62, 189-197.
- Emery, D., and B. M. Wagland, 1991, Vaccines against gastrointestinal nematode parasites of ruminants. *Parasitology Today* 7, 347-349.
- Ensminger, M.E., 2002, *Sheep and Goat Science*, Sixth Edition. Interstate Publishers, Inc., Danville, Illinois.
- Fernandez, A.S., Larsen, M., Henningsen, E., Nansen, P., Gronvold, J., Bjorn, H., and Wolstrup, J., 1999, Effect of *Duddingtonia flagrans* against *Ostertagia ostertagi* in cattle grazing at different stocking rates. *Parasitology*, 105-111.
- Finkelman, F.D., Urban, J.F., Jr., 1992, Cytokines: making the right choice. *Parasitol Today* 8, 311-314.

Gamble, H.R., Zajac, A.M., 1992, Resistance of St. Croix lambs to *Haemonchus contortus* in experimentally and naturally acquired infections. *Vet Parasitol* 41, 211-225.

Gasbarre, L.C., 1997, Effects of gastrointestinal nematode infection on the ruminant immune system. *Vet Parasitol* 72, 327-337; discussion 337-343.

Giacomin, P.R., Wang, H., Gordon, D.L., Botto, M., Dent, L.A., 2005, Loss of complement activation and leukocyte adherence as *Nippostrongylus brasiliensis* develops within the murine host. *Infect Immun* 73, 7442-7449.

Gill, H.S., 1991, Genetic control of acquired resistance to haemonchosis in Merino lambs. *Parasite Immunol* 13, 617-628.

Gill, H.S., Altmann, K., Cross, M.L., Husband, A.J., 2000, Induction of T helper 1- and T helper 2-type immune responses during *Haemonchus contortus* infection in sheep. *Immunology* 99, 458-463.

Goldsby, R.A., Kindt, T.J., Osborne, B.A., 2000a, *Kuby Immunology, Fourth Edition*. W.H. Freeman and Company, New York, 308-311 pp.

Goldsby, R.A., Kindt, T.J., Osborne, B.A., 2000b, *Kuby Immunology, Fourth Edition*. W.H. Freeman and Company, New York, 40 p.

Goldsby, R.A., Kindt, T.J., Osborne, B.A., 2000c, *Kuby Immunology, Fourth Edition*. W.H. Freeman and Company, New York, 44-45 pp.

Gomez-Munoz, M.T., Cuquerella, M., Gomez-Iglesias, L.A., Mendez, S., Fernandez-Perez, F.J., de la Fuente, C., Alunda, J.M., 1999, Serum antibody response of Castellana sheep to *Haemonchus contortus* infection and challenge: relationship to abomasal worm burdens. *Vet Parasitol* 81, 281-293.

Gon, S., Saito, S., Takeda, Y., Miyata, H., Takatsu, K., Sendo, F., 1997, Apoptosis and in vivo distribution and clearance of eosinophils in normal and *Trichinella spiralis*-infected rats. *J Leukoc Biol* 62, 309-317.

Gray, G.D., Barger, I.A., Le Jambre, L.F., Douch, P.G., 1992, Parasitological and immunological responses of genetically resistant Merino sheep on pastures contaminated with parasitic nematodes. *Int J Parasitol* 22, 417-425.

Gruner, L., Aumont, G., Getachew, T., Brunel, J.C., Pery, C., Cognie, Y., Guerin, Y., 2003, Experimental infection of Black Belly and INRA 401 straight and crossbred sheep with trichostrongyle nematode parasites. *Vet Parasitol* 116, 239-249.

Gurish, M.F., Bryce, P.J., Tao, H., Kisselgof, A.B., Thornton, E.M., Miller, H.R., Friend, D.S., Oettgen, H.C., 2004, IgE enhances parasite clearance and regulates mast cell responses in mice infected with *Trichinella spiralis*. *J Immunol* 172, 1139-1145.

- Ha, T.Y., Reed, N.D., Crowle, P.K., 1983, Delayed expulsion of adult *Trichinella spiralis* by mast cell-deficient W/W<sup>v</sup> mice. *Infect Immun* 41, 445-447.
- Haig, D.M., Jarrett, E.E., Tas, J., 1984, *In vitro* studies on mast cell proliferation in *N. brasiliensis* infection. *Immunology* 51, 643-651.
- Heatley, R.V., Befus, A.D., Bienenstock, J., 1982, *Nippostrongylus brasiliensis* infection in the rat: effects of surgical removal of Peyer's patches, mesenteric lymph nodes and spleen. *Int Arch Allergy Appl Immunol* 68, 397-398.
- Helmby, H., Grecis, R.K., 2003, Contrasting roles for IL-10 in protective immunity to different life cycle stages of intestinal nematode parasites. *Eur J Immunol* 33, 2382-2390.
- Hohenhaus, M.A., Outteridge, P.M., 1995, The immunogenetics of resistance to *Trichostrongylus colubriformis* and *Haemonchus contortus* parasites in sheep. *Br Vet J* 151, 119-140.
- Houdijk, J.G., Jackson, F., Coop, R.L., Kyriazakis, I., 2006, Rapid improvement of immunity to *Teladorsagia circumcincta* is achieved through a reduction in the demand for protein in lactating ewes. *Int J Parasitol* 36, 219-227.
- Houdijk, J.G., Kyriazakis, I., Jackson, F., Huntley, J.F., Coop, R.L., 2003, Is the allocation of metabolisable protein prioritised to milk production rather than to immune functions in *Teladorsagia circumcincta*-infected lactating ewes? *Int J Parasitol* 33, 327-338.
- Houdijk, J.G., Kyriazakis, I., Jackson, F., Huntley, J.F., Coop, R.L., 2005, Effects of protein supply and reproductive status on local and systemic immune responses to *Teladorsagia circumcincta* in sheep. *Vet Parasitol* 129, 105-117.
- Huntley, J.F., Newlands, G.F., Jackson, F., Miller, H.R., 1992, The influence of challenge dose, duration of immunity, or steroid treatment on mucosal mast cells and on the distribution of sheep mast cell proteinase in *Haemonchus*-infected sheep. *Parasite Immunol* 14, 429-440.
- Iqbal, Z., Sarwar, M., Jabbar, A., Ahmed, S., Nisa, M., Sajid, M.S., Khan, M.N., Mufti, K.A., Yaseen, M., 2007, Direct and indirect anthelmintic effects of condensed tannins in sheep. *Vet Parasitol* 144, 125-131.
- Ishizaka, T., Konig, W., Kurata, M., Mauser, L., Ishizaka, K., 1975, Immunologic properties of mast cells from rats infected with *Nippostrongylus brasiliensis*. *J Immunol* 115, 1078-1083.
- Jacobs, H.J., Wiltshire, C., Ashman, K., Meeusen, E.N., 1999, Vaccination against the gastrointestinal nematode, *Haemonchus contortus*, using a purified larval surface antigen. *Vaccine* 17, 362-368.
- Janeway, C.A., Travers, P., Walport, M., Sclomchik, M., 2004, *Immunobiology: The Immune System in Health and Disease*, Sixth Edition. Garland Publisher.

- Justus, D.E., Morakote, N., 1981, Mast cell degranulation associated with sequestration and removal of *Trichinella spiralis* antigens. *Int Arch Allergy Appl Immunol* 64, 371-384.
- Kabagambe, E.K., Barras, S.R., Li, Y., Pena, M.T., Smith, W.D., Miller, J.E., 2000, Attempts to control haemonchosis in grazing ewes by vaccination with gut membrane proteins of the parasite. *Vet Parasitol* 92, 15-23.
- Kagira, J., Kanyaria, P.W., 2001, The role of parasitic diseases as causes of mortality in small ruminants in a high-potential farming area in central Kenya. *J S Afr Vet Assoc* 72, 147-149.
- Kahiya, C., Mukaratirwa, S., Thamsborg, S.M., 2003, Effects of *Acacia nilotica* and *Acacia karoo* diets on *Haemonchus contortus* infection in goats. *Vet Parasitol* 115, 265-274.
- Kaplan, R.M., Burke, J.M., Terrill, T.H., Miller, J.E., Getz, W.R., Mobini, S., Valencia, E., Williams, M.J., Williamson, L.H., Larsen, M., Vatta, A.F., 2004, Validation of the FAMACHA eye color chart for detecting clinical anemia in sheep and goats on farms in the southern United States. *Vet Parasitol* 123, 105-120.
- Kaplan R.M., Burke, J.M., Howell, S.B., and Rocconi, J.R., 2005. Total Anthelmintic failure on a meat goat farm in Arkansas, USA. American Association of Veterinary Parasitologists, 50th Annual Meeting, Minneapolis, MN.
- King, S.J., Miller, H.R., Newlands, G.F., Woodbury, R.G., 1985, Depletion of mucosal mast cell protease by corticosteroids: effect on intestinal anaphylaxis in the rat. *Proc Natl Acad Sci USA* 82, 1214-1218.
- Kooyman, F.N., Van Kooten, P.J., Huntley, J.F., MacKellar, A., Cornelissen, A.W., Schallig, H.D., 1997, Production of a monoclonal antibody specific for ovine immunoglobulin E and its application to monitor serum IgE responses to *Haemonchus contortus* infection. *Parasitology* 114 ( Pt 4), 395-406.
- Krecek, R.C., Waller, P.J., 2006, Towards the implementation of the "basket of options" approach to helminth parasite control of livestock: Emphasis on the tropics/subtropics. *Vet Parasitol* 139, 270-282.
- Lacroux, C., Nguyen, T.H., Andreoletti, O., Prevot, F., Grisez, C., Bergeaud, J.P., Gruner, L., Brunel, J.C., Francois, D., Dorchies, P., Jacquet, P., 2006, *Haemonchus contortus* (Nematoda: Trichostrongylidae) infection in lambs elicits an unequivocal Th2 immune response. *Vet Res* 37, 607-622.
- Lange, K.C., Olcott, D.D., Miller, J.E., Mosjidis, J.A., Terrill, T.H., Burke, J.M., Kearney, M.T., 2006, Effect of sericea lespedeza (*Lepedeza cuneata*) fed as hay, on natural and experimental *Haemonchus contortus* infections in lambs. *Vet Parasitol* 141, 273-278.
- Larsen, M., Faedo, M., Waller, P., and Hennessy, D.R., 1998, The potential of nematophagus fungi to control the free-living stages of nematode parasites of sheep: Studies with *Duddingtonia flagrans*. *Veterinary Parasitology* 76, 121-128.

- Lee, G.B., Ogilvie, B.M., 1982, The intestinal mucus barrier to parasites and bacteria. *Adv Exp Med Biol* 144, 247-248.
- Levine, N.D., Clark, D.T., Bradley, R.E., Kantor, S., 1975, Relationship of pasture rotation to acquisition of gastrointestinal nematodes by sheep. *Am J Vet Res* 36, 1459-1464.
- Lin, T.J., Befus, A.D., 1997, Differential regulation of mast cell function by IL-10 and stem cell factor. *J Immunol* 159, 4015-4023.
- Mackenzie, C.D., Jungery, M., Taylor, P.M., Ogilvie, B.M., 1981, The *in-vitro* interaction of eosinophils, neutrophils, macrophages and mast cells with nematode surfaces in the presence of complement or antibodies. *J Pathol* 133, 161-175.
- Madden, K.B., Urban, J.F., Jr., Ziltener, H.J., Schrader, J.W., Finkelman, F.D., Katona, I.M., 1991, Antibodies to IL-3 and IL-4 suppress helminth-induced intestinal mastocytosis. *J Immunol* 147, 1387-1391.
- McDermott, J.R., Bartram, R.E., Knight, P.A., Miller, H.R., Garrod, D.R., Grencis, R.K., 2003, Mast cells disrupt epithelial barrier function during enteric nematode infection. *Proc Natl Acad Sci USA* 100, 7761-7766.
- Meeusen, E.N., Balic, A., Bowles, V., 2005, Cells, cytokines and other molecules associated with rejection of gastrointestinal nematode parasites. *Vet Immunol Immunopathol* 108, 121-125.
- Miller, H.R., 1984, The protective mucosal response against gastrointestinal nematodes in ruminants and laboratory animals. *Vet Immunol Immunopathol* 6, 167-259.
- Miller, J.E., Bahirathan, M., Lemarie, S.L., Hembry, F.G., Kearney, M.T., Barras, S.R., 1998, Epidemiology of gastrointestinal nematode parasitism in Suffolk and Gulf Coast Native sheep with special emphasis on relative susceptibility to *Haemonchus contortus* infection. *Vet Parasitol* 74, 55-74.
- Miller, J.E., Horohov, D.W., 2006, Immunological aspects of nematode parasite control in sheep. *J Anim Sci* 84 Suppl, E124-132.
- Mitchell, L.A., Wescott, R.B., Perryman, L.E., 1983, Kinetics of expulsion of the nematode, *Nippostrongylus brasiliensis*, in mast-cell deficient W/WV mice. *Parasite Immunol* 5, 1-12.
- Mugambi, J.M., Bain, R.K., Wanyangu, S.W., Ihiga, M.A., Duncan, J.L., Murray, M., Stear, M.J., 1997, Resistance of four sheep breeds to natural and subsequent artificial *Haemonchus contortus* infection. *Vet Parasitol* 69, 265-273.
- Nansen, P., Larsen, M., Gronvold, J., Wolstrup, P., Zorn, A., and Henriksen, S.A., 1995, Prevention of clinical trichostrongylidosis in calves by strategic feeding with the predacious fungus *Duddingtonia flagrans*. *Prasitology Research* 81, 371-374.

- Newlands, G.F., MacKellar, A., Miller, H.R., 1990, Intestinal mucosal mast cells in *Nippostrongylus*-infected mice: lack of sensitivity to corticosteroids. *Int J Parasitol* 20, 669-672.
- Newton, S.E., Meeusen, E.N., 2003, Progress and new technologies for developing vaccines against gastrointestinal nematode parasites of sheep. *Parasite Immunol* 25, 283-296.
- Ogilvie, B.M., Love, R.J., Jarra, W., Brown, K.N., 1977, *Nippostrongylus brasiliensis* infection in rats: The cellular requirement for worm expulsion. *Immunology* 32, 521-528.
- Olcott, D.D., 2006. Effect of vaccination in goat with H-GAL-GP and H-11 antigens from intestinal membrane cells of *Haemonchus contortus*. MS Thesis, Louisiana State University, Baton Rouge, LA, USA.
- Paolini, V., Bergeaud, J.P., Grisez, C., Prevot, F., Dorchies, P., Hoste, H., 2003, Effects of condensed tannins on goats experimentally infected with *Haemonchus contortus*. *Vet Parasitol* 113, 253-261.
- Pena, M.T., 2001. The role of immunity in resistance of Gulf Coast Native sheep to *Haemonchus contortus* infection. MS Thesis, Louisiana State University, Baton Rouge, LA, USA.
- Pena, M.T., 2004, Miller, J.E., Horohov, D.W., 2004, Effect of dexamethasone treatment on the immune response of Gulf Coast Native lambs to *Haemonchus contortus* infection. *Vet Parasitol* 119, 223-235.
- Pena, M.T., Miller, J.E., Horohov, D.W., 2006, Effect of CD4+ T lymphocyte depletion on resistance of Gulf Coast Native lambs to *Haemonchus contortus* infection. *Vet Parasitol* 138, 240-246.
- Perez, J., Garcia, P.M., Hernandez, S., Mozos, E., Camara, S., Martinez-Moreno, A., 2003, Experimental haemonchosis in goats: effects of single and multiple infections in the host response. *Vet Parasitol* 111, 333-342.
- Pernthaner, A., Cole, S.A., Morrison, L., Hein, W.R., 2005, Increased expression of interleukin-5 (IL-5), IL-13, and tumor necrosis factor alpha genes in intestinal lymph cells of sheep selected for enhanced resistance to nematodes during infection with *Trichostrongylus colubriformis*. *Infect Immun* 73, 2175-2183.
- Pernthaner, A., Stankiewicz, M., Bisset, S.A., Jonas, W.E., Cabaj, W., Pulford, H.D., 1995, The immune responsiveness of Romney sheep selected for resistance or susceptibility to gastrointestinal nematodes: lymphocyte blastogenic activity, eosinophilia and total white blood cell counts. *Int J Parasitol* 25, 523-529.
- Peter, J.W., Chandrawathani, P., 2005, *Haemonchus contortus*: Parasite problem No. 1 from Tropics - Polar Circle. Problems and prospects for control based on epidemiology. *Trop Biomed* 22, 131-137.

- Presson, B.L., Gray, G.D., Burgess, S.K., 1988, The effect of immunosuppression with dexamethasone on *Haemonchus contortus* infections in genetically resistant Merino sheep. *Parasite Immunol* 10, 675-680.
- Preston, J.M., Allonby, E.W., 1978, The influence of breed on the susceptibility of sheep and goats to a single experimental infection with *Haemonchus contortus*. *Vet Rec* 103, 509-512.
- Preston, J.M., Allonby, E.W., 1979, The influence of breed on the susceptibility of sheep of *Haemonchus contortus* infection in Kenya. *Res Vet Sci* 26, 134-139.
- Prichard, R.K., 1990, Anthelmintic resistance in nematodes: Extent, recent understanding and future directions for control and research. *International Journal for Parasitology* 20, 515-523.
- Rahman, W.A., 1994, Resistance to benzimidazole anthelmintics by *Haemonchus contortus* in goats in peninsular Malaysia. *Vet Parasitol* 55, 155-157.
- Rainbird, M.A., Macmillan, D., Meeusen, E.N., 1998, Eosinophil-mediated killing of *Haemonchus contortus* larvae: effect of eosinophil activation and role of antibody, complement and interleukin-5. *Parasite Immunol* 20, 93-103.
- Sangster, N. C., 1999. Anthelmintic resistance: past, present and future. *Int. J. Parasitol.* 29:115-124.
- Schallig, H.D., Van Leeuwen, M.A., 1997, Protective immunity to the blood-feeding nematode *Haemonchus contortus* induced by vaccination with parasite low molecular weight antigens. *Parasitology* 114 (Pt 3), 293-299.
- Schallig, H.D., van Leeuwen, M.A., Hendrikx, W.M., 1995, Isotype-specific serum antibody responses of sheep to *Haemonchus contortus* antigens. *Vet Parasitol* 56, 149-162.
- Schopf, L.R., Hoffmann, K.F., Cheever, A.W., Urban, J.F., Jr., Wynn, T.A., 2002, IL-10 is critical for host resistance and survival during gastrointestinal helminth infection. *J Immunol* 168, 2383-2392.
- Shaik, S.A., Terrill, T.H., Miller, J.E., Kouakou, B., Kannan, G., Kaplan, R.M., Burke, J.M., Mosjidis, J., 2006. *Sericea lespedeza* hay as a natural deworming agent against *Haemonchus contortus* infection in goats. *Vet Parasitol* 139, 150-157.
- Sher, A., Gazzinelli, R.T., Oswald, I.P., Clerici, M., Kullberg, M., Pearce, E.J., Berzofsky, J.A., Mosmann, T.R., James, S.L., Morse, H.C., 3rd, 1992, Role of T-cell derived cytokines in the downregulation of immune responses in parasitic and retroviral infection. *Immunol Rev* 127, 183-204.
- Shin, E.H., Osada, Y., Sagara, H., Takatsu, K., Kojima, S., 2001, Involvement of complement and fibronectin in eosinophil-mediated damage to *Nippostrongylus brasiliensis* larvae. *Parasite Immunol* 23, 27-37.

- Smith, W.D., 1977a, Anti-larval antibodies in the serum and abomasal mucus of sheep hyperinfected with *Haemonchus contortus*. Res Vet Sci 22, 334-338.
- Smith, W.D., 1977b, Serum and mucus antibodies in sheep immunised with larval antigens of *Haemonchus contortus*. Res Vet Sci 22, 128-129.
- Smith, W.D., Angus, K.W., 1980, *Haemonchus contortus*: attempts to immunise lambs with irradiated larvae. Res Vet Sci 29, 45-50.
- Smith, W.D., Christie, M.G., 1979, *Haemonchus contortus*: some factors influencing the degree of resistance of sheep immunized with attenuated larvae. J Comp Pathol 89, 141-150.
- Smith, W.D., Jackson, F., Jackson, E., Williams, J., Willadsen, S.M., Fehilly, C.B., 1984, Resistance to *Haemonchus contortus* transferred between genetically histocompatible sheep by immune lymphocytes. Res Vet Sci 37, 199-204.
- Soulsby, E.J.L., 1982, Helminths, Arthropods and protozoa of domesticated animals, 7th Edition. Lea and Febiger, 233-252 pp.
- Stankiewicz, M., Jonas, W.E., Douch, P.C., Rabel, B., Bisset, S., Cabaj, W., 1993, Globule leukocytes in the lumen of the small intestine and the resistance status of sheep infected with parasitic nematodes. J Parasitol 79, 940-945.
- Stankiewicz, M., Pernthaner, A., Cabaj, W., Jonas, W.E., Douch, P.G., Bisset, S.A., Rabel, B., Pfeffer, A., Green, R.S., 1995, Immunization of sheep against parasitic nematodes leads to elevated levels of globule leukocytes in the small intestine lumen. Int J Parasitol 25, 389-394.
- Stear, M.J., Murray, M., 1994, Genetic resistance to parasitic disease: particularly of resistance in ruminants to gastrointestinal nematodes. Vet Parasitol 54, 161-176.
- Strain, S.A., Stear, M.J., 2001, The influence of protein supplementation on the immune response to *Haemonchus contortus*. Parasite Immunol 23, 527-531.
- Su, Z., Segura, M., Morgan, K., Loredó-Osti, J.C., Stevenson, M.M., 2005, Impairment of protective immunity to blood-stage malaria by concurrent nematode infection. Infect Immun 73, 3531-3539.
- Taylor, M.A., Hunt, K.R., Wilson, C.A. and Quick, J.M., 1990, Clinical observations, diagnosis and control of *H. contortus* infection in periparturient ewes. Veterinary Record 126, 555-556.
- Terefe, G., Yacob, H.T., Grisez, C., Prevot, F., Dumas, E., Bergeaud, J.P., Dorchies, P., Hoste, H., Jacquiet, P., 2005, *Haemonchus contortus* egg excretion and female length reduction in sheep previously infected with *Oestrus ovis* (Diptera: Oestridae) larvae. Vet Parasitol 128, 271-283.
- Torres-Acosta, J.F., Jacobs, D.E., Aguilar-Caballero, A., Sandoval-Castro, C., May-Martinez, M., Cob-Galera, L.A., 2004, The effect of supplementary feeding on the resilience and resistance

of browsing Criollo kids against natural gastrointestinal nematode infections during the rainy season in tropical Mexico. *Vet Parasitol* 124, 217-238.

Uber, C.L., Roth, R.L., Levy, D.A., 1980, Expulsion of *Nippostrongylus brasiliensis* by mice deficient in mast cells. *Nature* 287, 226-228.

Urban, J.F., Jr., Ishizaka, T., Ishizaka, K., 1977, IgE formation in the rat following infection with *Nippostrongylus brasiliensis*. III. Soluble factor for the generation of IgE-bearing lymphocytes. *J Immunol* 119, 583-590.

Urban, J.F., Jr., Katona, I.M., Paul, W.E., Finkelman, F.D., 1991, Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. *Proc Natl Acad Sci USA* 88, 5513-5517.

Urban, J.F., Jr., Maliszewski, C.R., Madden, K.B., Katona, I.M., Finkelman, F.D., 1995, IL-4 treatment can cure established gastrointestinal nematode infections in immunocompetent and immunodeficient mice. *J Immunol* 154, 4675-4684.

Urban, J.F., Jr., Noben-Trauth, N., Schopf, L., Madden, K.B., Finkelman, F.D., 2001, Cutting edge: IL-4 receptor expression by non-bone marrow-derived cells is required to expel gastrointestinal nematode parasites. *J Immunol* 167, 6078-6081.

Urquhart, G.M., Armour, J., Ducan, J.L., Dunn, A.M., Jennings, F.W., 1996a, *Veterinary Parasitology*, Second Edition. Blackwell Science, 1-22 pp.

Urquhart, G.M., Armour, J., Duncan, J.L., Dunn, A.M., Jennings, F.W., 1996b, *Veterinary Parasitology*, Second Edition. Blackwell Science, 97-99 pp.

Vallance, B.A., Blennerhassett, P.A., Deng, Y., Matthaei, K.I., Young, I.G., Collins, S.M., 1999, IL-5 contributes to worm expulsion and muscle hypercontractility in a primary *T. spiralis* infection. *Am J Physiol* 277, G400-408.

van Wyk, J.A., Hoste, H., Kaplan, R.M., Besier, R.B., 2006, Targeted selective treatment for worm management--how do we sell rational programs to farmers? *Vet Parasitol* 139, 336-346.

Vardhani, V.V., 2003, Eosinophil relationship in gut anaphylaxis during experimental ancylostomosis. *Vet Parasitol* 115, 25-33.

Vatta, A.F., Krecek, R.C., Letty, B.A., van der Linde, M.J., Grimbeek, R.J., de Villiers, J.F., Motswatswe, P.W., Molebiemang, G.S., Boshoff, H.M., Hansen, J.W., 2002, Incidence of *Haemonchus* spp. and effect on haematocrit and eye colour in goats farmed under resource-poor conditions in South Africa. *Vet Parasitol* 103, 119-131.

Vlassoff, A., McKenna, P.B., 1994, Nematode parasites of economic importance in New Zealand. *New Zealand Journal of Zoology* 21, 1-8.

Waller, P.J., 1987, Anthelmintic resistance and the future for round worm control. *Veterinary Parasitology* 25, 177-191.

Waller, P.J., 2003, Global perspectives on nematode parasite control in ruminant livestock: the need to adopt alternatives to chemotherapy, with emphasis on biological control. *Anim Health Res Rev* 4, 35-43.

Waller, P.J., Rudby-Martin, L., Ljungstrom, B.L., Rydzik, A., 2004, The epidemiology of abomasal nematodes of sheep in Sweden, with particular reference to over-winter survival strategies. *Vet Parasitol* 122, 207-220.

Waller, P.J., Rydzik, A., Ljungstrom, B.L., Tornquist, M., 2006, Towards the eradication of *Haemonchus contortus* from sheep flocks in Sweden. *Vet Parasitol* 136, 367-372.

Watanabe, K., Hamano, S., Yada, S., Noda, K., Kishihara, K., Nomoto, K., Tada, I., 2001, The effect of interleukin-4 on the induction of intestinal mast cells and chronological cytokine profiles during intestinal nematode *Strongyloides ratti* infection. *Parasitol Res* 87, 149-154

Winter, M.D., Wright, C., Lee, D.L., 1997, The effect of dexamethasone on resistance of older lambs to infection with *Nematodirus battus*. *J Helminthol* 71, 133-138.

Wolstrup, J., Gronvold, J., Henriksen, S.A., Nansen, P., Larsen, M., Bogh, H.O., Ilsoe, B., 1994, An attempt to implement the nematode-trapping fungus *Duddingtonia flagrans* in biological control of trichostrongyle infections of first year grazing calves. *J Helminthol* 68, 175-180.

Wooster, M.J., Woodgate, R. G., and Chick, B. F., 2001, Reduced efficacy of ivermectin, abamectin and moxidectin against field isolates of *Haemonchus contortus*. *Aust Vet J* 79, 840-842.

Yakoob, A., Holmes, P.H., Armour, J., 1983, Pathophysiology of gastrointestinal trichostrongyles in sheep: plasma losses and changes in plasma pepsinogen levels associated with parasite challenge of immune animals. *Res Vet Sci* 34, 305-309.

Yazwinski, T.A., Goode, L., Moncol, D.J., Morgan, G.W., Linnerud, A.C., 1980, *Haemonchus contortus* resistance in straightbred and crossbred Barbados Blackbelly sheep. *J Anim Sci* 51, 279-284.

Zhong, S., Dobson, C., 1996, *Heligmosomoides polygyrus*: resistance in inbred, outbred, and selected mice. *Exp Parasitol* 82, 122-131.

## CHAPTER 2

### EVALUATION OF SELECTED IMMUNE RESPONSES TO ARTIFICIAL INFECTIONS OF *HAEMONCHUS CONTORTUS* IN GULF COAST NATIVE COMPARED WITH SUFFOLK LAMBS

#### 2.1. Introduction

*Haemonchus contortus* is a gastrointestinal hematophagus nematode parasite of sheep, goats and wild ruminants (Davidson et al., 1980; Urquhart et al., 1996). This parasite is worldwide in its distribution and causes one of the most economically important disease conditions in tropical and subtropical regions of the world where the climate is warm and humid. Economic losses are incurred mainly due to loss in production, mortality and cost of prophylaxis and curative treatments. Chemical anthelmintics have been used for control; however, evolution of resistance against the most commonly used anthelmintics (Miller and Horohov, 2006) and public demand for animal products free of chemical residues (Waller, 2003) has a negative impact on their use and alternative approaches for control are needed.

The development of vaccines and breeding of resistant animals to *H. contortus* are among the most ideal alternative strategies for controlling this nematode. A lack of understanding of the immune components and the mechanism responsible for resistance is partially responsible for the unavailability of a commercial vaccine. A better understanding may also facilitate finding reliable genetic markers that can be used in selection of animals for breeding of resistant animals.

Studies have revealed that some breeds of sheep demonstrate a relatively high degree of resistance to nematode parasite infection, *H. contortus* being one, and they do well without much management intervention than other more susceptible breeds. Gulf Coast Native (Native) sheep that originated in the gulf coast region (southeast) of the US, is one such breed that has demonstrated resistant characteristics compared with Suffolk sheep. Resistance has been shown to develop initially in suckling lambs which was maintained through adulthood (Bahirathan et

al., 1996; Miller et al., 1998). This resistance has been shown to have an immunologic component (Pena et al., 2004, 2006) hence is genetically controlled and highly variable among individuals within a breed (Miller et al., 2006).

Protective response to nematode infections in rodents has been reported to be one of two types (Th1 or Th2), however, it is not so clear in ruminants. Resistance to nematode parasites in ruminants involves increased populations of inflammatory cells mainly eosinophils, mast cells, and globule leukocytes. Likewise the increase in immunoglobulins, especially IgE, IgG1 and IgG2, has been reported to be associated with protection against gastrointestinal nematodiasis in ruminants. Specifically, repeated infection of *H. contortus* in sensitized Castellana sheep showed a partial resistance with a longer prepatent period, lowered fecal egg count (FEC) and fewer worms (Gomez-Munoz et al., 1999). However, these parameters were not correlated to serum levels of IgG, IgG1, IgG2, IgM or IgA. In *Trichostrongylus axei* sensitized sheep, mucosal mastocytosis, globule leukocytosis, and increased eosinophil count were accompanied by increased inhibition of larval migration in abomasal mucosa upon re-exposure compared with naïve controls (Pfeffer et al., 1996). In contrast to the findings of Gomez-Munoz et al., (1999), serum level of *T. axei*-specific total and IgG1 and peripheral eosinophil counts were reported to have increased in these sheep. Likewise immunizing infections with *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* in sheep have resulted in increased peripheral and tissue eosinophil number and increased tissue globule leukocyte in abomasum upon challenge infections (Stankiewicz et al., 1995). *H. contortus* infection in repeatedly exposed sheep demonstrated a clear Th2 type of response with increased recruitment of eosinophils, mast cells and globule leukocytes in abomasal mucosa (Lacroux et al., 2006). In contrast, Florida Native lambs, a known resistant breed, did not show significant difference in eosinophils, mast cells and globule leukocyte count compared with susceptible Rambouillet and their F2 offsprings

(Amarante et al., 1999). However, there was a negative correlation of cell count and with worm burden and FEC. Sensitized Texel sheep have responded with lowered FEC and increased serum level of IgG1 followed by IgG2 to *H. contortus* infection (Schallig et al., 1995). In another study, Schallig et al (1994) have reported IgA against L3 and IgG1 and IgG2 against adult antigens. However, *H. contortus* specific IgG1 and IgE levels were reported to be higher by Gill et al., (2000). These findings indicate that there is a complex immune response and variable components may be active in resistance against the nematodes involved.

Native sheep have proven to be more resistant than Suffolk sheep; therefore, identifying immunological components that may be responsible for imparting this resistance will lead to a better understanding of the immunological process which in turn may facilitate development of efficient vaccines and/or help to identify genetic markers that can be used to identify resistant animals. The objective of this study was to determine serum immunoglobulin isotype profiles and cellular recruitment profiles in abomasal mucosa of experimentally infected Native and Suffolk lambs with *H. contortus*.

## **2.2. Materials and Methods**

### **2.2.1. Location and Animals**

The study was conducted (July to September, 2005) at the School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA. Sixty 5-6 month old (30 Suffolk and 30 Native) lambs were maintained in concrete floor pens and fed a lamb growing ration and water was provided ad libitum. The protocol was approved by the Louisiana State University Institutional Care and Use Committee.

### **2.2.2. Experimental Design and Sampling Scheme**

Lambs were dewormed with levamisole (12 mg/kg, AgriLabs, St. Joseph, MO) and albendazole (20 mg/kg, Valbazen<sup>®</sup>, Pfizer, New York, NY) on two consecutive days during each

of 3 weeks to achieve as low a FEC as possible. Within breed, lambs were randomly assigned to 6 different groups with 5 animals each. Six groups (three of each breed) were designated as early infection and 6 as late infection to be necropsied at 14 and 21 days post infection, respectively. Two infection protocols were used, a one time bolus infection and trickle infections administered over time. For early infections, one group from each breed was given a bolus infection dose of 18,000 freshly cultured *H. contortus* infective third stage larvae (L3) on day 0 and one from each breed was given 6000 L3s on day 0 and 2000 L3 every other day for three times for a total 12000 L3s. The remaining 2 groups were untreated control groups. The same infection protocol was followed for the late infection groups except the trickle infected lambs received 3 doses of 2000 L3s every other day for a second week for a total of 18000 L3 per lamb. The groups were identified by dose they received, early or late infection and breed: Bolus Early Suffolk and Native (BES and BEN), Trickle Early Suffolk and Native (TES and TEN), Control Early Suffolk and Native (CES and CEN), Bolus Late Suffolk and Native (BLS and BLN), Trickle Late Suffolk and Native (TLS and TLN) and Control Late Suffolk and Native (CLS and CLN).

Fecal material for FEC was collected directly from the rectum each week and put into styrofoam cups labeled with animal number and date. Blood samples for serum separation and immunoglobulin isotypes were collected each week in red top 10 ml plain vacutainer tubes (No Additive, Becton, Dickinson, & Co.). Blood samples for PCV and differential leukocyte counts were collected in purple top 7 ml EDTA coated vacutainer tubes on days 0, 2, 7 and weekly thereafter. All blood samples were collected by jugular venipuncture. At the time of necropsy (14 or 21 days post infection), abomasa, small and large intestines were washed individually in separate buckets and wash samples were collected for worm recovery, identification and enumeration. Abomasa and small intestines were then soaked overnight and soak samples were collected for worm recovery, identification and enumeration. In addition, approximately 1 inch

long pieces of abomasal mucosa were clipped from the 3 different regions (cardiac, fundic and pyloric) of the abomasum for histopathology. Abomasal and prescapular lymph nodes were also collected.

### **2.2.3. Techniques Employed**

#### **2.2.3.1. Fecal Culture**

Feces from a sheep infected with *H. contortus* was collected rectally 3 times daily for 1 week. Each day, feces were pooled and crushed, mixed with water and an approximately equal amount of vermiculite until a moist crumbly consistency was achieved. The pan was then covered with aluminum foil with several holes to facilitate air circulation. The mixture was incubated at room temperature for 14 days with periodic turning (every 4-5 days) and adding water as necessary to prevent desiccation.

L3s were harvested from the fecal culture using a Baermann technique. A large funnel supported by a stand, connected to a short rubber tube into which a 15 ml plastic test tube was inserted. A wire mesh screen with an aperture of 0.15 mm was placed in the funnel and lukewarm water was added until the level of water was about the level of the screen. A square piece of cheese cloth was placed over the wire mesh screen and the culture material was placed over the cheesecloth and wrapped. Lukewarm water was added until it completely covered the culture material and cheese cloth and was left over night. Live motile L3 were collected in the test tube attached to the funnel. The L3 were baermannized again through kimwipe sheets to filter out residual culture debris and provide a clean L3 population. L3 were identified microscopically as 98% *H. contortus* and the remainder being *Cooperia* spp. and *Trichostrongylus* spp. L3 were stored in tissue culture bottles at room temperature. A few drops of penicillin G were added to prevent bacterial growth. Prior to each infection, L3 were baermannized again over night to provide active viable L3 for infection purposes. The

appropriate numbers of L3 for all experimental infections were given in 12 ml of water in plastic syringes with a metal nozzle extender attached.

#### **2.2.4. Fecal Egg Count**

##### **2.2.4.1. McMaster Technique**

FEC was conducted using a modified McMaster technique (Whitlock, 1948). Two grams of feces was weighed and crushed in a polythene cup. Thirty ml of saturated salt solution (737g of salt dissolved in 3000 ml of water) was added and the solution was mixed thoroughly with an electric mixer. A small amount of the solution was pipetted out immediately and placed in McMaster slide chambers (Chalex Corporation, Issaquah, WA). All the Trichostrongyle-type eggs were counted inside the grids of each chamber and the total number obtained was multiplied by a factor of 50 and was expressed as eggs per gram (EPG). If the FEC by McMaster technique was found to be 0, the sample was subjected to a sugar floatation technique.

##### **2.2.4.2. Sugar Floatation Technique**

Two grams of feces was weighed, crushed and mixed in approximately 15 ml of water. The solution was strained through tea strainer into a 15 ml centrifuge tube and centrifuged for 10 minutes at 1200 rpm. The supernatant was poured off and the tube was filled half way with sugar solution (S.G. 1.20) and the sediment was mixed thoroughly. The tube was filled with sugar solution to a convex meniscus, placed in the centrifuge and a cover slip was placed on the top of the tube. Tubes were centrifuged at 800 rpm for 10 minutes. Cover slips were transferred on microscopic slides and all the eggs were counted under a microscope and reported as EPG.

##### **2.2.5. Blood Packed Cell Volume (PCV)**

Blood collected by venipuncture in EDTA coated tube was mixed well by gentle rocking and transferred to microhematocrit capillary tubes. One end of the tube was sealed and then

centrifuged (Autocrit Ultra 3 Microhematocrit Centrifuge, Becton, Dickinson, & Co.) for 5 minutes. The PCV was read by means of the Hematocrit tube reader located inside the machine.

#### **2.2.6. Leukocyte Differential**

EDTA blood was used to prepare thin smears on clean grease free microscopic slides and labeled with animal identification number and date. The smears were stained using the Giemsa-Wright's technique and read under 1000x power using mineral oil. The first 100 leukocytes were counted and classified according to their morphological characters as neutrophils, eosinophils, basophils, monocytes and lymphocytes. .

#### **2.2.7. Necropsy and Sample Collection**

Lambs were euthanized with Beuthanasia-D Special (Pentobarbital Sodium 390 mg/ml and Phenytoin Sodium 50 mg/ml, Schering, Wayne, NJ) at a dose rate of 10 ml/100 lb body weight intravenously via the jugular vein. The abdominal cavity was opened. Abomasal and prescapular lymph nodes were removed and weighed. Lymph nodes were cut in half and preserved in 10% phosphate buffered formalin for histopathological examination.

Each organ (abomasum, small and large intestine) was removed and opened. The contents were emptied and washed with tap water into a bucket to make up 5 liters. After thorough stirring, a 500 ml aliquot (10%) was collected into pre-labeled plastic bottles. After settling for 5-6 hrs, approximately 50 ml was decanted and replaced with approximately 50 ml formalin as a preservative. The washed abomasa and small intestines were soaked in water over night at room temperature and rewashed in 5 liters of water. Subsequently, the collected samples were processed as described above.

#### **2.2.8. Worm Recovery, Identification and Enumeration**

Each abomasum and small intestine sample bottle was mixed well and a 100 ml aliquot was filtered through a No. 200 wire sieve (USA Standard sieve series, ASTM designation E 11,

Newark wire cloth company, Newark, New Jersey 07104) for recovery of worms. The sediment containing worms was repeatedly washed in the sieve with tap water, collected in a beaker and diluted with water. The solution was stirred and small amounts were poured into a Petri dish. A drop of Lugol's iodine was added to stain the worms red and then the background was de-stained with a household bleach solution. The first 100 worms were recovered using a tuberculin syringe needle and mounted on microscopic slides with 5 under each cover slip in a drop of lactophenol. If there were less than 10 worms found in 100 ml, a second 100 ml aliquot was examined. All of the 500 ml aliquot from each large intestine was filtered through a No. 35 wire sieve (US Standard sieve series, ASTM E 11, American Scientific Products, Division of American Hospital Supply Corporation, McGraw Park IL 60085) and the worms were removed and processed as above. All excess worms over 100 were counted and recorded. The mounted worms were identified to species, sex, and stage of development and recorded. The number recovered was extrapolated to estimate the number present per animal.

#### **2.2.9. Histopathological Technique**

Fixed abomasal tissue samples were trimmed, embedded in paraffin, sectioned (3 $\mu$ m thick) and one set was stained with hematoxylin and eosin for enumeration of eosinophils and neutrophils using the LEICA TP 1050 Automatic Tissue Processor and Leica Autostainer XL staining procedure. The reagents used were hematoxylin (Anatech, Ltd., Battle Creek, MI), eosin Y (Anatech, Ltd., Battle Creek, MI), Propar-Anatech, Ltd., Battle Creek, MI) and absolute alcohol (AAPER Alcohol and Chemical Co., Shelbyville, KY). Another set of tissue sections was stained with toulidine blue for enumeration of mast cells. The number of cells in each of ten 0.3 mm<sup>2</sup> fields were counted in the lamina propria of the 3 respective regions of the abomasum and recorded as the mean number per mm<sup>2</sup>.

### **2.2.10. Enzyme Linked Immunosorbent Assay (ELISA)**

Serum was separated from whole blood and stored at -20°C until evaluated by ELISA. For IgG whole molecule, ELISA was performed on individual samples using rabbit anti-goat IgG horse radish peroxidase conjugate (Sigma-Aldrich Co.). Levels of immunoglobulin isotypes were determined from pooled serum samples. Serum was pooled because sufficient quantities of monoclonal antibodies against all the sheep immunoglobulin isotypes were not available. Samples were thawed and 500 µl from each individual in a group were mixed in a tube for each sampling time point and 1% sodium azide solution (Sigma-Aldrich Co.) was added and then refrigerated. A serum sample from a colostrum deprived lamb was used as a negative control. The results of ELISA are presented as optical densities.

High binding polystyrene plates (Costar EIA/RIA, Corning, Inc.) were coated with 100 µl of *H. contortus* whole worm antigen (WWA) in 0.05M carbonate bicarbonate buffer, pH 9.6 (Sigma-Aldrich Co.) with a concentration of 5 µg/ml. The plates were sealed with a micro titer plate sealer (SealPlate Microplate Adhesive Film, E & K Scientific) and refrigerated overnight for coating. The plates were washed 4 times with phosphate buffered saline (PBS, Sigma-Aldrich Co.) and patted dry. The wells were blocked for 5 minutes with 200 µl of blocking buffer (Starting Block (PBS) Blocking Buffer, Pierce Biotechnology) then emptied. Serum samples were diluted for each isotype (1:25 for IgG, 1:10 for IgA, IgG1 and IgE and 1:50 for IgM and IgG2) based on the strength of monoclonal antibodies in blocking solution (Starting Block T20 (PBS) Blocking Buffer, Pierce Biotechnology). One hundred µl of diluted serum, appropriate positive (pooled serum from the 5 ewes with the highest reactivity) and negative control sera were added in duplicate to each well in the plate, sealed and incubated at room temperature for 1 hr. Plates were washed 4 times with PBS and patted dry. For total IgG, 100 µl/well of rabbit anti-goat IgG horse radish peroxidase conjugate (Sigma-Aldrich Co.) was

diluted with T20 blocking buffer at 1:25,000 dilution and incubated for 1 hr before chromagen 1-Step Ultra TMB-ELISA (Pierce Biotechnology) substrate was added. For isotypes IgM, IgA, and IgE, before the addition of conjugate, 100 µl of mouse anti IgM, IgA and IgE at 1:5 dilution and for IgG2 and IgM at 1:10 dilution of their respective monoclonal antibodies produced in mouse were added and incubated at room temperature for 1 hr. Goat anti-mouse IgG conjugated with horse radish peroxidase was diluted at 1:25000 and 100 µl was added to each well in plate after washing 4 times with PBS. Plates were washed 4 times with PBS and patted dry and 100 µl/well of the chromagen substrate, 1-Step Ultra TMB-ELISA (Pierce Biotechnology) was added and incubated without a seal for 30 min in the dark at room temperature. The reaction was stopped with the addition of 100 µl/well of 2M H<sub>2</sub>SO<sub>4</sub>. Absorbencies were read at 450-550 nm by an ELISA plate reader (ELX800 Universal Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT) and are presented as optical density (OD) for each sample.

#### **2.2.11. Statistical Analysis**

Data was analyzed by the SAS statistical package version 9.1.3. FEC and worm count were log transformed to stabilize variance. Repeated measures analysis of variance was used for comparisons of FEC, PCV reduction percentage, peripheral eosinophil percentage, and ELISA OD for each immunoglobulin over each time point. Split-plot arrangement of treatments with treatment groups and animals within treatment groups were main plot effects and time by group interactions were subplot effects. A P value of <0.05 was set as level of significance. When a significant difference overall was found, a Scheffé's test was used for main effect with pair wise comparison. Worm recovery, adult immature ratios and cellular enumeration in different regions of abomasum data were compared by unpaired student t test and significant P value was set as <0.05.

## 2.3. Results

### 2.3.1. Fecal Egg Count

FECs were close to 0 when the study started as the lambs were not completely clear of infection in spite of repeated anthelmintic treatment (Table 2.1). There was no significant ( $p>0.05$ ) difference found among early infection groups regardless of their infection regime, status and breed characters. However, the BLN group had a significantly ( $p<0.05$ ) lower mean FEC compared with the corresponding BLS group and both of these group means were significantly ( $p<0.05$ ) different from the uninfected control group of their respective breeds. The TLN and TLS lambs were significantly different ( $p<0.05$ ) from the uninfected controls but not from each other, however, the TLN lambs tended to be lower.

Table 2.1. Mean weekly fecal egg count in artificially infected groups belonging to Native and Suffolk breeds.

Group	0DPI	7DPI	14DPI	21DPI
Bolus Early Suffolk	1	7	1	
Bolus Early Native	2	4	2	
Control Early Suffolk	0	1	3	
Control Early Native	8	6	2	
Trickle Early Suffolk	5	6	3	
Trickle Early Native	0	0	2	
Bolus Late Suffolk	10	5	4	4310 <sup>a</sup>
Bolus Late Native	3	3	2	305 <sup>b</sup>
Control Late Suffolk	1	1	0	0 <sup>c</sup>
Control Late Native	0	0	3	0 <sup>c</sup>
Trickle Late Suffolk	6	8	1	5930 <sup>a</sup>
Trickle Late Native	0	0	0	1930 <sup>a</sup>

Note: Significantly different group means are indicated by different superscripted letters. DPI= Days post infection. (n= 5).

### 2.3.2. Blood PCV Reduction Percentage

Mean group PCV reduction percentage was determined by  $((\text{Day 0 PCV} - \text{Sample Day PCV} / \text{Day 0 PCV}) \times 100)$  (Table 2.2.). Although there was no significant ( $p>0.05$ ) difference between infection groups, there was a tendency for the Suffolk group to have greater PCV reduction percentages than the Native group in both early and late infection groups for both

infection protocols. The reduction percentages were greater on DPI 21 compared with DPI 14 in both the breeds.

Table 2.2. Mean packed cell volume (PCV) reduction percentage and standard error of the mean (SEM) by sampling time points.

Group	PCV reduction percentage and Standard Error of the mean							
	2DPI	SEM	7DPI	SEM	14DPI	SEM	21DPI	SEM
Bolus Early Suffolk	1.9	3.1	4.2	3.4	20.5	5.1		
Bolus Early Native	6.8	5.2	12.2	2.1	15.5	5.8		
Control Early Suffolk	-1.7	10.7	15	5.4	15.6	4.6		
Control Early Native	3.2	6	3	3.4	6.5	4.1		
Trickle Early Suffolk	8.6	5.8	19.4	5.6	6.4	7.1		
Trickle Early Native	-5.9	2.8	0.9	2.8	-4.8	4.5		
Bolus Late Suffolk	1.1	4.8	-1.9	3.5	25.8	3.6	59.8	6.3
Bolus Late Native	2.5	4.9	1.6	5.1	17	7.6	46.8	0.8
Control Late Suffolk	9.6	5.5	7.9	5.9	20.9	4.2	57.5	4.1
Control Late Native	-0.5	6.1	-2	8.8	15.7	5.1	44.4	2
Trickle Late Suffolk	11.4	7.5	15.6	6.8	12.7	5.1	21.1	6.1
Trickle Late Native	2.3	6.9	2.4	11.9	15.6	3.7	41.6	4.3

Note: DPI=Days post infection, SEM=Standard error of the mean. (n=5)

### 2.3.3. Lymph Node Hypertrophy

There was no significant difference among weights of prescapular lymph nodes of uninfected control, bolus and trickle infected lambs necropsied at day 14 post infection within or between the two breeds (Table 2.3). Prescapular lymph nodes were significantly ( $p<0.05$ ) larger in BLS lambs necropsied on day 21 post infection compared with their uninfected control group ( $p<0.05$ ) and other bolus and trickle infected groups. Uninfected control and trickle infected groups did not have any significant differences in their prescapular lymph node weights.

Mean Abomasal lymph node weights were not significantly different ( $p>0.05$ ) between the corresponding infected groups regardless of the infection regimes. However, they were significantly greater ( $p<0.05$ ) in infected groups compared with the uninfected control groups of their corresponding breeds except between trickle infected Native lambs and uninfected control.

Table 2.3. Mean weight of the prescapular and abomasal lymph nodes in grams.

Group	Prescapular LN	SEM	Abomasal LN	SEM
Bolus Early Suffolk	3.52 <sup>a</sup>	0.58	1.8 <sup>a</sup>	0.42
Bolus Early Native	2.48 <sup>a</sup>	0.34	1.88 <sup>a</sup>	0.57
Control Early Suffolk	3.0 <sup>a</sup>	0.36	0.5 <sup>b</sup>	0.06
Control Early Native	2.3 <sup>a</sup>	0.22	0.74 <sup>ab</sup>	0.07
Trickle Early Suffolk	3.1 <sup>a</sup>	0.47	2.0 <sup>a</sup>	0.26
Trickle Early Native	1.9 <sup>a</sup>	0.07	1.4 <sup>a</sup>	0.54
Bolus Late Suffolk	5.92 <sup>b</sup>	0.59	2.64 <sup>b</sup>	0.60
Bolus Late Native	4.22 <sup>a</sup>	0.85	2.4 <sup>b</sup>	0.42
Control Late Suffolk	4.2 <sup>a</sup>	0.19	0.94 <sup>a</sup>	0.13
Control Late Native	4.42 <sup>a</sup>	0.88	0.98 <sup>a</sup>	0.17
Trickle Late Suffolk	4.08 <sup>a</sup>	1.08	3.14 <sup>a</sup>	0.39
Trickle Late Native	3.52 <sup>a</sup>	0.70	2.08 <sup>a</sup>	0.36

Note: Significant differences are indicated by different letters. LN=Lymph node, SEM=Standard error of the mean, (n=5).

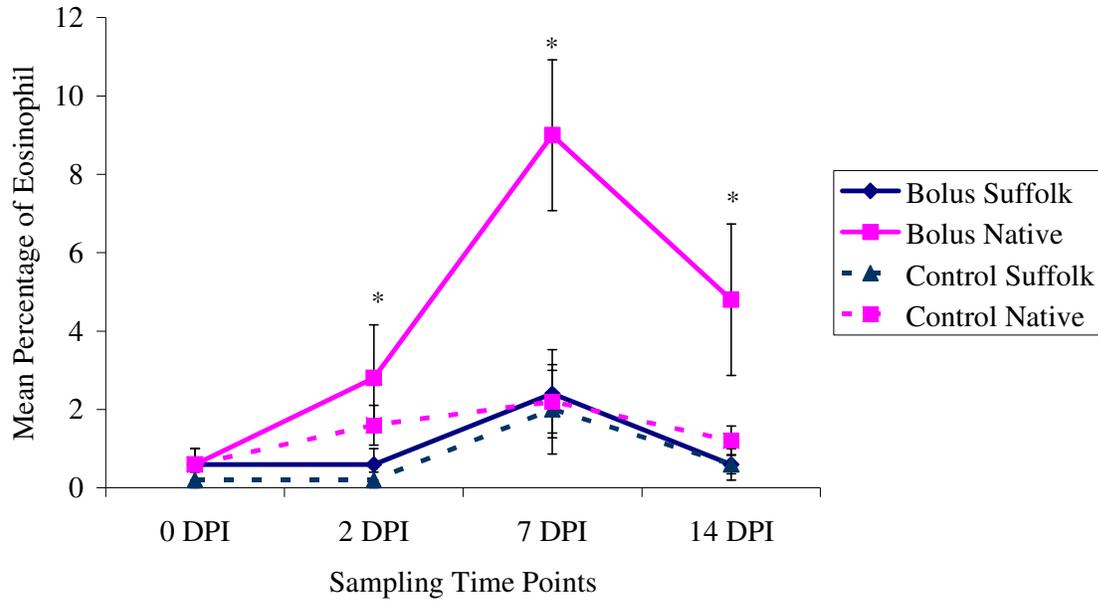
#### 2.3.4. Peripheral Eosinophil Percentage

Peripheral eosinophil percentages for BEN, TEN, BLN and TLN groups were significantly ( $p < 0.05$ ) greater than their corresponding Suffolk groups (Figures 2.1.A. & B, and 2.2.A & B). Mean eosinophil percentage for the Suffolk groups, regardless of infection regimen, remained near the basal (control) level throughout the study. There were no differences in peripheral eosinophil percentages among uninfected control groups. A consistent observation in the infected Native groups was that peak eosinophilia occurred at 7 days (early infection) and 14 days (late infection) post infection then declined the following week.

#### 2.3.5. Worm Recovery

No worms were recovered from any of the uninfected control groups. There was no significant ( $p > 0.05$ ) difference in total worm count between the corresponding infected groups of the two breeds regardless of the infection regime (Figures 2.3, 2.4.A & B, and 2.5). However, there was a trend for fewer *H. contortus* recovered from infected Native groups compared with Suffolk groups.

A. Peripheral Eosinophil Percentage  
Bolus Early Groups



B. Peripheral Eosinophil Percentage  
Trickle Early Groups

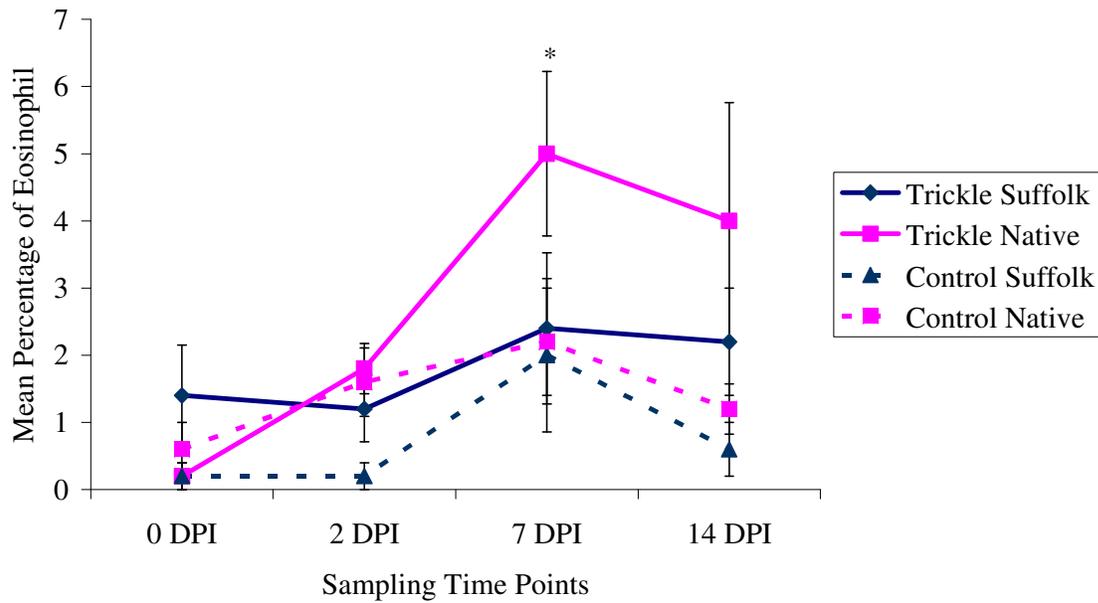
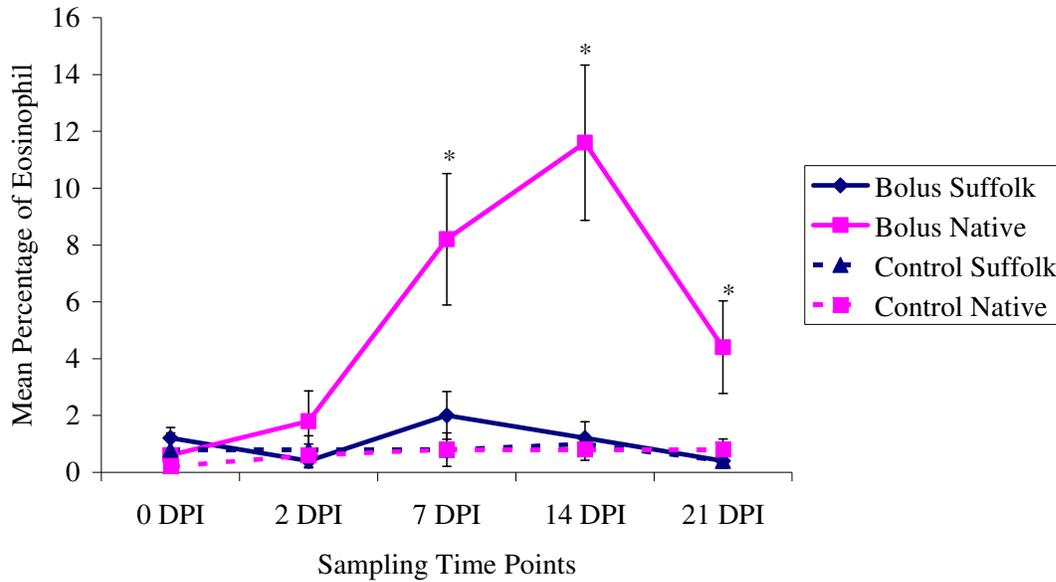


Fig 2.1. Mean peripheral eosinophil percentages in Native and Suffolk lambs. A) Bolus Early Groups B) Trickle Early Groups. Significant differences ( $p < 0.05$ ) are indicated by asterisk marks. DPI=Days post infection, ( $n=5$ ).

A. Peripheral Eosinophil Percentage  
Bolus Late Groups



B. Peripheral Eosinophil Percentage  
Trickle Late Groups

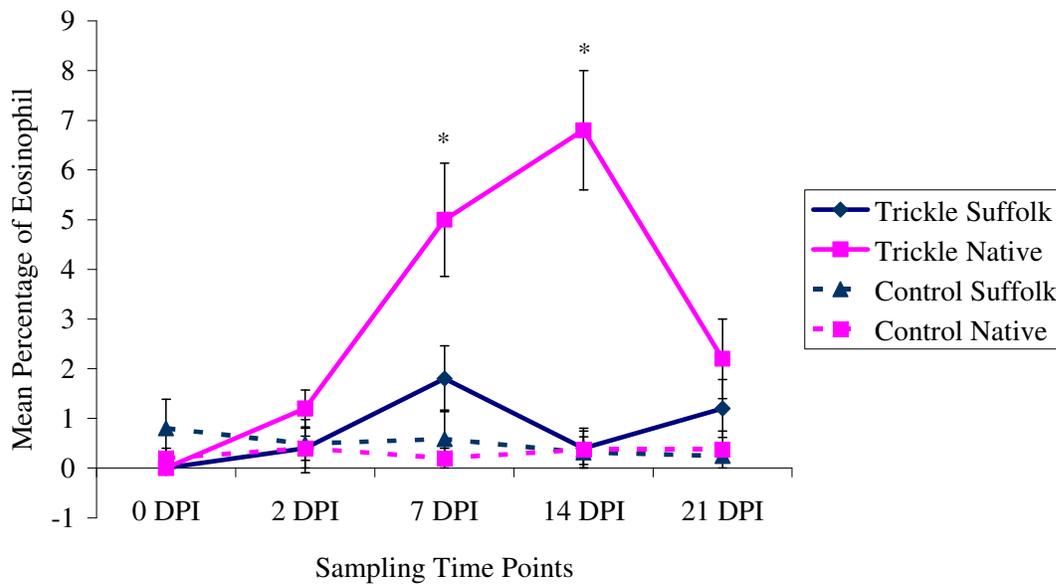


Figure 2.2. Mean peripheral eosinophil percentages in Native and Suffolk lambs. A) Bolus Late Groups and B) Trickle Late Groups. Significant differences ( $p < 0.05$ ) are indicated by asterisk marks. DPI=days post infection, ( $n=5$ ).

Of particular note, the number of immature worms in relation to adults recovered was significantly greater ( $p < 0.05$ ) in the BLN group (Figure 2.4.B). Although, no other groups demonstrated significant differences in adult to immature ratio, the Suffolk groups tended to have a greater number of adult worms than immature worms. Similarly, male to female ratios were not significantly ( $p > 0.05$ ) different among groups except in the trickle late infection groups ( $p < 0.05$ ).

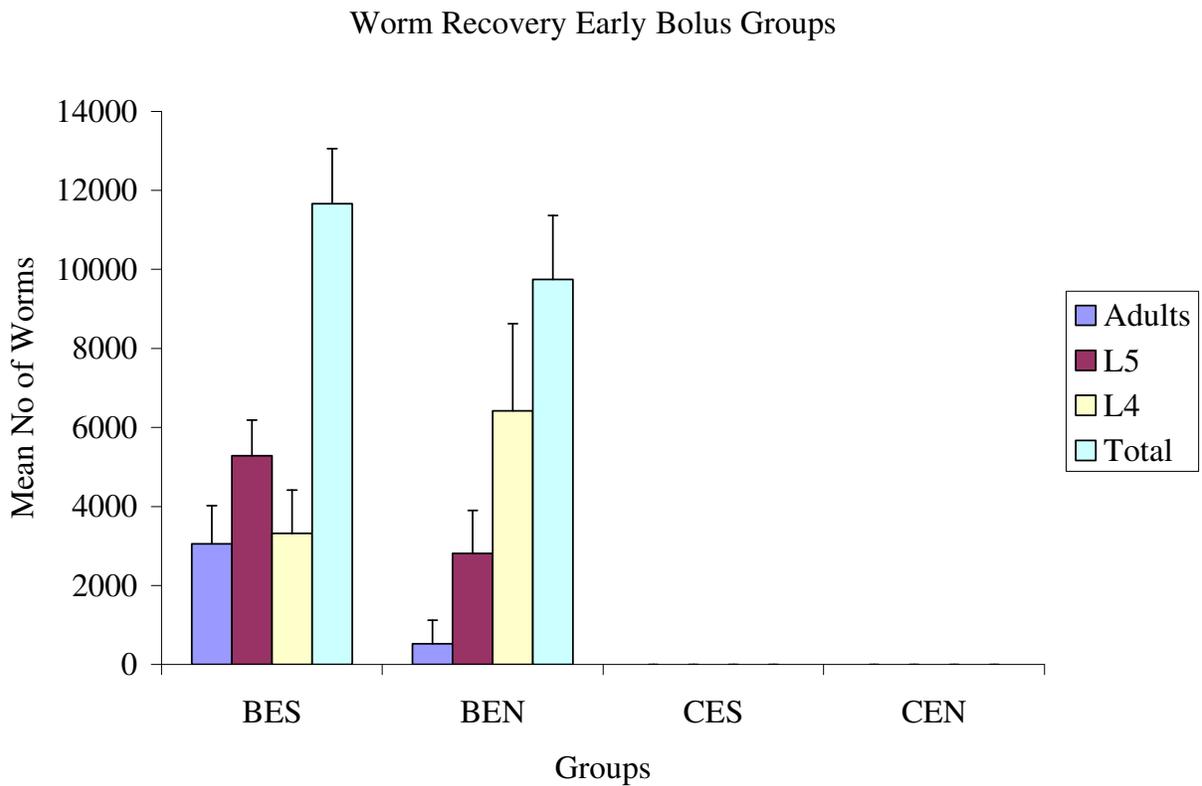
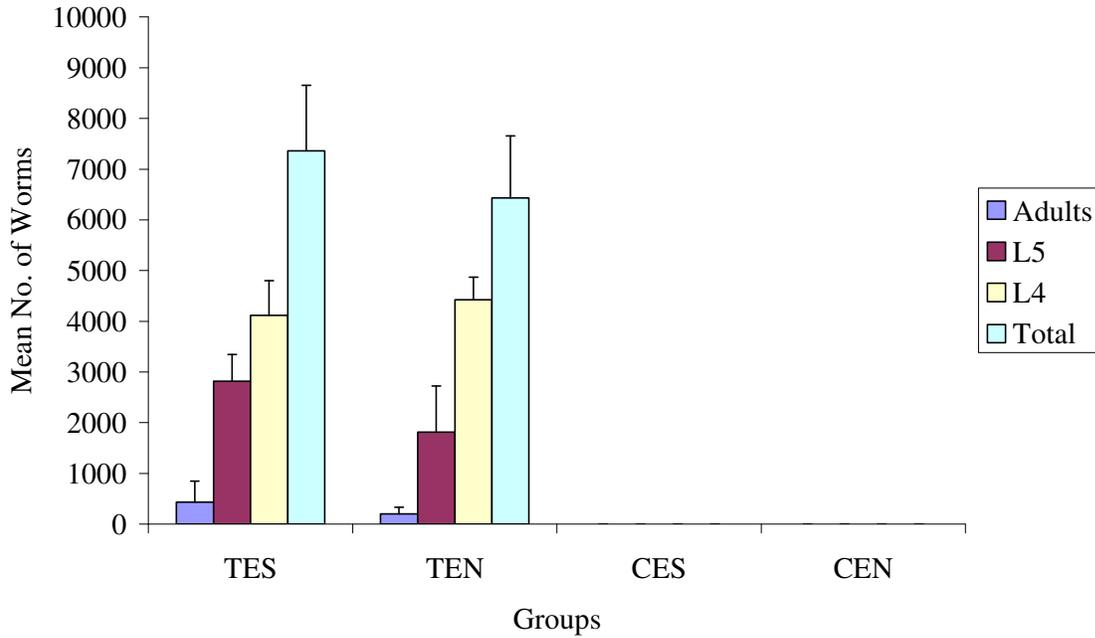


Figure 2.3. Mean *H. contortus* recovery from the abomasa of Bolus Early Groups. BES=Bolus Early Suffolk, BEN=Bolus Early Native, CES=Control Early Suffolk, CEN=Control Early Native, L5=Larval stage 5, L4=Larval stage 4, (n=5).

A. Worm Recovery Trickle Early Groups



B. Worm Recovery Bolus Late Groups

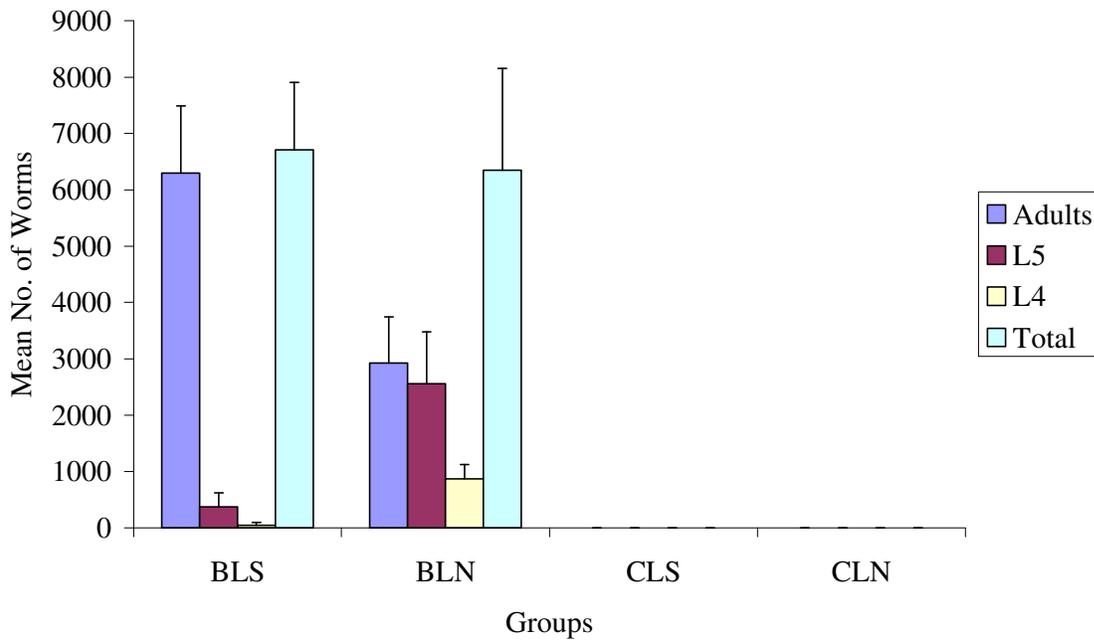


Figure 2.4. Mean *H. contortus* recovery from the abomasa. A) Trickle Early groups B) Bolus Late groups. TES=Trickle Early Suffolk, TEN=Trickle Early Native, CES=Control Early Suffolk, CEN=Control Early Native, BLS=Bolus Late Suffolk, Bolus Late Native, CLS=Control Late Suffolk, CLN=Control Late Native, L5=Larval stage 5, L4=Larval stage 4, (n=5).

### Worm Recovery Trickle Late Groups

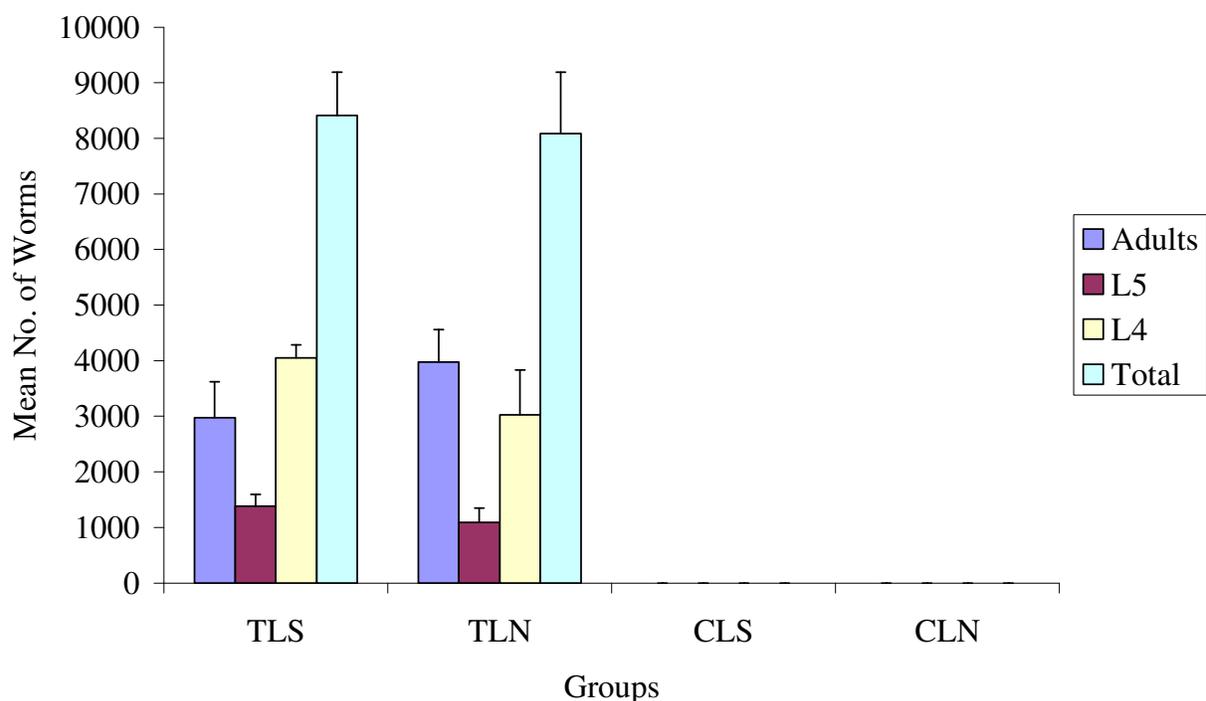


Figure 2.5. Mean *H. contortus* recovery from the abomasa of Trickle Late Groups. TLS=Trickle Late Suffolk, TLN=Trickle Late Native, CLS=Control Late Suffolk, CLN=Control Late Native, L5=Larval stage 5, L4=Larval stage 4, (n=5).

#### 2.3.6. Eosinophil, Neutrophil and Mast Cell Numbers in Abomasal Mucosa

Eosinophil, neutrophil and mast cell numbers were significantly ( $p < 0.05$ ) greater in all infected groups compared with their respective untreated control groups (Table 2.4). Overall, eosinophil, neutrophil and mast cell numbers were greater for Native groups compared with Suffolk groups regardless of their infection regimes; however, difference between breeds was not always significant ( $p > 0.05$ ). Neutrophils were observed to be in greater numbers in early infection groups while they were fewer in late infection groups regardless of the breed and regime of infection. Among corresponding groups infected with similar regimes, significantly ( $p < 0.05$ ) greater numbers of eosinophils were observed in the cardiac and fundic regions of BEN and TEN groups than their corresponding Suffolk groups. Similarly, neutrophil numbers were

found to be significantly ( $p < 0.05$ ) greater in the cardiac region of TEN, fundic region in BEN, TEN and BLN, and pyloric region of BEN groups than from their corresponding Suffolk groups. Likewise, mast cell numbers were found to be significantly ( $p < 0.05$ ) greater in the cardiac region of TEN and BLN, fundic region of BLN, and pyloric region of TEN, BEN and TLN groups than their corresponding Suffolk groups. For the remaining groups, regardless of infection regime, the cell numbers for all cell types tended to be greater in Native groups compared with Suffolk groups.

Table 2.4. Mean eosinophil (E), neutrophil (N) and mast cell (M) number per  $\text{mm}^2$  of cardiac, fundic and pyloric regions of abomasum in infected and uninfected control lambs.

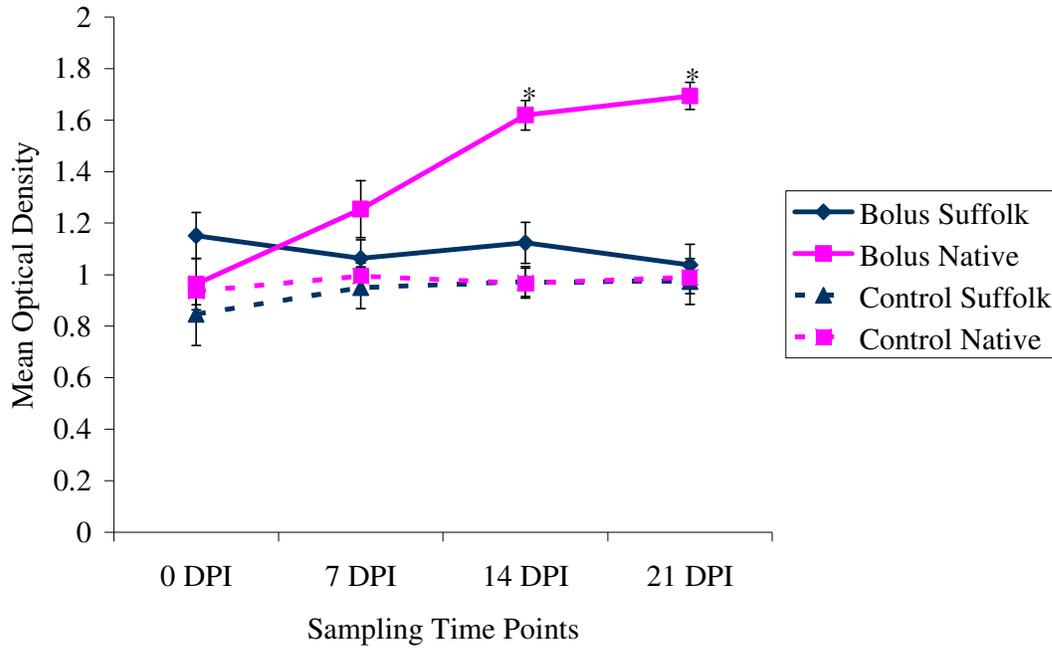
Treatment Groups	Cardiac Abomasum			Fundic Abomasum			Pyloric Abomasum		
	Mean count/mm			Mean count/mm			Mean count/mm		
	E	N	M	E	N	M	E	N	M
Bolus Early Suffolk	16 <sup>a</sup>	5 <sup>a</sup>	4 <sup>a</sup>	27 <sup>a</sup>	8 <sup>a</sup>	4 <sup>a</sup>	13 <sup>a</sup>	3 <sup>a</sup>	6 <sup>a</sup>
Bolus Early Native	68 <sup>b</sup>	17 <sup>a</sup>	9 <sup>a</sup>	63 <sup>b</sup>	16 <sup>b</sup>	5 <sup>a</sup>	47 <sup>a</sup>	1 <sup>b</sup>	10 <sup>a</sup>
Trickle Early Suffolk	27 <sup>a</sup>	9 <sup>a</sup>	3 <sup>a</sup>	30 <sup>a</sup>	7 <sup>a</sup>	4 <sup>a</sup>	20 <sup>a</sup>	4 <sup>a</sup>	5 <sup>a</sup>
Trickle Early Native	55 <sup>b</sup>	15 <sup>b</sup>	13 <sup>b</sup>	95 <sup>b</sup>	20 <sup>b</sup>	10 <sup>b</sup>	46 <sup>a</sup>	7 <sup>a</sup>	14 <sup>b</sup>
Control Early Suffolk	0 <sup>c</sup>	2 <sup>c</sup>	1 <sup>c</sup>	1 <sup>c</sup>	2 <sup>c</sup>	0 <sup>c</sup>	2 <sup>c</sup>	1 <sup>c</sup>	1 <sup>c</sup>
Control Early Native	0 <sup>c</sup>	1 <sup>c</sup>	0 <sup>c</sup>	0 <sup>c</sup>	1 <sup>c</sup>	1 <sup>c</sup>	0 <sup>c</sup>	0 <sup>c</sup>	0 <sup>c</sup>
Bolus Late Suffolk	8 <sup>a</sup>	1 <sup>a</sup>	4 <sup>a</sup>	4 <sup>a</sup>	1 <sup>a</sup>	6 <sup>a</sup>	18 <sup>a</sup>	2 <sup>a</sup>	8 <sup>a</sup>
Bolus Late Native	13 <sup>a</sup>	2 <sup>a</sup>	13 <sup>b</sup>	22 <sup>a</sup>	2 <sup>b</sup>	13 <sup>b</sup>	24 <sup>a</sup>	2 <sup>a</sup>	23 <sup>b</sup>
Trickle Late Suffolk	7 <sup>a</sup>	1 <sup>a</sup>	5 <sup>a</sup>	11 <sup>a</sup>	1 <sup>a</sup>	3 <sup>a</sup>	21 <sup>a</sup>	11 <sup>a</sup>	4 <sup>a</sup>
Trickle Late Native	18 <sup>a</sup>	2 <sup>a</sup>	10 <sup>a</sup>	26 <sup>a</sup>	3 <sup>a</sup>	7 <sup>a</sup>	15 <sup>a</sup>	1 <sup>a</sup>	19 <sup>b</sup>
Control Late Suffolk	0 <sup>c</sup>	0 <sup>c</sup>	1 <sup>c</sup>	0 <sup>c</sup>	0 <sup>c</sup>	0 <sup>c</sup>	1 <sup>c</sup>	0 <sup>c</sup>	1 <sup>c</sup>
Control Late Native	0 <sup>c</sup>	0 <sup>c</sup>	0 <sup>c</sup>	0 <sup>c</sup>	0 <sup>c</sup>	1 <sup>c</sup>	1 <sup>c</sup>	0 <sup>c</sup>	2 <sup>c</sup>

Note: Significant differences are indicated by different superscripted letters. (n=5).

### 2.3.7. Total Serum IgG Level

All groups started with similar levels of serum IgG. Levels for early infection groups, regardless of their infection regimen and breed, were not significantly ( $p > 0.05$ ) different from each other (data not presented). Levels for the BLN group, increased significantly ( $p < 0.05$ ) compared with all other groups including the corresponding BLS group (Figure 2.6.A). The response for the TLN group was also greater than the other groups, but the difference was not as obvious (Figure 2.6.B).

A. Total IgG ELISA Bolus Late Groups



B. Total IgG ELISA Trickle Late Groups

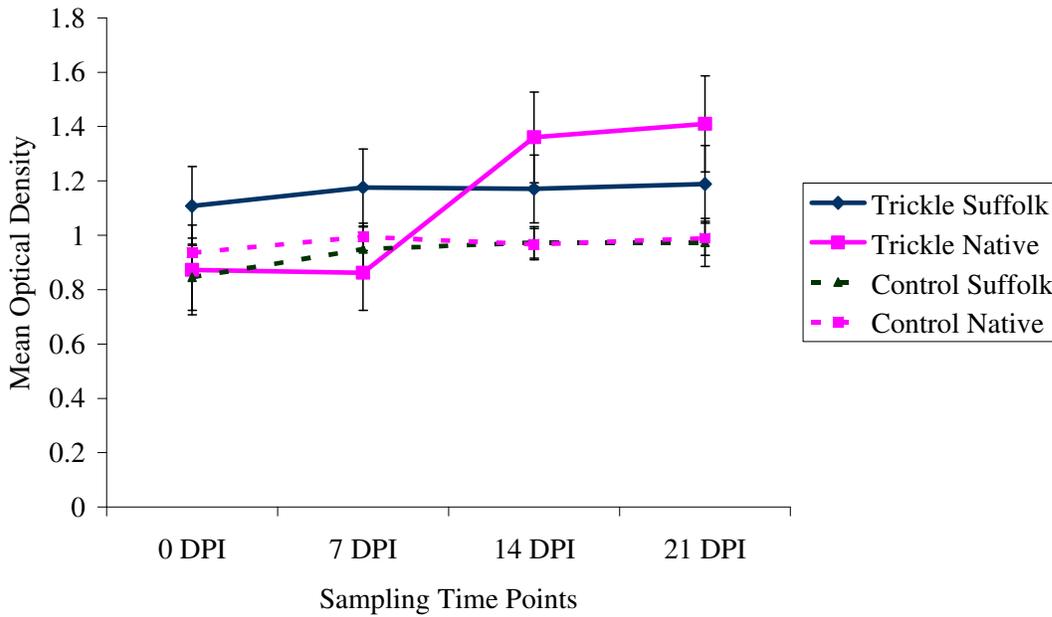


Figure 2.6. Mean optical densities representing the serum level of IgG against *H. contortus* whole worm antigen. A) Bolus Late Groups and B) Trickle Late Groups. An asterisk mark indicates significant difference. DPI=Days post infection. (n=5).

### 2.3.8. Serum IgM Level

There was no trend in IgM level for either early or late infection, infection regimen or breed. All remained relatively stable (data not presented).

### 2.3.9. Serum IgG1 Level

Serum IgG1 levels for early infection groups, regardless of their infection regimen and breed, were not significantly different from each other (data not presented). Levels for the BLN group, increased steadily compared with all other groups including the corresponding BLS group with the increase more prominent on Day 21 (Figure 2.7.). Similarly, the response for the TLN group increased steadily being more prominent on Days 14 and 21 than the other groups (Figure 2.8.).

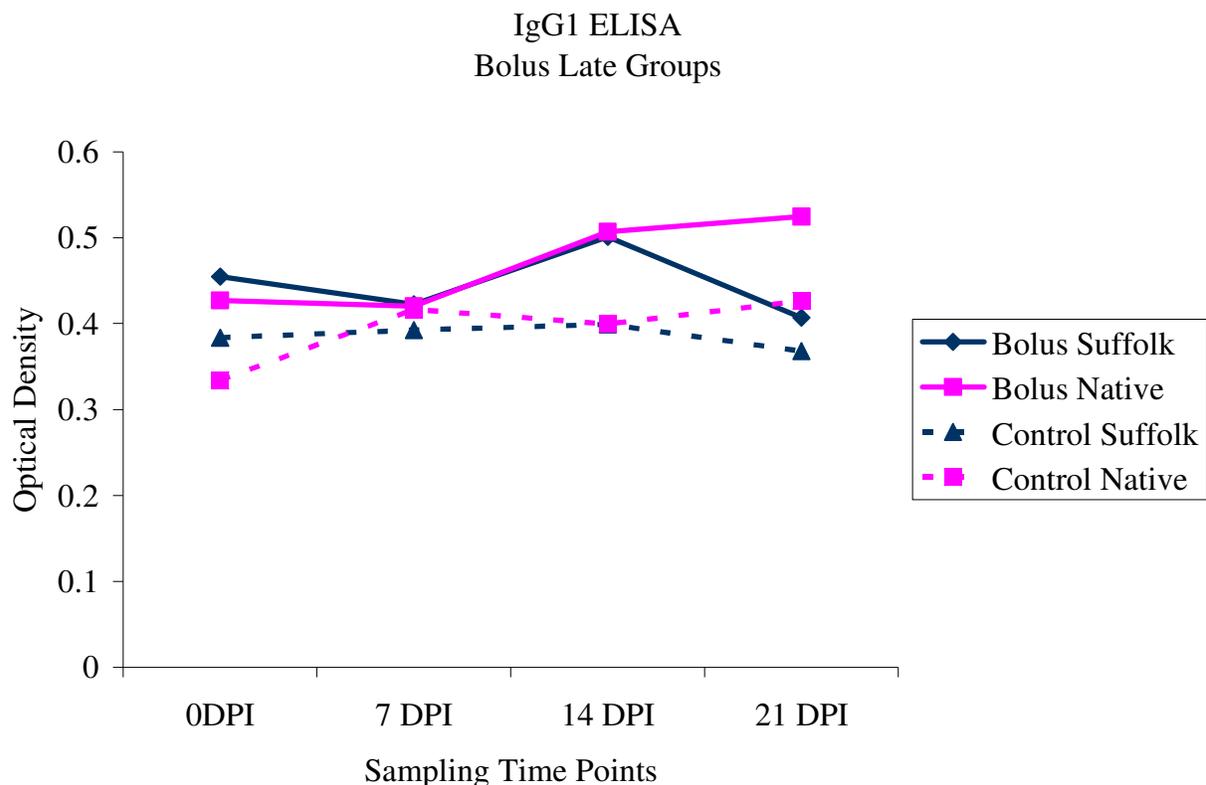


Figure 2.7. Optical densities representing the serum level of IgG1 against *H. contortus* whole worm antigen in pooled serum samples from the bolus late groups. DPI=Days post infection, (n=5).

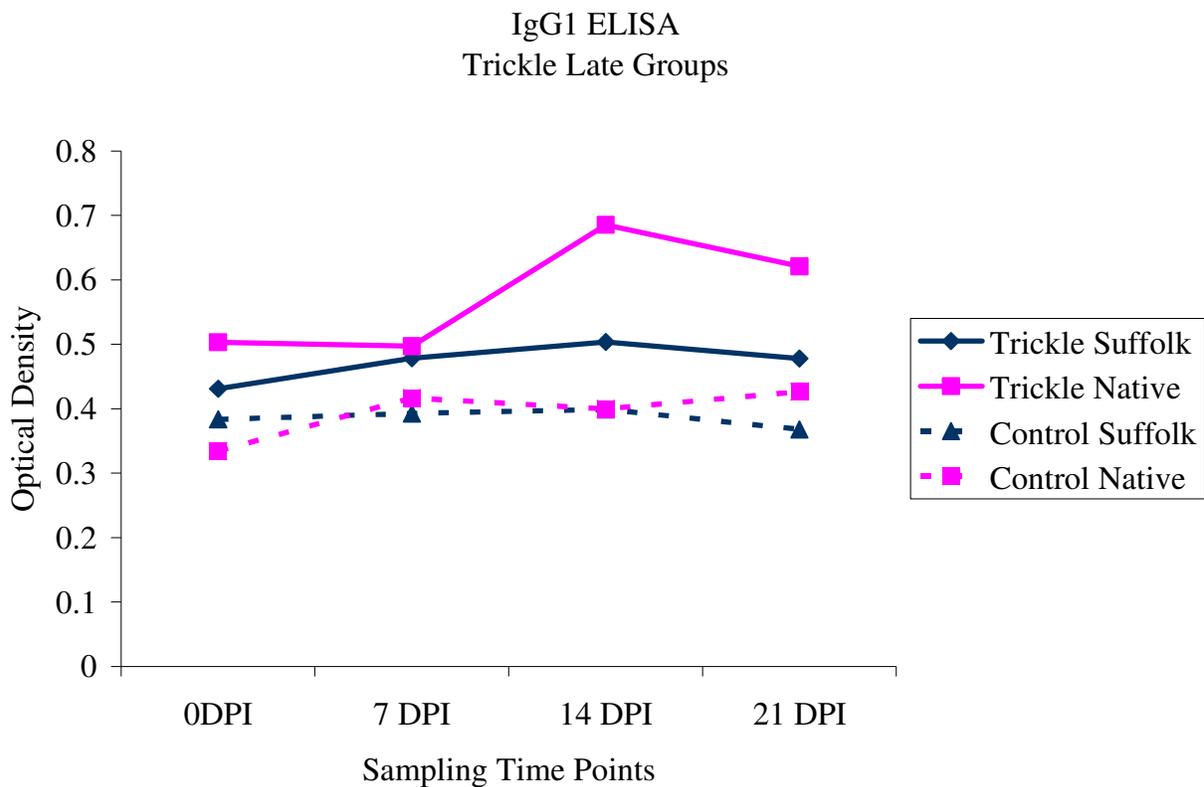


Figure 2.8. Optical densities representing the serum level of IgG1 against *H. contortus* whole worm antigen in pooled serum sample from the trickle late groups. DPI=Days post infection, (n=5).

### 2.3.10. Serum IgG2 Level

Optical densities for serum IgG2 levels for early infection groups, regardless of their infection regimen and breed, were not significantly different from each other (data not presented).

The BLN group showed an increase in optical densities for serum IgG2 level and remained greater through out the study (Figure 2.9) when compared with bolus treated Suffolk lambs. All other groups regardless of breed or infection regime did not show any change in their serum IgG2 level.

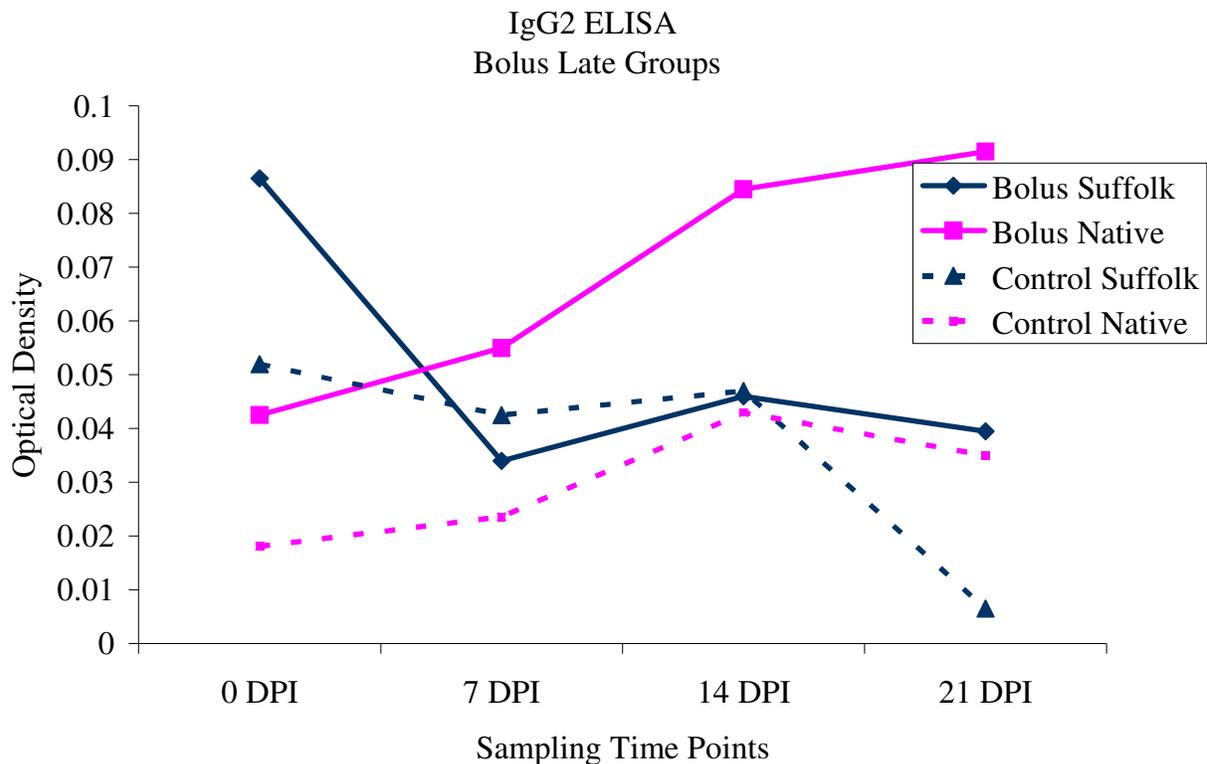


Figure 2.9. Optical densities representing the serum level of IgG2 against *H. contortus* whole worm antigen in pooled serum samples from the bolus late groups. DPI=Days post infection, (n=5).

### 2.3.11. Serum IgA Level

The early groups of lambs and the trickle infected late groups of both breeds regardless of their infection regime did not demonstrate any discernible trend in IgA level (data not presented).

BLN, BLS and CLS groups started out with similar optical densities for serum IgA levels, however, the optical densities in BLN lambs increased after infection peaking at day 14 post infection and Suffolk lambs regardless of their infection status and regime do not show any change in optical densities for serum IgA level (Figure 2.10). The CLN lambs demonstrated a greater level of initial IgA which was maintained throughout the study.

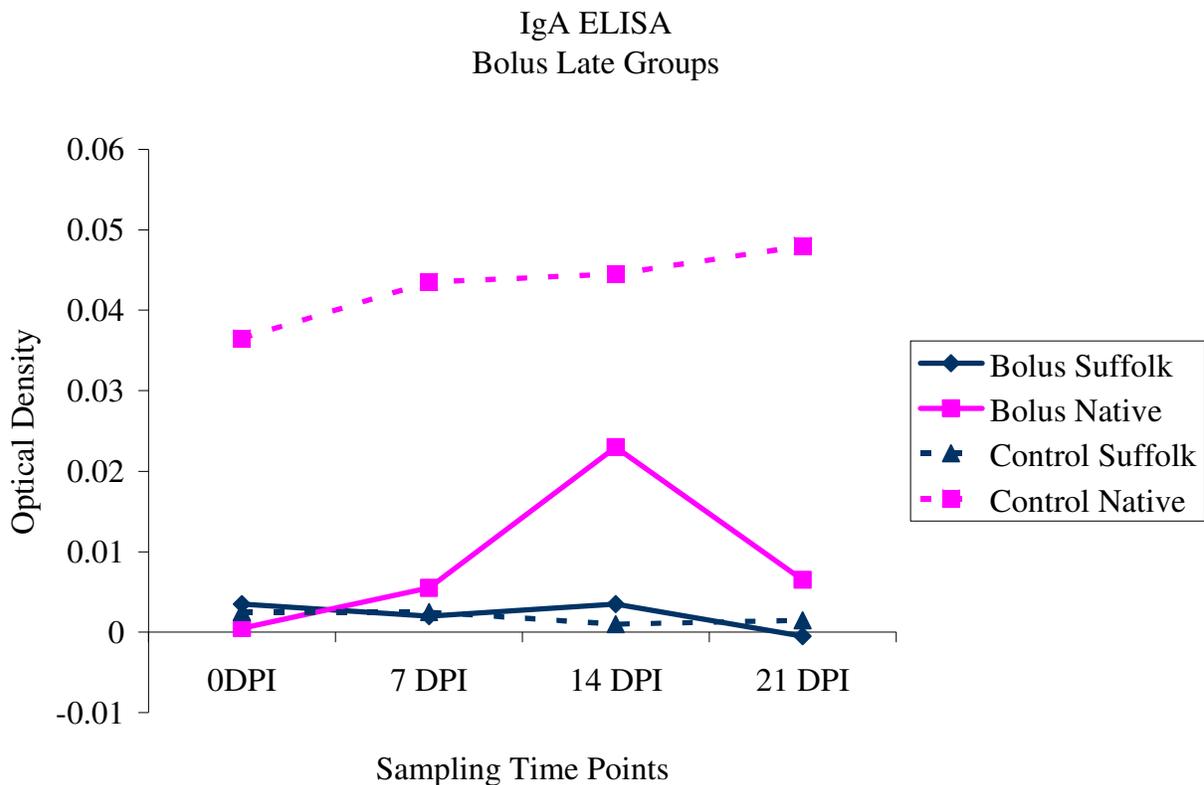


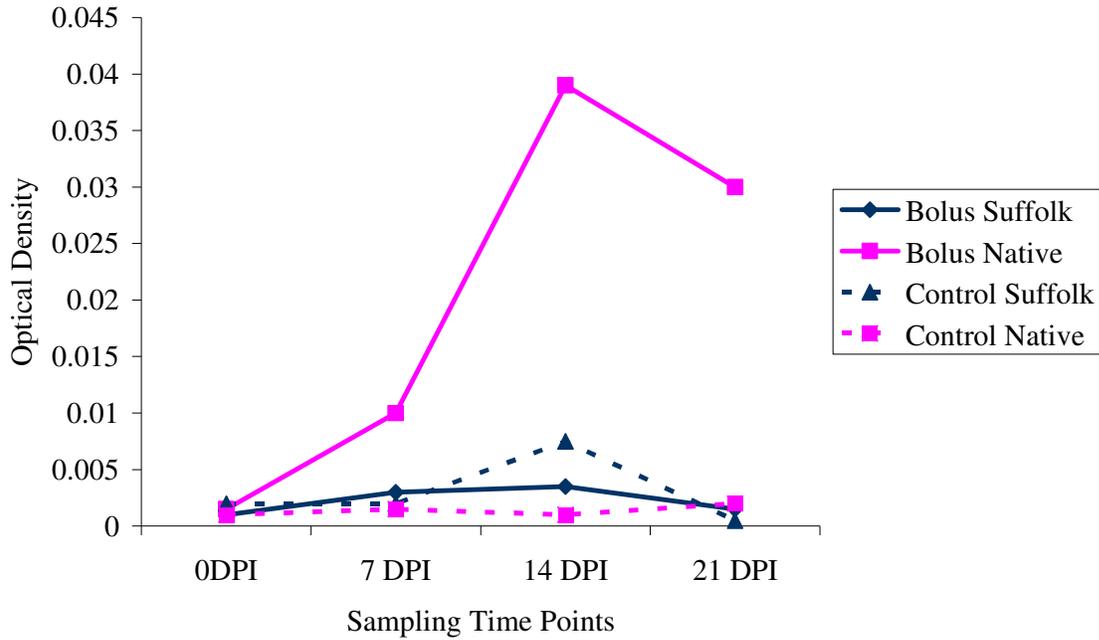
Figure 2.10. Optical densities representing the serum level of IgA against *H. contortus* whole worm antigen in pooled serum samples from the bolus late groups. DPI=Days post infection, (n=5).

### 2.3.12. Serum IgE Level

The early groups regardless of breed or infection regime, failed to show any kind of trend in IgE levels (data not presented).

For late bolus infection groups, level of IgE was similar at the beginning of the study however, after 7 day post infection the BLN group increased and peaked at 14 days (Figure 2.11.A). The other groups remained around the same level including the BLS lambs. For late trickle infection groups, regardless of their infection status, regime and breed level of IgE was similar at the beginning of the study however; the TLN lambs increased and peaked at 14 day post infection while all other groups remained around the baseline level (Figure 2.11.B).

A. IgE ELISA Bolus Late Groups



B. IgE ELISA Trickle Late Groups

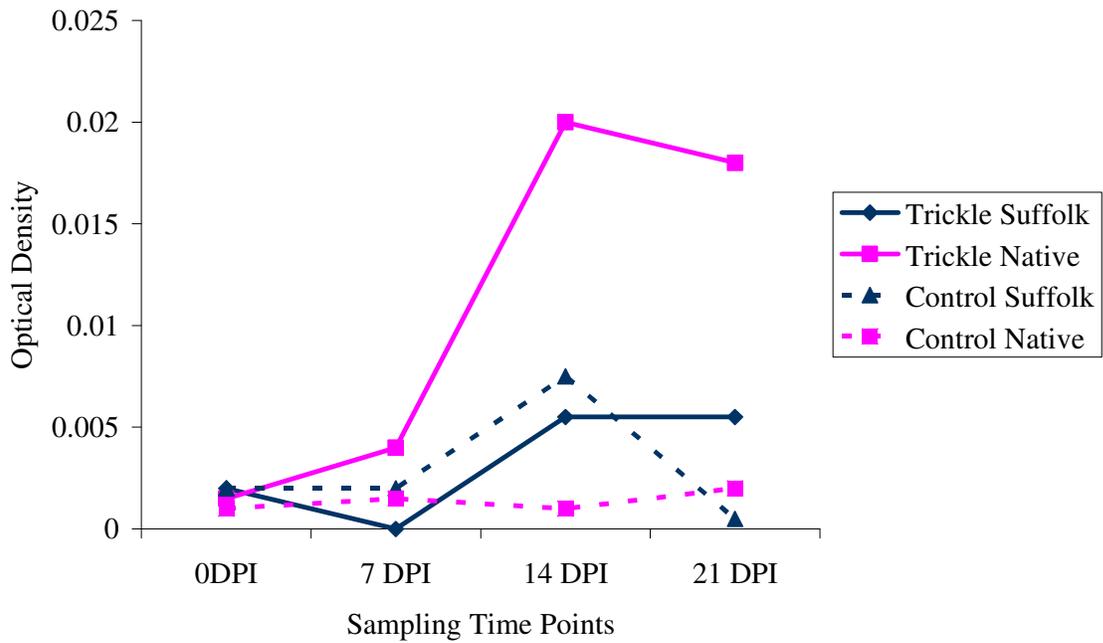


Figure 2.11. Optical densities representing the serum level of IgE against *H. contortus* whole worm antigen in pooled serum samples. A) Bolus Late Groups and B) Trickle Late Groups. DPI=Days post infection, (n=5).

## 2.4. Discussion

The results show that prior to day 14 of infection, few trichostrongyle type eggs continued to be present and that may be due to incomplete clearing of the previous infection by the anthelmintic treatments. Since these animals were exposed to previous *H. contortus* infection, the results obtained are a secondary response. The lack of FEC increase during this period (0-14 days) was expected as the prepatent period (i.e. time to reach the adult) of this parasite in sheep is 15-18 days (Soulsby, 1982). On day 21 post infection, FEC increased substantially in all the late infected groups compared with uninfected controls and both bolus and trickle infected Native groups were lower than the Suffolk counterparts. The increase in FEC resulted from the increase in the adult population of *H. contortus* in the abomasum. The late infected Suffolk groups also had all adult worms and a very few immature stages regardless of the infection regimen, whereas a substantial number of immature worms and fewer adults were recovered from the infected Native groups which accounted for the lower FEC. On day 14, all the worms recovered were immature with fewer, though not significant, recovered from Native lambs. It was unexpected that the total worm population (adult and/or immature) in each breed, regardless of infection regimen would be similar, but there was a trend for consistently fewer in Native lambs. This is in contrast with other findings in resistant breeds of sheep including the Native sheep where significantly fewer worms were recovered (Woolaston et al., 1990; Miller et al., 1998; Amarante et al., 1999). It should be noted that the previous studies with Native sheep were conducted under natural grazing conditions over periods of 4 to 16 weeks and not short term experimental infections (Miller et al., 1998). Also, worms recovered were predominantly adults with no appreciable larvae as was found in this study. It is possible that the larvae observed in this study may eventually have been eliminated and not reached maturity or developed slowly to replace adults which would result in FEC and worm burdens remaining low

(Amarante et al., 2005; Aumont et al., 2003). If that is the case, then the adult population in the Native lambs would not increase substantially, thus supporting previous observations. Also, if these larvae would have eventually matured adding to the adult population, there would have been equivalent worm burdens in each breed. Since it is not known what level of infection is necessary to stimulate a good protective response, the level of infection administered may have been high enough to overcome some component of the protective response that the Native lambs could mount during the short infection period.

Lymph node weight has been reported to increase by 20 fold in helminth infection which was attributed to the anatomical structure of the abomasum that is not favorable for efficient antigen presentation (Gasbarre, 1997). However, the results are in line with the findings of Balic et al., (2000) who did not find any difference in LN weights between *H. contortus* infected and uninfected lambs. In this study, both the BES and TES lambs had hypertrophied abomasal lymph nodes. For Native lambs on the other hand, a significant difference was found only between the BLN and the uninfected controls. Among the corresponding infected groups, there was no significant difference found between abomasal lymph node weights. Balic et al. (2002) reported a change in cellular profile of the draining lymph nodes in lambs which had larvae present in tissue compared with those which did not. In this study, no parasite larvae were detected in the abomasal mucosal tissue.

Adult and L4 worms feed on blood and it is been reported that each worm can consume about 0.05 ml of blood every day (Urquhart et al., 1996). When engorged worms stop feeding and leave the wound, the raw ulcer created keeps bleeding which results in more blood loss. This combination leads to anemia. *H. contortus* worms are reported to produce calreticulin, a calcium binding protein, which interferes with blood clotting and facilitates blood feeding (Suchitra and Joshi, 2005). In this study, the PCV reduction percentage observed can be attributable to

establishment of infection. There were about 15 and 20% reductions in mean group PCV by the end of the second week for Native and Suffolk lambs respectively, and by the end of the third week that increased to 59.8 and 38.6% in bolus infected and 57.5 and 44.4% in trickle infected Suffolk and Native lambs, respectively, which resulted in anemia (more severe in Suffolk lambs). Although not significant, Native lambs had a consistently lower mean PCV reduction percentage than Suffolk lambs which reflected the lower L4 and adult worm burden.

Increased peripheral eosinophilia may be an indication for greater responsiveness of hosts to helminth infections (Dawkins et al., 1989), and it has been reported to be correlated with protection against *H. contortus* (Terefe et al., 2005). The results of this study show that peripheral eosinophilia was induced as early as 2 days after infection in Native lambs regardless of infection regimen. The peak eosinophilia was seen on day 14 and declined thereafter. However, level remained greater than the infected Suffolk lambs at all time points. At the peak, the peripheral eosinophil percentage was as much as 15 to 25 fold greater in Native lambs compared with 2 to 4 fold in Suffolk lambs. The findings are in contrast to what was reported by Wildblood et al., (2005) where eosinophilia was induced due to chemoattractant characteristics possessed by ovine trichostrongyle parasites not a characteristic of the host. In this study, the Suffolk lambs had fewer eosinophils in peripheral circulation compared with their corresponding Native lambs, although the infection rate was similar. Findings of this study therefore are in agreement with conclusions made by Dawkins et al., (1989).

Eosinophilia was in general induced in the infected abomasal tissue along with increased MMC count and neutrophilia to some extent. This is in agreement with Perez et al., (2001) that also reported that at three weeks post infection, lymphocytes and macrophages congregated around the necrotic granuloma in *Haemonchus* infected goats. There was no necrotic granuloma observed in any of abomasal section samples examined in this study. Hunter and Mackenzie

(1982) reported a few eosinophils present by the seventh day of *Haemonchus* infection in lambs, and by day 12 there were no mast cells or globule leukocytes noted. In lambs infected for the first time, eosinophils were observed in the abomasal mucosa where they did not seem to increase later in adult worm infected lambs, but greater numbers of MMC and globule leukocyte was reported in such lambs (Balic et al., 2000). No difference in eosinophils, mast cells or globule leukocytes was observed after 12 weeks of immunizing infection, however eosinophil recruitment was rapid and they were observed around L4 in lambs (Balic et al., 2002). Sheep that were challenged 9 weeks after immunization demonstrated eosinophils adhering on to the surface of *Haemonchus* larvae specially in gastric pits and larvae showed considerable damage (Balic et al., 2006). Similarly, in another in vitro study, eosinophils from *Haemonchus* sensitized sheep were seen to immobilize *Haemonchus* larvae in the presence of anti-haemonchus antibodies (Rainbird et al., 1998). The increased number of MMC following the infection in all the lambs in this study supports a close association with nematode infection and may be involved in parasite elimination (Pennock and Grecis, 2006). Enzymes produced by mast cells are known to play an important role in nematode elimination. Rat mast cell protease-1 is known to function as an inducer of IgE and IgG1 production (Yoshikawa et al., 2001). In *T. spiralis* infection, mastocytosis has been correlated with degradation of occludin, a tight cellular junction protein, which resulted in increased permeability of the epithelial membrane (McDermott et al., 2003). High concentration of mouse intestinal mast cell protein and rat mast cell proteinase-I have been associated with lower nematode burden in rats and mice (King et al., 1986; Newlands et al., 1990). A greater number of eosinophils, and MMC in Native lambs in general was associated with increased immature population in our study, which indicated a possible contribution to inhibition of the nematode population. Lower numbers of these cells in Suffolk lambs may not

have been able to create an adverse environment for inhibition of growth and development of these parasites.

Neutrophils are known to be involved in innate immunity (Goldsby et al., 2000). The results from this study demonstrate a significantly greater number in abomasal mucosa in infected Native lambs in both groups on day 14 of early infection and the bolus infected late group on day 21. Neutrophils were greater on day 14 of late infections within the same breed which indicated there may be a role played by these cells in initial phases of *H. contortus* infection.

Among all the immunoglobulin isotypes, IgE is the most investigated in nematode infections and it has been found to increase the most during infection. Results of this study indicated that the level of serum IgE increased up to 39 fold in Native lambs whereas it was non-significant in Suffolk lambs. Most of the IgE produced is tissue bound to the Fcε receptors on MMC, eosinophils and basophils. Upon subsequent exposure to the antigen, degranulation of the granulocytes is triggered and a variety of mediators like histamine, leukatrienes, prostaglandins and enzymes like chymase are released that bring about changes in gut physiology. Similarly, eosinophils and other cells are seen attached to the surface of larvae and degranulation has been observed to take place while they adhered to the worm surface (Mackenzie et al., 1981). Ability of sensitized ovine eosinophils to immobilize *H. contortus* larvae in vitro was potentiated by addition of antibody against L3 surface proteins (Rainbird et al., 1998). The immobilization increased with the addition of complement with different levels of damage to their surfaces. However it is also reported that eosinophils although adhered to the surface of the *T. muris* larvae, were unable to kill after 48 hours of incubation (Preston et al., 1986).

Although individual animal serum immunoglobulin isotypes could not be done, pooled samples showed all five isotypes of immunoglobulins specific to *H. contortus* WWA were

present in the serum. BLN lambs demonstrated a distinct trend where IgE increased by 13 fold at its peak after two weeks, which was maintained at about the same level. Similarly, serum IgA increased in the BLN lambs which peaked at about 2 weeks. IgA is the major immunoglobulin isotype in mucosal immunity (Abbas and Lichtman, 2005) and is found in abomasal mucus in nematode infections (Amarante et al., 2005; Harrison et al., 2003). IgA level in abomasal mucus at a greater concentration in s was not measured; however, serum IgA may be an indication of the presence of IgA in abomasal mucous. In contrast, IgE and IgA levels in infected Suffolk lambs remained around the baseline throughout the study. Therefore, production of IgE and IgA may be important in resistance against the establishment of *H. contortus*.

The level of IgG2 initially started at a greater level in bolus infected Suffolk lambs compared with Native lambs however following infection it showed a complete opposite trend. In Suffolk, the serum level of IgG2 decreased while in Natives it increased. In vaccinated sheep, the production of IgG2 has been reported to be at a greater level (Knox et al., 2005), but Gill et al., (1994) reported a lower number of IgG2 bearing cells in *H. contortus* infected sheep abomasa. Serum IgM level decreased post infection in Suffolk lambs, whereas in Native lambs it was maintained at around the same level. IgG1 level remained about the same until 14 dpi in both the bolus infected groups however it declined in Suffolk but showed an increased trend in Natives which is in agreement with the findings reported by Gill et al., (1994). The IgG level in Natives lambs whether bolus or trickle infected increased but in Suffolk it remained around the same level. Immunized sheep B cells are reported to produce IgM and IgG in vitro (Coyne and Brake, 2001). Mucus collected from a hyperimmunized sheep was found to have IgA and IgG and it was suggested that IgG was of systemic origin whereas IgA was locally produced (Smith, 1977). Gomez-Munoz et al., (1999), have found resistant animals to produce greater levels of all the isotypes. The level of these immunoglobulins was found to be associated with decreased FEC

and worm load. From the results of this study and reported by others, it is plausible to speculate that increased serum levels of immunoglobulin isotypes IgE, IgA, and IgG1 are indicative of their proportionately increased production and recruitment of inflammatory cells, or secretions such as abomasal mucus. The histological observation of increased cellular infiltration of eosinophils and mastocytosis in the resistant Native breed of sheep accompanied by greater number of delayed development of *H. contortus* larvae compared with susceptible lambs is indicative of their protective role. The speculation that the secondary exposure of the parasitic antigens may have triggered the degranulation of sensitized MMC and eosinophils that have IgE bound to their surfaces which resulted in release of mediators earlier than in the naïve animals, thus the growth and development of *H. contortus* in Native lambs was adversely affected.

In conclusion, our findings indicated that immune components such as mast cell hyperplasia, peripheral and tissue eosinophilia, increased serum IgE, IgA, IgG1 are associated with and may be responsible for increased resistance of Native lambs against *H. contortus* infection indicated by inhibition of its growth and development.

## **2.5. Conclusions**

It appears that the cellular and immunoglobulin responses evoked by the Native lambs may be involved in the observed response.

## **2.6. References**

Abbas, A.K., Lichtman, A.H., 2005, Cellular and Molecular Immunology, Fifth Edition. Elsevier Saunders, 342-343 pp.

Amarante, A.F., Bricarello, P.A., Huntley, J.F., Mazzolin, L.P., Gomes, J.C., 2005, Relationship of abomasal histology and parasite-specific immunoglobulin A with the resistance to *Haemonchus contortus* infection in three breeds of sheep. *Vet Parasitol* 128, 99-107.

Amarante, A.F., Craig, T.M., Ramsey, W.S., El-Sayed, N.M., Desouki, A.Y., Bazer, F.W., 1999, Comparison of naturally acquired parasite burdens among Florida native, Rambouillet and crossbreed ewes. *Vet Parasitol* 85, 61-69.

- Aumont, G., Gruner, L., Hostache, G., 2003, Comparison of the resistance to sympatric and allopatric isolates of *Haemonchus contortus* of Black Belly sheep in Guadeloupe (FWI) and of INRA 401 sheep in France. *Vet Parasitol* 116, 139-150.
- Bahirathan, M., Miller, J.E., Barras, S.R., Kearney, M.T., 1996, Susceptibility of Suffolk and Gulf Coast Native suckling lambs to naturally acquired strongylate nematode infection. *Vet Parasitol* 65, 259-268.
- Balic, A., Bowles, V.M., Meeusen, E.N., 2000, Cellular profiles in the abomasal mucosa and lymph node during primary infection with *Haemonchus contortus* in sheep. *Vet Immunol Immunopathol* 75, 109-120.
- Balic, A., Bowles, V.M., Meeusen, E.N., 2002, Mechanisms of immunity to *Haemonchus contortus* infection in sheep. *Parasite Immunol* 24, 39-46.
- Balic, A., Cunningham, C.P., Meeusen, E.N., 2006, Eosinophil interactions with *Haemonchus contortus* larvae in the ovine gastrointestinal tract. *Parasite Immunol* 28, 107-115.
- Coyne, C.P., Brake, D., 2001, Characterisation of *Haemonchus contortus*-derived cell populations propagated in vitro in a tissue culture environment and their potential to induce protective immunity in sheep. *Int J Parasitol* 31, 359-376.
- Davidson, W.R., McGhee, M.B., Nettles, V.F., Chappell, L.C., 1980, Haemonchosis in white-tailed deer in the southeastern United States. *J Wildl Dis* 16, 499-508.
- Dawkins, H.J., Windon, R.G., Eagleson, G.K., 1989, Eosinophil responses in sheep selected for high and low responsiveness to *Trichostrongylus colubriformis*. *Int J Parasitol* 19, 199-205.
- Gasbarre, L.C., 1997, Effects of gastrointestinal nematode infection on the ruminant immune system. *Vet Parasitol* 72, 327-337; discussion 337-343.
- Gill, H.S., Altmann, K., Cross, M.L., Husband, A.J., 2000, Induction of T helper 1- and T helper 2-type immune responses during *Haemonchus contortus* infection in sheep. *Immunology* 99, 458-463.
- Gill, H.S., Husband, A.J., Watson, D.L., Gray, G.D., 1994, Antibody-containing cells in the abomasal mucosa of sheep with genetic resistance to *Haemonchus contortus*. *Res Vet Sci* 56, 41-47.
- Goldsby, R.A., Kindt, T.J., Osborne, B.A., 2000, *Kuby Immunology*, Fourth Edition. W.H. Freeman and Company, New York, 44-45 pp.
- Gomez-Munoz, M.T., Cuquerella, M., Gomez-Iglesias, L.A., Mendez, S., Fernandez-Perez, F.J., de la Fuente, C., Alunda, J.M., 1999, Serum antibody response of Castellana sheep to *Haemonchus contortus* infection and challenge: relationship to abomasal worm burdens. *Vet Parasitol* 81, 281-293.

- Harrison, G.B., Pulford, H.D., Hein, W.R., Barber, T.K., Shaw, R.J., McNeill, M., Wakefield, S.J., Shoemaker, C.B., 2003, Immune rejection of *Trichostrongylus colubriformis* in sheep; a possible role for intestinal mucus antibody against an L3-specific surface antigen. *Parasite Immunol* 25, 45-53.
- Hunter, A.R., MacKenzie, G., 1982, The pathogenesis of a single challenge dose of *Haemonchus contortus* in lambs under six months of age. *J Helminthol* 56, 135-144.
- King, S.J., Miller, H.R., Woodbury, R.G., Newlands, G.F., 1986, Gut mucosal mast cells in *Nippostrongylus*-primed rats are the major source of secreted rat mast cell protease II following systemic anaphylaxis. *Eur J Immunol* 16, 151-155.
- Knox, D.P., Smith, S.K., Redmond, D.L., Smith, W.D., 2005, Protection induced by vaccinating sheep with a thiol-binding extract of *Haemonchus contortus* membranes is associated with its protease components. *Parasite Immunol* 27, 121-126.
- Lacroux, C., Nguyen, T.H., Andreoletti, O., Prevot, F., Grisez, C., Bergeaud, J.P., Gruner, L., Brunel, J.C., Francois, D., Dorchies, P., Jacquet, P., 2006, *Haemonchus contortus* (Nematoda: Trichostrongylidae) infection in lambs elicits an unequivocal Th2 immune response. *Vet Res* 37, 607-622.
- Mackenzie, C.D., Jungery, M., Taylor, P.M., Ogilvie, B.M., 1981, The *in-vitro* interaction of eosinophils, neutrophils, macrophages and mast cells with nematode surfaces in the presence of complement or antibodies. *J Pathol* 133, 161-175.
- McDermott, J.R., Bartram, R.E., Knight, P.A., Miller, H.R., Garrod, D.R., Grecis, R.K., 2003, Mast cells disrupt epithelial barrier function during enteric nematode infection. *Proc Natl Acad Sci U S A* 100, 7761-7766.
- Miller, J.E., Bahirathan, M., Lemarie, S.L., Hembry, F.G., Kearney, M.T., Barras, S.R., 1998, Epidemiology of gastrointestinal nematode parasitism in Suffolk and Gulf Coast Native sheep with special emphasis on relative susceptibility to *Haemonchus contortus* infection. *Vet Parasitol* 74, 55-74.
- Miller, J.E., Bishop, S.C., Cockett, N.E., McGraw, R.A., 2006, Segregation of natural and experimental gastrointestinal nematode infection in F(2) progeny of susceptible Suffolk and resistant Gulf Coast Native sheep and its usefulness in assessment of genetic variation. *Vet Parasitol*.
- Miller, J.E., Horohov, D.W., 2006, Immunological aspects of nematode parasite control in sheep. *J Anim Sci* 84 Suppl, E124-132.
- Newlands, G.F., MacKellar, A., Miller, H.R., 1990, Intestinal mucosal mast cells in *Nippostrongylus*-infected mice: lack of sensitivity to corticosteroids. *Int J Parasitol* 20, 669-672.

Pena, M.T., Miller, J.E., Horohov, D.W., 2004, Effect of dexamethasone treatment on the immune response of Gulf Coast Native lambs to *Haemonchus contortus* infection. *Vet Parasitol* 119, 223-235.

Pena, M.T., Miller, J.E., Horohov, D.W., 2006, Effect of CD4+ T lymphocyte depletion on resistance of Gulf Coast Native lambs to *Haemonchus contortus* infection. *Vet Parasitol* 138, 240-246.

Pennock, J.L., Grencis, R.K., 2006, The mast cell and gut nematodes: damage and defence. *Chem Immunol Allergy* 90, 128-140.

Perez, J., Garcia, P.M., Hernandez, S., Martinez-Moreno, A., Martin de las Mulas, J., Camara, S., 2001, Pathological and immunohistochemical study of the abomasum and abomasal lymph nodes in goats experimentally infected with *Haemonchus contortus*. *Vet Res* 32, 463-473.

Pfeffer, A., Douch, P.G., Shaw, R.J., Gatehouse, T.K., Rabel, B., Green, R.S., Shirer, C.L., Jonas, W.E., Bisset, S., 1996, Sequential cellular and humoral responses in the abomasal mucosa and blood of Romney sheep dosed with *Trichostrongylus axei*. *Int J Parasitol* 26, 765-773.

Preston, C.M., Jenkins, T., McLaren, D.J., 1986, Surface properties of developing stages of *Trichuris muris*. *Parasite Immunol* 8, 597-611.

Rainbird, M.A., Macmillan, D., Meeusen, E.N., 1998, Eosinophil-mediated killing of *Haemonchus contortus* larvae: effect of eosinophil activation and role of antibody, complement and interleukin-5. *Parasite Immunol* 20, 93-103.

Schallig, H.D., van Leeuwen, M.A., Bernadina, W.E., Hendrikx, W.M., 1994, Serum antibody responses of Texel sheep experimentally infected with *Haemonchus contortus*. *Res Vet Sci* 57, 63-68.

Schallig, H.D., van Leeuwen, M.A., Hendrikx, W.M., 1995, Isotype-specific serum antibody responses of sheep to *Haemonchus contortus* antigens. *Vet Parasitol* 56, 149-162.

Smith, W.D., 1977, Anti-larval antibodies in the serum and abomasal mucus of sheep hyperinfected with *Haemonchus contortus*. *Res Vet Sci* 22, 334-338.

Soulsby, E.J.L., 1982, Helminths, Arthropods and protozoa of domesticated animals, 7th Edition. Lea and Febiger, 233-252 pp.

Stankiewicz, M., Pernthaner, A., Cabaj, W., Jonas, W.E., Douch, P.G., Bisset, S.A., Rabel, B., Pfeffer, A., Green, R.S., 1995, Immunization of sheep against parasitic nematodes leads to elevated levels of globule leukocytes in the small intestine lumen. *Int J Parasitol* 25, 389-394.

Suchitra, S., Joshi, P., 2005, Characterization of *Haemonchus contortus* calreticulin suggests its role in feeding and immune evasion by the parasite. *Biochim Biophys Acta* 1722, 293-303.

Terefe, G., Yacob, H.T., Grisez, C., Prevot, F., Dumas, E., Bergeaud, J.P., Dorchies, P., Hoste, H., Jacquiet, P., 2005, *Haemonchus contortus* egg excretion and female length reduction in sheep previously infected with *Oestrus ovis* (Diptera: Oestridae) larvae. *Vet Parasitol* 128, 271-283.

Urquhart, G.M., Armour, J., Duncan, J.L., Dunn, A.M., Jennings, F.W., 1996, *Veterinary Parasitology*, Second Edition. Blackwell Science, 1-22 pp.

Waller, P.J., 2003, Global perspectives on nematode parasite control in ruminant livestock: the need to adopt alternatives to chemotherapy, with emphasis on biological control. *Anim Health Res Rev* 4, 35-43.

Whitlock, H.R. 1948, Some modifications of the McMaster helminth egg-counting technique apparatus. *Journal of Council of Scientific Research* 21, 177-180.

Wildblood, L.A., Kerr, K., Clark, D.A., Cameron, A., Turner, D.G., Jones, D.G., 2005, Production of eosinophil chemoattractant activity by ovine gastrointestinal nematodes. *Vet Immunol Immunopathol* 107, 57-65.

Woolaston, R.R., Barger, I.A., Piper, L.R., 1990, Response to helminth infection of sheep selected for resistance to *Haemonchus contortus*. *Int J Parasitol* 20, 1015-1018.

Yoshikawa, T., Imada, T., Nakakubo, H., Nakamura, N., Naito, K., 2001, Rat mast cell protease-I enhances immunoglobulin E production by mouse B cells stimulated with interleukin-4. *Immunology* 104, 333-340.

## CHAPTER 3

### TH2 TYPE OF IMMUNE COMPONENTS ARE ASSOCIATED WITH INCREASED RESISTANCE TO *HAEMONCHUS CONTORTUS* IN NATURALLY INFECTED GULF COAST NATIVE LAMBS

#### 3.1. Introduction

*Haemonchus contortus* is a gastrointestinal hematophagus nematode parasite of sheep, goats and wild ruminants etc (Davidson et al., 1980; Urquhart et al., 1996). This parasite is worldwide in its distribution especially in the tropical and subtropical regions. These are favorable for growth and development of this parasite. Haemonchosis is a disease condition caused by infection with *H. contortus* and is one of the most economically important disease conditions in small ruminants. Economic losses are incurred mainly due to loss in production, mortality and cost of prophylaxis and curative treatments. Use of chemical anthelmintics is the best known method for control; however, evolution of resistance against the common anthelmintics and public demand for animal products free of chemical residues (Waller, 2003) had a negative impact on their use and alternative approaches for control are needed.

The development of vaccines and breeding of resistant animals to *H. contortus* are among the most ideal alternative strategies for controlling this nematode. A lack of understanding of the immune components and the mechanism(s) responsible for resistance is partially responsible for the unavailability of a commercial vaccine in the market. Similarly, research efforts to identify reliable genetic markers for resistance are on going which will facilitate marker assisted genetic selection programs for breeding of resistant animals.

Immunological responses in resistant as well as immunized rodents against various nematode parasites such as *Nippostrongylus brasiliensis*, *Heligomosoides polygyrus*, *Trichinella spiralis*, *Trichuris muris* etc. have been extensively evaluated (Ogilvie et al., 1980; Urban et al., 2001; Mahida, 2003; Finkelman et al., 2004; Doligalska et al., 2006). In rodent models,

activation of CD4+ T cells produce two distinct sets of cytokines called Th1 and Th2. The response mediated by Th2 cytokines IL-4, IL-5, IL-10 and IL-13 accompanied by mucosal mastocytosis, tissue eosinophilia and globule leukocytosis was found to be protective against nematode infections (Garside et al., 2000; Gill et al., 2000), whereas induction of the Th1 response mediated by cytokines IFN- $\gamma$  and IL-12 resulted in increased susceptibility (Helmby and Grecis, 2003b). Production of IgE (Urban et al., 1991), IgA and IgG1 in the early response of the immune system to infection and IgG2 during the later response (Koyama et al., 1999) has been implicated to play various roles during the course of nematode infection. However, the immune response elicited by nematode parasites has not always been clearly one of Th1 or Th2 and overlapping between these two sets of responses is a common observation. IL-10, for example, has been observed to have different effects on different stages of parasites. In *T. spiralis* infected mice, IL-10 producing mice were protected against the adult worms whereas in IL-10 knockout mice IFN- $\gamma$  levels increased which resulted in protection against larval stages (Helmby and Grecis, 2003a). *T. muris* infection was terminated in mice with a different mechanism involving increased epithelial turn over induced by IL-13 and chemokine CXCL10 (Cliffe et al., 2005). Other factors such as immunoglobulins were reported to be not necessary during this process. There appears to be a different set of immune components responsible for resistance against different parasites indicating that the interaction of immune components is both complex and variable.

Recently, protection against *Ostertagia ostertagi* and *Cooperia oncophora* in cattle was reported to involve local inflammation of the abomasum mediated by Tumor Necrosis Factor alpha (TNF- $\alpha$ ), IL-1 $\beta$  and Macrophage Inflammatory Protein-1alpha (MIP-1 $\alpha$ ) (Li et al., 2006). There were no changes in the expression levels of CXCL6, CXCL10, IL-2, IL-4, IFN- $\gamma$ , IL-6, IL-8, IL-12 p40, IL-13, IL-15 and IL-18 cytokines. Likewise, infection of sensitized Castellana

sheep with *H. contortus* resulted in partial resistance characterized by increased prepatent period, lowered fecal egg count (FEC) and fewer worms (Gomez-Munoz et al., 1999). However, there was no relation of resistance to serum levels of IgG, IgG1, IgG2, IgM and IgA. In another study with *Trichostrongylus axei*, an abomasal parasite of sheep, increased numbers of mucosal mast cells, globule leukocytes, eosinophils count were accompanied by increased inhibition of larval migration in abomasal mucosa of the repeatedly exposed sheep compared to the controls (Pfeffer et al., 1996). Likewise, higher serum level of *T. axei*-specific total and IgG1 and higher peripheral eosinophil numbers were reported in these sheep. Sheep immunized by infection with *Teladorsagia circumcincta*, and *Trichostrongylus colubriformis* demonstrated increased peripheral and tissue eosinophil number and increased tissue globule leukocytes in the abomasum (Stankiewicz et al., 1995). Lacroux et al. (2006) reported a clear Th2 type of immune response in repeatedly exposed lambs with *H. contortus*. These lambs recruited eosinophils, mast cells and globule leukocytes earlier than the primary infected lambs. In Florida Native lambs, there was no difference observed in eosinophil, mast cell or globule leukocyte count compared with susceptible Rambouillet and their F2 offsprings (Amarante et al., 1999a). However there was a negative correlation with worm burden and FEC. Similarly, Texel sheep have responded with lowered FEC and increased serum level of IgG1 followed by IgG2 to a secondary infection (Schallig et al., 1995). IgM and IgA were reported less dominant in these sheep. In another study, Schallig et al (1994) reported IgA response to L3 and IgG1 and IgG2 response to adult antigens. However, *H. contortus* specific IgG1 and IgE levels were reported to be higher by Gill et al. (2000).

Studies have revealed that some breeds of sheep demonstrate a relatively high degree of resistance to nematode parasite infections, *H. contortus* being one, such that they do well without much management intervention that other more susceptible breeds require. Gulf Coast Native

(Native) sheep, native to the gulf coast southeastern region in the US, is one such breed that has demonstrated resistance characteristics. Resistance has been shown to develop initially in suckling lambs which is maintained through adulthood (Bahirathan et al., 1996; Miller et al., 1998). Results from previous experiments demonstrated that Native lambs treated with dexamethasone and anti-CD4+T cell antibodies resulted in increased susceptibility to *H. contortus* infection (Pena et al., 2004, 2006). These findings indicate a vital role played by CD4+ T cells in upregulating various immunologic components in greater resistance in Native lambs. Cross breeding of Native lambs with Suffolk lambs also indicated that resistance is genetically controlled and highly variable among individuals within the breed (Miller et al., 2006).

In a previous study, Native lambs responded to artificial infection of *H. contortus* with peripheral as well as abomasal eosinophilia, mastocytosis, increased level of serum IgG, IgE compared with infected Suffolk lambs (Chapter 2). However, there was a high degree of variability among individual animals within the Native breed in both types of infection regimens. It was also found that, there was a significantly greater immature adult ratio among the worms recovered from bolus infected Native lambs compared to Suffolk lambs 21 days post infection which indicated a delayed growth and development in Native breed. However, this finding was not consistent across all the Native lamb groups tested under different infection regimens and time points after infection examined. Epidemiological studies have indicated increased resistance in Native lambs based on the FEC, PCV and number of anthelmintic treatment required. Therefore, this study was designed to evaluate and determine components of the immune response that are associated with resistance in naturally infected Native lambs compared with Suffolk lambs.

## **3.2. Materials and Methods**

### **3.2.1. Location and Animals**

The experiment was conducted at the Small Ruminant Farm, Louisiana State University Agricultural Experiment Station, Central Research Station, Baton Rouge, LA 70803. Five to six month old Native and Suffolk lambs (10 each) were randomly selected from the lambs that were born in 2006. The experimental protocol was approved by the Louisiana State University Institutional Care and Use Committee.

### **3.2.2. Experimental Design and Sampling Scheme**

The lambs were repeatedly treated with levamisole (AgriL<sup>®</sup>, St. Joseph, MO) and albendazole (Valbazen<sup>®</sup>, Pfizer, New York, NY) at 12 mg/kg and 20 mg/kg body weight per orum, respectively, for two consecutive days each week for three weeks until FEC was zero or close to zero as described in Chapter 2. The lambs were housed on a dirt floor shed at night and were allowed to graze in a multi-species nematode parasite contaminated pasture where *H. contortus* was the most prevalent nematode.

#### **3.2.2.1. Fecal Sample Collection**

Fecal sample was collected on a weekly basis starting on the day the lambs were allowed to graze the contaminated pasture. Samples were taken directly from the rectum and put in Styrofoam cups labeled with the corresponding animal identification number and the date of collection.

#### **3.2.2.2. Blood Collection for Packed Cell Volume (PCV) and Differential Leukocyte Count**

Fresh blood was collected on days 0, 2, 4, 7, 10, 14, 21, 28, 35 and 42 post exposure by jugular venipuncture into 7 ml purple top vacutainer tube pre-coated with K3 EDTA (Becton, Dickinson, & Co.) and inverted several times to assure thorough mixing of blood with

anticoagulant. A thin blood smear was prepared and PCV was determined as described in Chapter 2.

### **3.2.2.3. Blood Collection for RNA Extraction**

Two and a half ml of blood was drawn by jugular venipuncture into a 3 ml plastic syringe and was mixed in Paxgene blood RNA tubes (Qiagen) pre-filled with a reagent for cytolysis and stabilization of RNA. Samples were collected on days 0, 2, 4, 7, 10, 14, 21, 28, 35 and 42 post exposures. After adding the blood, the tube was inverted at least ten times to make sure that the blood cells were completely lysed. These tubes were preserved at -20°C until they were processed for extraction of RNA according to the manufacturer's directions (Appendix A and B).

### **3.2.2.4. Serum Collection**

Blood was collected by jugular venipuncture into red top 10 ml plain vacutainer tubes (No Additive, Becton, Dickinson, & Co.) and serum was separated after the blood was clotted. The tubes were centrifuged for ten minutes at 1000 rpm and serum was transferred into 5 ml Eppendorf tubes and preserved at -20° C until analyzed by ELISA.

### **3.2.2.5. Necropsy**

The lambs were euthanized with (Beuthanasia<sup>®</sup>-D, 1 ml/lb body weight) and necropsied as described in Chapter 2. Five animals randomly selected from each group were necropsied on day 35 and the remaining five animals from each group on day 42 post exposure.

### **3.2.2.6. Worm Recovery, Enumeration and Speciation**

At necropsy, the abomasum, small intestines and large intestines (cecum and proximal colon) were removed and processed as described in Chapter 2 for recovery, enumeration and speciation of worms from each organ.

### **3.2.2.7. Histopathological Samples Collection**

At necropsy, the abomasal and prescapular lymph nodes were removed as soon as the abdominal cavity was opened and processed as presented in Chapter 2. Soon after the abomasal contents were emptied approximately one inch long pieces of abomasal mucosa were taken from the cardiac, fundic and pyloric regions and processed as explained in Chapter 2 for histopathological examination.

### **3.2.3. Techniques Employed**

#### **3.2.3.1. Fecal Egg Count**

Fecal samples were processed using modified McMaster and sugar floatation techniques to determine FEC as described in Chapter 2.

#### **3.2.3.2. Blood PCV**

Blood PCV was determined by microhematocrit method as described in Chapter 2.

#### **3.2.3.3. Differential Leukocyte Count**

Peripheral leukocyte composition was determined in thin blood smears stained with Wright-Giemsa's stain and cells were enumerated and identified as described in Chapter 2.

#### **3.2.3.4. RNA Extraction and cDNA Synthesis**

RNA in each peripheral blood sample was extracted using a RNA extraction kit (Paxgene and Versagene) according to the manufacturer's recommendation (Appendix A and B). The RNA was quantified by spectrophotometric reading absorbency at 260/280 ratios which were greater than 1.8 for all samples. Complementary DNA (cDNA) strand was synthesized with one  $\mu\text{g}$  of RNA and reverse transcribed in an 80  $\mu\text{l}$  reaction containing 20 unit AMV Rtase, 0.5 $\mu\text{g}$  oligo dT primers, 40 units of RNAsin and 5mM  $\text{MgCl}_2$  (Promega, Madison, WI)(Horohov et al., 2005). The temperature and cycle length for making cDNA was 42°C for 15 minutes, followed by 95°C for 5 minutes, and 4°C for 15 minutes.

### 3.2.3.5. Quantitative Polymerase Chain Reaction (qPCR) Analysis for Cytokine mRNA Expression

Forward and reverse primer sequences for cytokines IL-4, IFN- $\gamma$  and IL-10 mRNA (Table 3.1) were purchased from IDT (Coralville, IA), based on previously published sequences for ovine IL-4, IL-10 and IFN- $\gamma$  (Hein et al., 2004) and housekeeping gene ATPase (Budhia et al., 2006).

Table 3.1. Primer sequences for ovine cytokines and house keeping gene used for SYBR Green real-time PCR.

Primer name	Forward sequence (5'-3')	Reverse sequence (5'-3')
OvATPase	TTCCTACTGCCCTGGATG	CACGAAGATGAGAAGCGAGT
OvIL-4	GGAGCTGCCTGTAGCAGACG	TTCTCAGTTGCGTTCTTTGGG
OvIL-10	TGCTGGATGACTTTAAGGGTTACC	TCATTTCCGACAAGGCTTGG
OvIFN- $\gamma$	TGA TTCAAATTCCGGTGGATG	TTCATTGATGGCTTTGCGC

Cytokine mRNA expression was detected by real time PCR using the MyiQ single color real time PCR detection system (BioRad). The reactions were carried out in 25 $\mu$ l volumes in duplicate in 96 well PCR plates as described by Budhia et al. (2006). Briefly, iQ<sup>TM</sup>SYBR<sup>®</sup> Green Supermix (BioRad) containing dNTP, iTaq DNA polymerase, SYBR Green I, fluorescein and KCl, Tris-HCl and MgCl<sub>2</sub>, 1 $\mu$ M each of reverse and forward primers, and 5 $\mu$ l of cDNA template were added to each plate. Pooled non-reverse transcribed template and blank samples were used in duplicate in each plate as a negative control. The amplification protocol included 95°C for 10 minutes, 40 cycles of 95°C for 20 seconds for annealing and 45 seconds for extension at 60°C. That was followed by one minute each of annealing and extension at 95°C and 55°C, respectively. Melting curves were generated at the end of the each run by running the product through temperature 55°C and increasing by 0.5°C after every 15 seconds until the final temperature of 95°C was reached.

Cycle thresholds (Ct) were calculated by the Mycycler software (mean  $\pm$  ten times the standard deviation of background) to determine the threshold values. An arbitrary cut-off point

was made if values were within 2 cycles of the corresponding RT negative samples on a plate. Each gene was analyzed individually and the highest threshold required to accurately analyze one was used to analyze all the genes in that plate in order to avoid low thresholds that were readily crossed by baselines that sloped upwards. Automatic baseline settings generated were used throughout the analysis. The whole plate was analyzed based on the threshold settings calculated.

First, mean Cts for each sample were calculated from the duplicate wells, and  $\Delta$ Cts were obtained by subtracting the Ct for housekeeping gene ATPase, from the Cts for each cytokine gene being analyzed. Outlier samples were ignored when traces showed clear irregularities. Some of the samples had ATPase detection delayed indicating a poor cDNA yield causing the genes of interest to be delayed in their detection. In order to avoid over-estimation of the amount of cytokine in target, the samples with low levels of ATPase expression were discarded from analysis.  $\Delta$ Cts were calibrated by determining the mean of all the individual  $\Delta$ Cts at all the sampling time points and deducting the individual  $\Delta$ Cts from the mean to obtain the  $\Delta\Delta$ Cts. The relative quantitation value for each mRNA level was calculated as  $2^{-2\Delta\Delta Ct}$ .

#### **3.2.3.6. Histopathological Examination**

Abomasal mucosa sections were stained with Hematoxyline & Eosin and were examined under the microscope at 400x for eosinophils, neutrophils, and globule leukocytes. Sections stained with toulidine blue were examined for mucosal mast cell (MMC) enumeration. All the cells were counted in 10 fields of  $0.3 \text{ mm}^2$  area of mucosa and presented as number of cells per square mm.

#### **3.2.3.7. Parasite Recovery**

Parasites were recovered from 100 ml of wash and soak samples from abomasa and small intestines after straining through a 200 pore size strainer as described in Chapter 2. If there were

less than 10 worms recovered from the first aliquot, then a second aliquot of 100 ml was examined. All the 500 ml of sample from large intestines was examined. The number obtained was extrapolated to estimate the total number of worms in each organ.

### **3.2.3.8. Immunoglobulin Isotype Profiling by ELISA**

Serum level of immunoglobulin isotypes, IgM, IgE, IgG1 and IgG2 was determined by ELISA following the protocol as described in Chapter 2 with the following modifications. Serum dilution for all the immunoglobulin isotypes assayed was 1:25, mAb 1:10, horse radish peroxidase (HRP) conjugate was used at 1:25000 dilution and color developing reaction time was one hour for isotypes IgG1, IgG2 and IgE, and 30 minutes for isotype IgM. Mouse anti-ovine IgM monoclonal antibody was adsorbed with whole worm antigen (WWA) at the ratio of 100  $\mu$ l to 75 $\mu$ g before it was used for capturing the ovine IgM isotype bound to the antigen in the ELISA plate. That was done in order to minimize non specific bindings of the monoclonal antibody showed during the assay. However there was no such reaction noted for other monoclonal antibodies used.

### **3.2.3.9. Whole Worm Antigen (WWA) Preparation**

WWA was prepared using adult *H. contortus* worms collected from lambs used in this experiment and preserved at -20°C. Worms were hand picked and washed in phosphate buffered saline solution (PBS) several times. Worms were ground and homogenized in 25 ml of PBS using a Tissumizer (Tekmar Co, Cincinnati, OH). The homogenate was centrifuged at 5000xg for 20 minutes and the supernatant was collected (Kabagambe, 1997). The soluble protein concentration in the supernatant solution was determined by a microtiter plate-adapted bicinchoninic acid assay (Pierce, Rockford, IL).

#### **3.2.4. Statistical Analysis**

The data obtained were analyzed by the SAS statistical package version 9.1.3. FEC and parasite recovery data was log transformed to stabilize variance. Repeated measures analysis of variance was used for comparisons of FEC, PCV reduction percentage, peripheral eosinophil percentage, immunoglobulin (ELISA OD) and cytokine mRNA expression levels over each sampling time point with split-plot arrangement of treatments with treatment group and animals within treatment groups as main plot effects and time by group interaction as subplot effects. When a significant difference overall was found, a Scheffé's test was used for main effect with pair wise comparison. Parasite recovery and abomasal cell enumeration data were compared by unpaired student t test. For all tests, a p value of  $\leq 0.05$  was set as level of significance.

### **3.3. Results**

#### **3.3.1. FEC**

Mean FEC is presented in Figure 3.1. The prior nematode infection was not completely cleared despite repeated administration of anthelmintics and the animals continued to show a low FEC. However, trichostrongyle type ova increased significantly in both the groups 3 weeks after they started grazing. The FEC started to increase rapidly and was significantly higher in Suffolk lambs compared with the Natives ( $p < 0.05$ ) until the end of the experiment. The FEC declined after the 4<sup>th</sup> week in Native lambs while it continued to increase in Suffolk lambs until the 5<sup>th</sup> week and declined in the 6<sup>th</sup> week.

#### **3.3.2. Blood PCV Reduction Percentage**

The starting mean blood PCV in the two groups was significantly different with Native lambs being higher ( $p < 0.05$ ) compared to Suffolk lambs. Therefore, they were converted into reduction percentages based on the initial PCV values on day zero of the exposure ((Day 0 PCV - Sample Day PCV / Day 0 PCV) x 100).

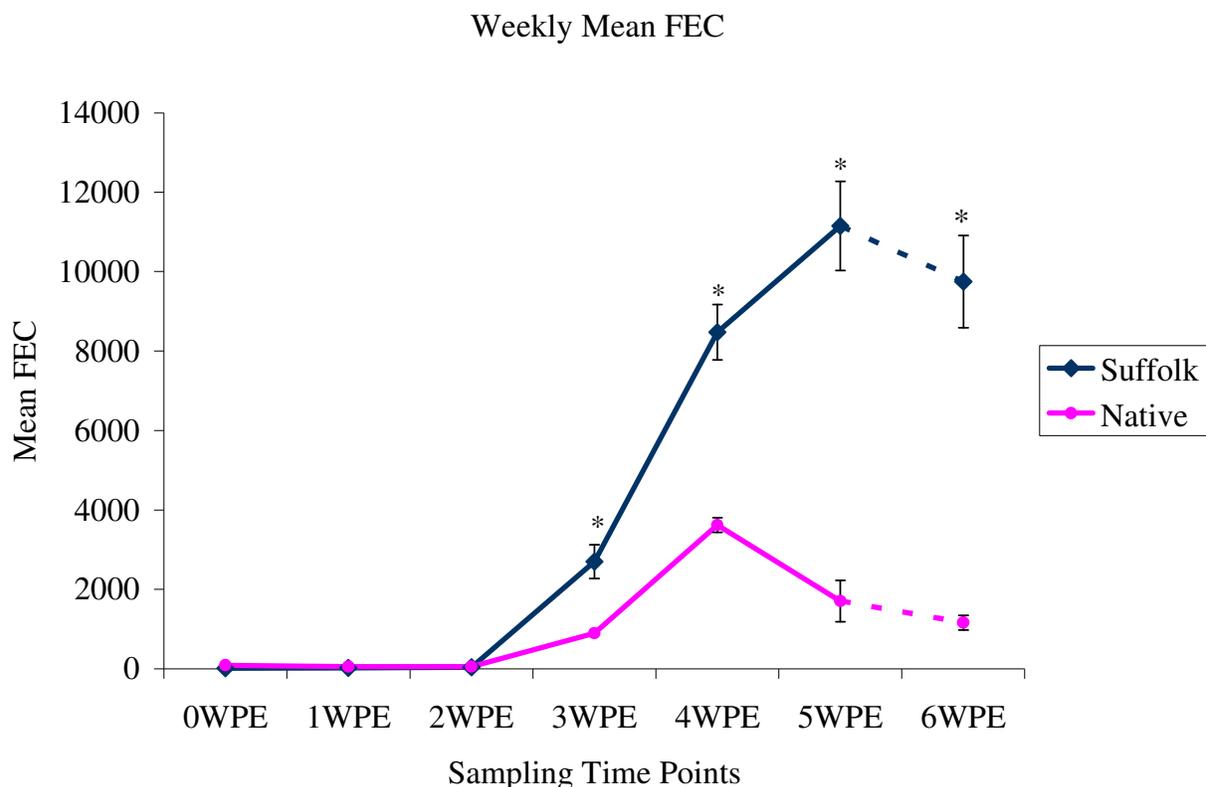


Figure 3.1. Mean weekly FEC and standard error of the mean in naturally infected Native and Suffolk lambs. Significant differences ( $p < 0.05$ ) are indicated by asterisk marks. Solid line indicates 10 lambs while dashed line indicates 5 lambs. (WPE= weeks post exposure).

The mean PCV reduction percentage remained similar in both the groups until two weeks post exposure as presented in Figure 3.2. The reduction percentage dramatically increased in Suffolk lambs and reached a peak in the 5<sup>th</sup> week compared with Native lambs ( $p < 0.05$ ). In Native lambs, the mean reduction percentage was maintained around the same level between the 4<sup>th</sup> and the 5<sup>th</sup> week. The PCV reduction percentage decreased for both Native and Suffolk groups on week 6 and was more pronounced for the Native group. At 6<sup>th</sup> week post exposure, the mean PCV reduction percentage in Native lambs was not significantly different ( $p < 0.05$ ) from the initial observation on 0 WPE whereas, it was significantly different ( $p < 0.05$ ) in Suffolk groups of lambs.

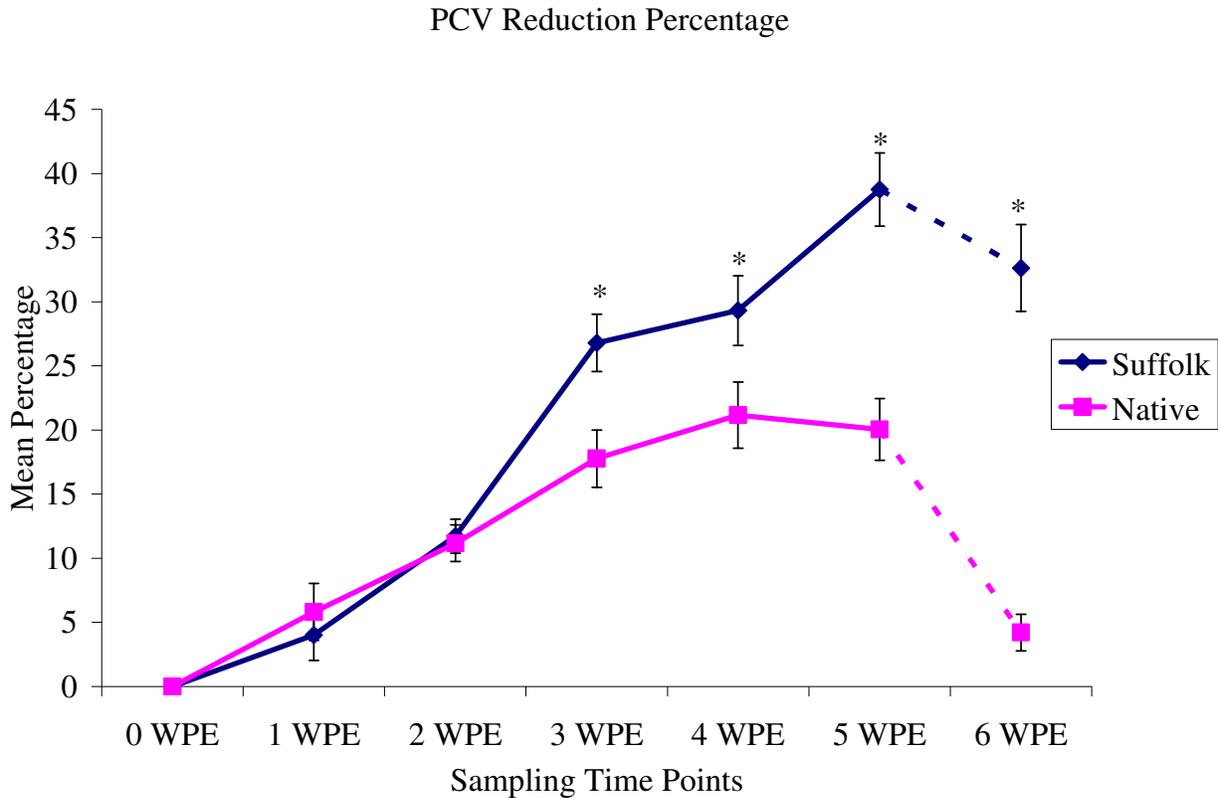


Figure 3.2. Weekly mean blood PCV reduction percentages and standard error of the mean in naturally infected Native and Suffolk lambs. Significant differences ( $p < 0.05$ ) are indicated by asterisk marks. Solid line indicates 10 lambs while dashed line indicates 5 lambs. (WPE=weeks post exposure).

### 3.3.3. Peripheral Eosinophil Percentage

Mean peripheral eosinophil percentages in both the groups were similar from the day they were infected until day 7 (Figure 3.3). From day 7, the Native lambs demonstrated a greater percentage of peripheral eosinophils through day 42 with two significant peaks, one each on days 14 and 35 ( $p < 0.05$ ). By contrast, Suffolk lamb eosinophil percentages remained around the base line with a slight increase after day 28 which continued until the end of the experiment.

### Peripheral Eosinophil Percentage

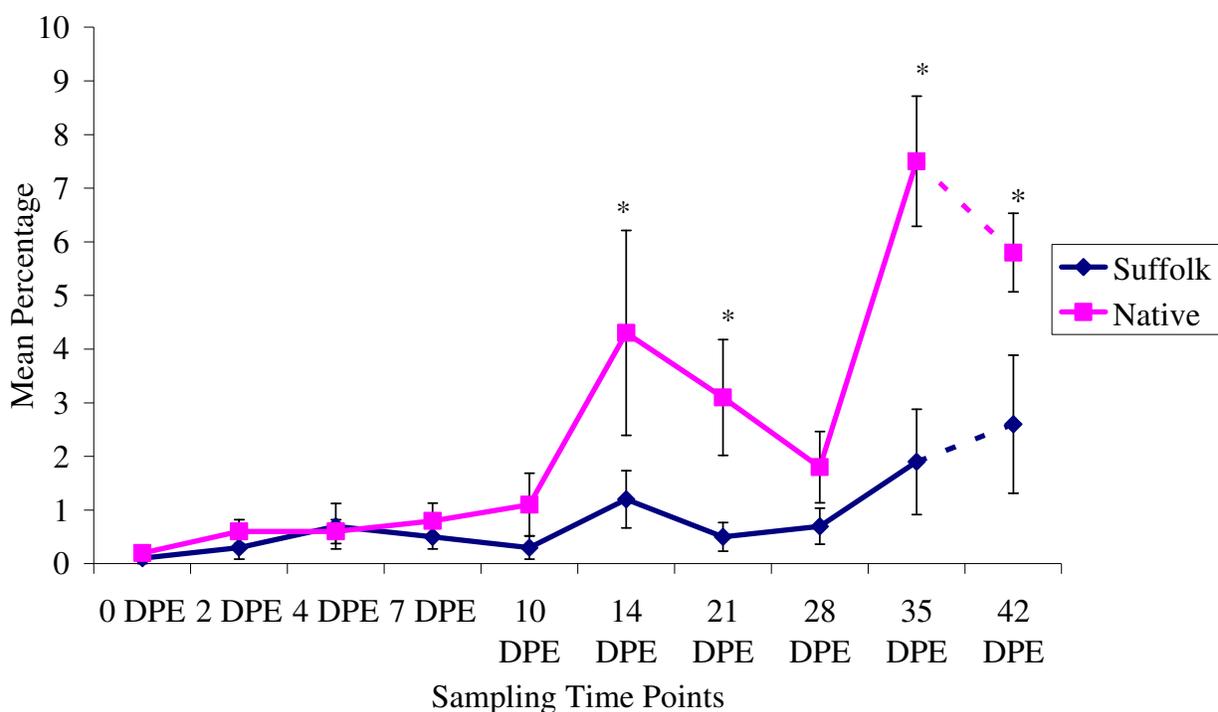
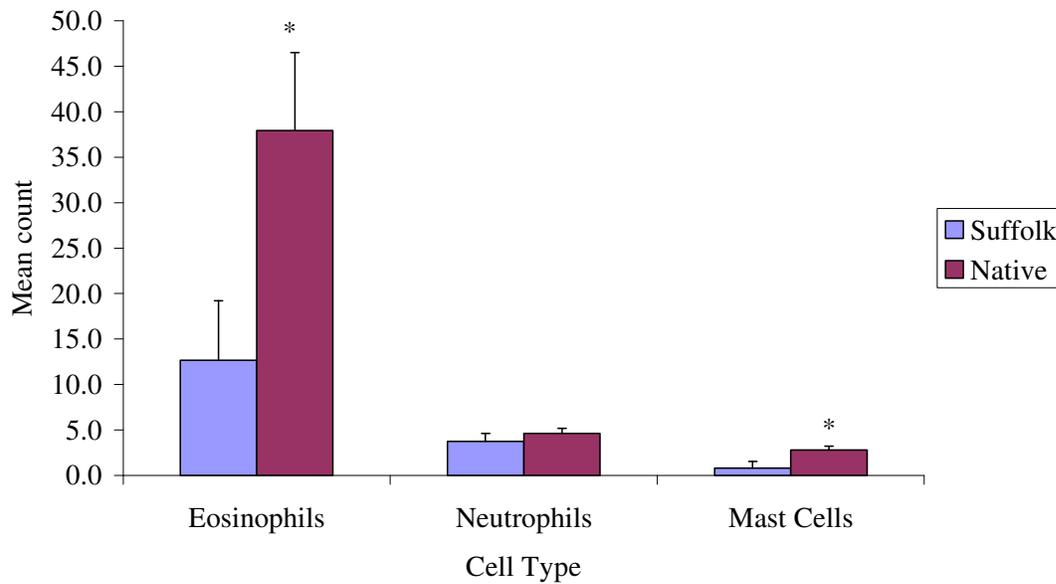


Figure 3.3. Mean peripheral eosinophil percentage and standard error of the mean in naturally infected Native and Suffolk lambs. Significant differences ( $p < 0.05$ ) are indicated by asterisk marks. Solid line indicates 10 lambs while dashed line indicates 5 lambs. (DPE=Days post exposure).

#### 3.3.4. Number of Eosinophils, Neutrophils and Mast Cells in the Cardiac Region of Abomasal Mucosa

The number of eosinophils and mast cells in the cardiac mucosa of the abomasum on day 35 post exposure was significantly ( $p < 0.05$ ) greater in Native compared to Suffolk lambs, whereas neutrophil number was not significantly different ( $p > 0.05$ ) (Figure 3.4.A). A similar trend with a similar magnitude in eosinophil and neutrophil count was observed ( $p < 0.05$  for both) on day 42 in both groups of lambs (Figure 3.4.B). However a greater elevation in mucosal mastocytosis was observed in both groups of lambs on day 42 of infection compared with day 35.

A. Cell Count in Cardiac Abomasum  
Day 35 Post Exposure



B. Cell Count Cardiac Abomasum  
Day 42 Post Exposure

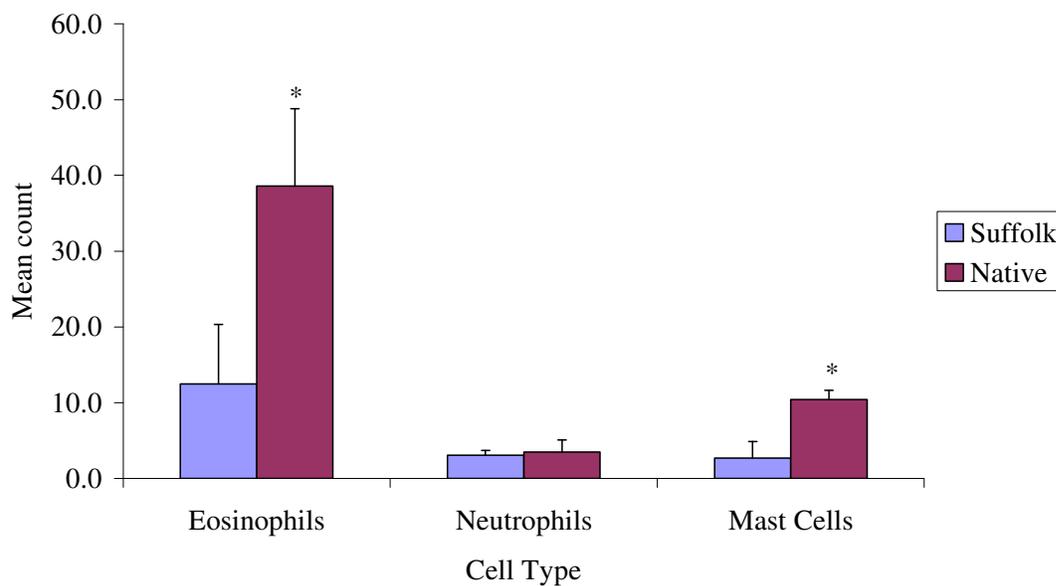


Figure 3.4. Mean eosinophil, mast cell and neutrophil count in the cardiac region of abomasal mucosa and standard error of the mean in naturally infected Native and Suffolk lambs. A) Day 35, and B) Day 42 post exposure. Significant differences ( $p < 0.05$ ) are indicated by asterisk marks. ( $n=5$ ).

### 3.3.5 Number of Eosinophils, Neutrophils and Mast Cells in the Fundic Region of Abomasal Mucosa

There were significantly ( $p < 0.05$ ) greater numbers of eosinophils, neutrophils and mast cells into the fundic abomasal mucosa in Native compared with Suffolk lambs on day 35 (Figure 3.5.). On day 42, however, only the mast cells were significantly ( $p < 0.05$ ) greater, but there was a trend for greater numbers of eosinophils and neutrophils (Figure 3.6.). As in the cardiac region, mast cell numbers were greater in both the group of lambs compared with their respective groups on day 35.

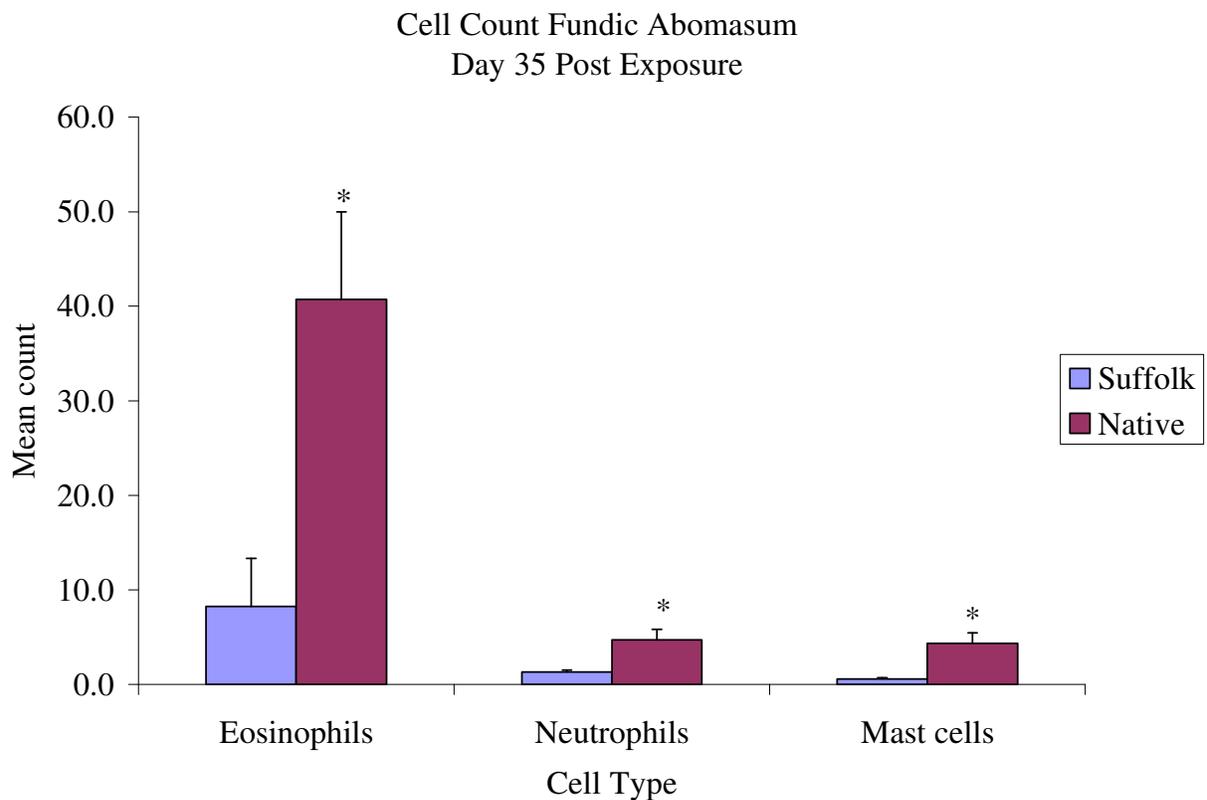


Figure 3.5. Mean eosinophil, neutrophil and mast cell count in the fundic region of abomasal mucosa and standard error of the mean in naturally infected Native and Suffolk lambs necropsied on day 35 post exposure. Significant differences ( $p < 0.05$ ) are indicated by asterisk marks. ( $n=5$ ).

Cell Count Fundic Abomasum  
Day 42 Post Exposure

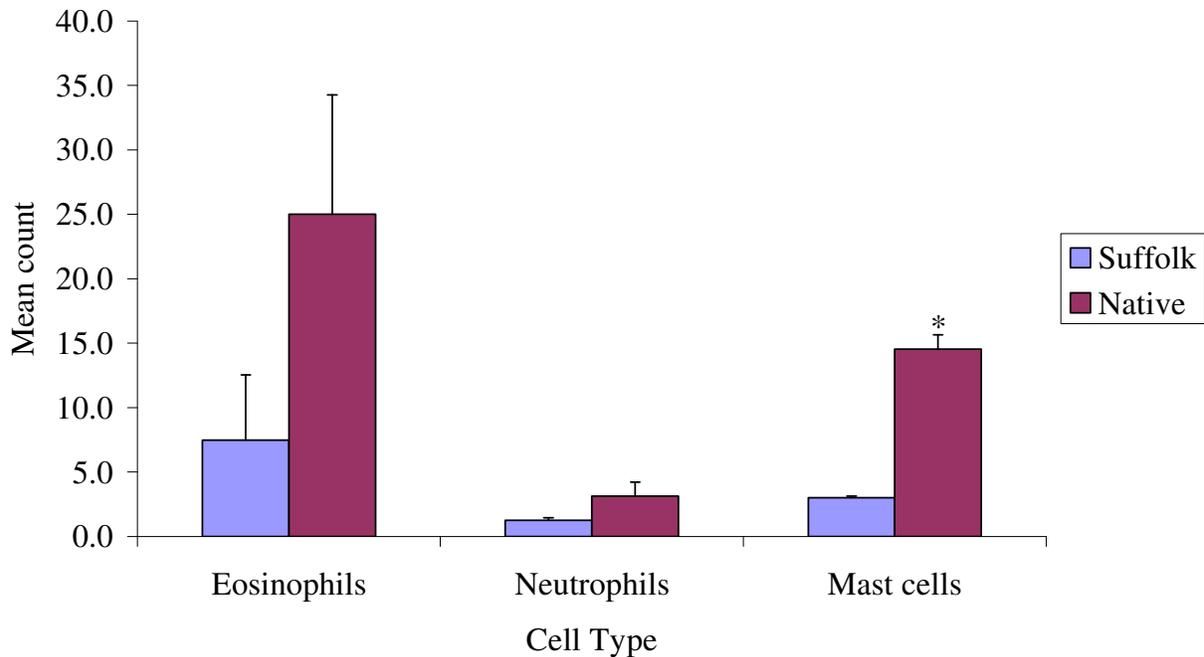
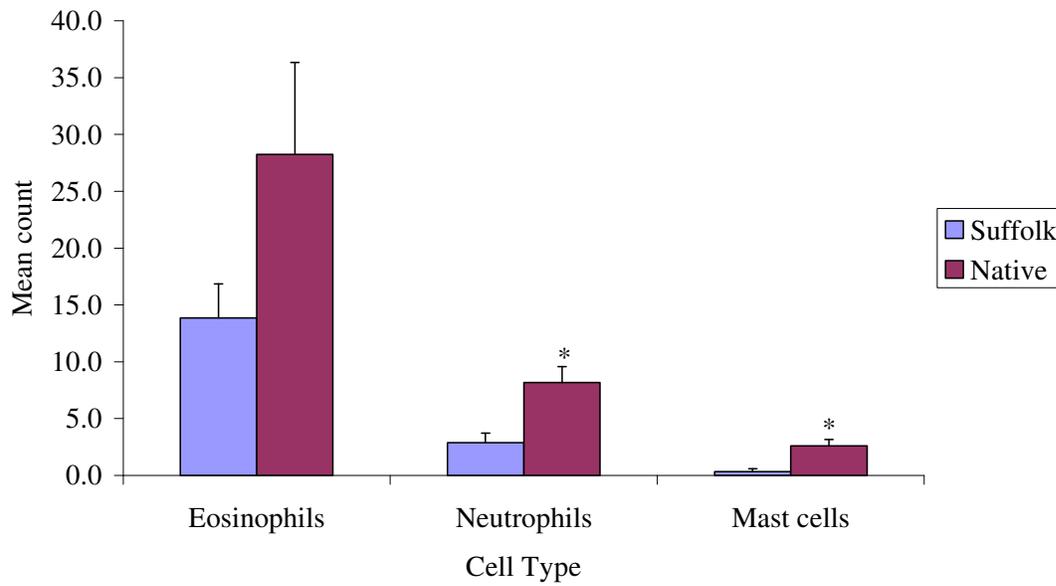


Figure 3.6. Mean eosinophil, mast cell and neutrophil count in the fundic region of abomasal mucosa and standard error of the mean in naturally infected Native and Suffolk lambs necropsied on day 42 post exposure. Significant difference ( $p < 0.05$ ) is indicated by an asterisk mark. ( $n = 5$ ).

**3.3.6. Number of Eosinophils, Neutrophils and Mast Cells in the Pyloric Region of Abomasal Mucosa**

A significantly ( $p < 0.05$ ) greater number of neutrophils and mast cells was observed in pyloric mucosa of the Native lambs compared with Suffolk lambs on day 35 post exposure (Figure 3.7.A). Eosinophil count, although not significant ( $p > 0.05$ ), demonstrated a tendency to be in greater numbers in Native lambs. Similarly, mucosal mast cell count was significantly ( $p < 0.05$ ) greater in pyloric mucosa of Native lambs compared with Suffolk lambs on day 42 post exposure while there was no difference ( $p > 0.05$ ) in neutrophil and eosinophil count although Native numbers were greater (Figure 3.7.B). As with the cardiac and fundic regions, mastocytosis on day 42 was greater than on day 35 post infection in both the groups of lambs.

A. Cell Count Pyloric Abomasum  
Day 35 Post Exposure



B. Cell Count Pyloric Abomasum  
Day 42 Post Exposure

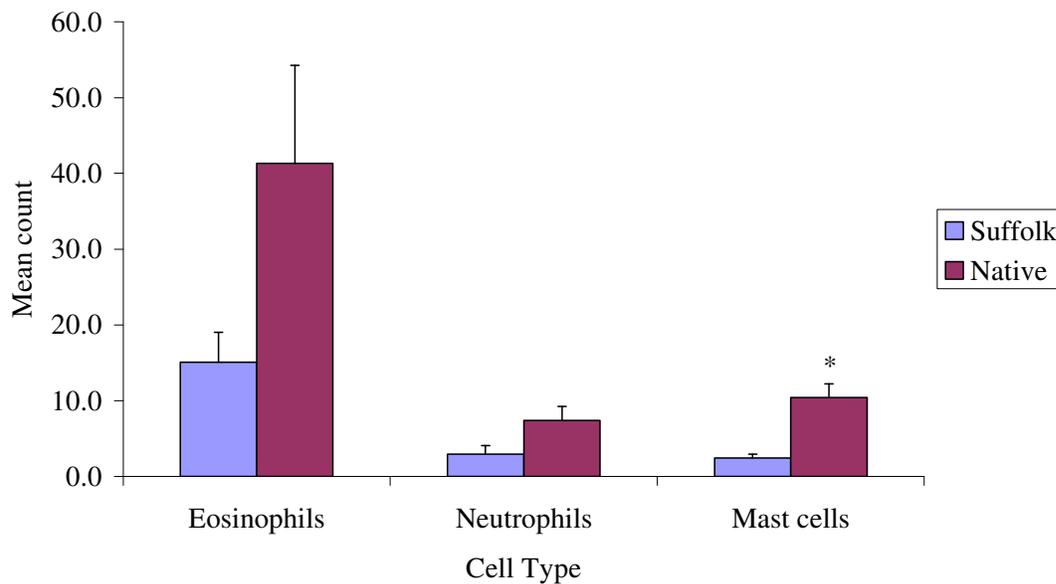


Figure 3.7. Mean eosinophil, mast cell and neutrophil count in the pyloric region of abomasal mucosa and standard error of the mean in naturally infected Native and Suffolk lambs. A) Day 35, and B) Day 42 post exposure. Significant differences ( $p < 0.05$ ) are indicated by asterisk marks. ( $n=5$ ).

### 3.3.7. Number of Globule Leukocytes in Abomasal Mucosa

The globule leukocyte count in the cardiac region of abomasal mucosa was significantly ( $p < 0.05$ ) greater in Native lambs on both days 35 and 42 post exposure compared with their corresponding Suffolk lambs (Figures 3.8. and 3.9.). Native lambs demonstrated only a trend for greater counts in the fundic region as the differences were not significant ( $p > 0.05$ ). However, total number of globule leukocytes in abomasum as a whole was significantly greater ( $p < 0.05$ ) in the Native group of lambs compared with Suffolk lambs. There were no globule leukocytes observed in the pyloric region on either sampling time points.

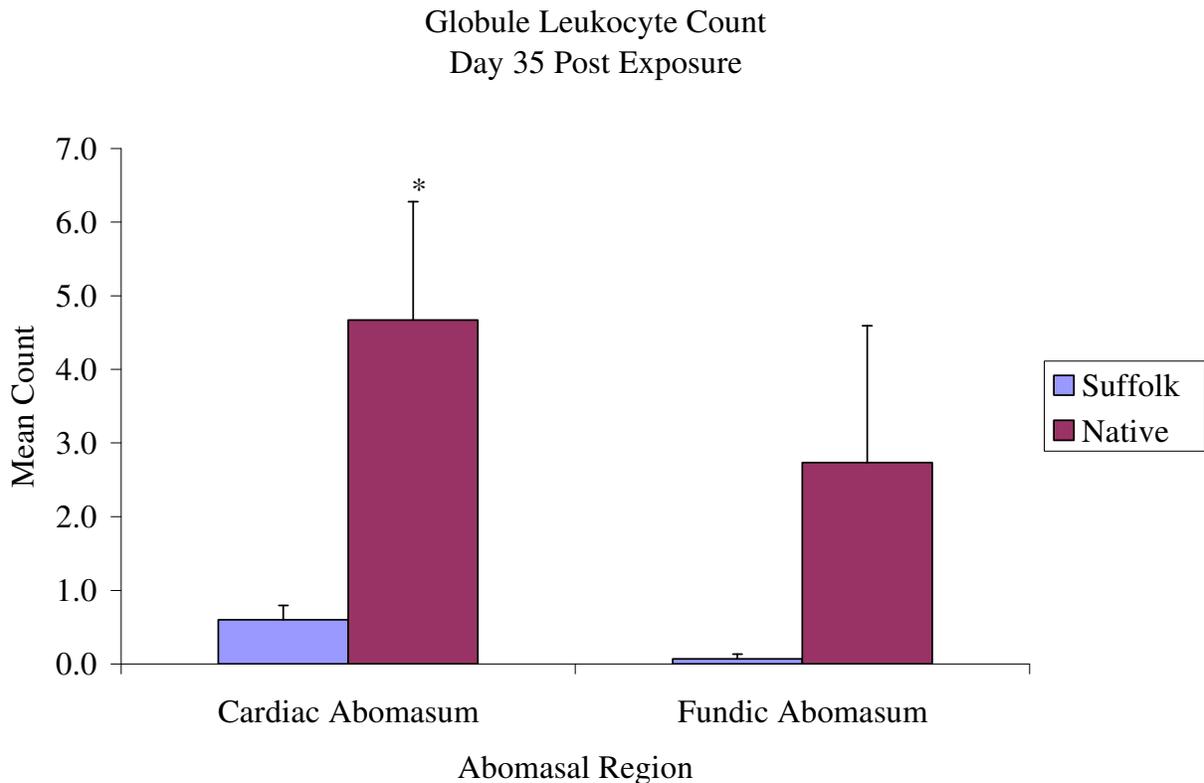


Figure 3.8. Mean globule leukocyte count in the cardiac and fundic regions of abomasal mucosa and standard error of the mean in naturally infected Native and Suffolk lambs necropsied on day 35 post exposure. Significant difference is indicated by an asterisk mark. (n=5).

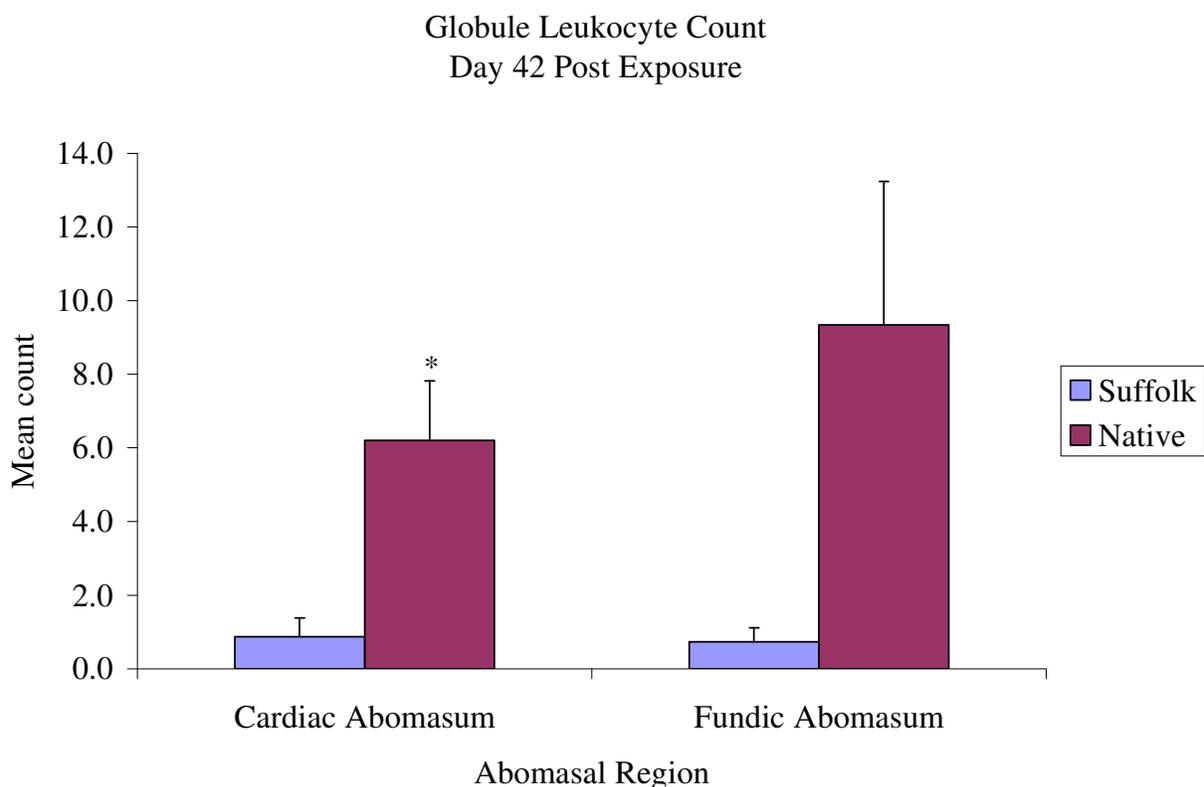


Figure 3.9. Mean globule leukocyte count in the cardiac and fundic regions of abomasal mucosa and standard error of the mean in naturally infected Native and Suffolk lambs necropsied on day 42 post exposure. Significant difference ( $p < 0.05$ ) is indicated by an asterisk mark. ( $n=5$ ).

### 3.3.8. *Haemonchus contortus* Recovery

*H. contortus* was the only species recovered from the abomasum (Figure 3.10.). About six fold greater numbers ( $p < 0.05$ ) of *H. contortus* were recovered in Suffolk compared to Native lambs on day 35 and over 4 fold greater ( $p < 0.05$ ) on day 42 post exposure. *Cooperia* and *Trichuris* spp. were the other two nematode parasites recovered. The mean number of *Cooperia* spp. recovered from small intestines was found to be 990 and 715 in Native and Suffolk lambs, respectively, on day 35. There were fewer recovered (250 and 240, respectively) on day 42 post exposure. The mean number of *Trichuris* spp. recovered from large intestine of Native lambs sacrificed on day 35 was 2 and from Suffolk sacrificed on 42 was 4.

### Mean *Haemonchus contortus* Recovery

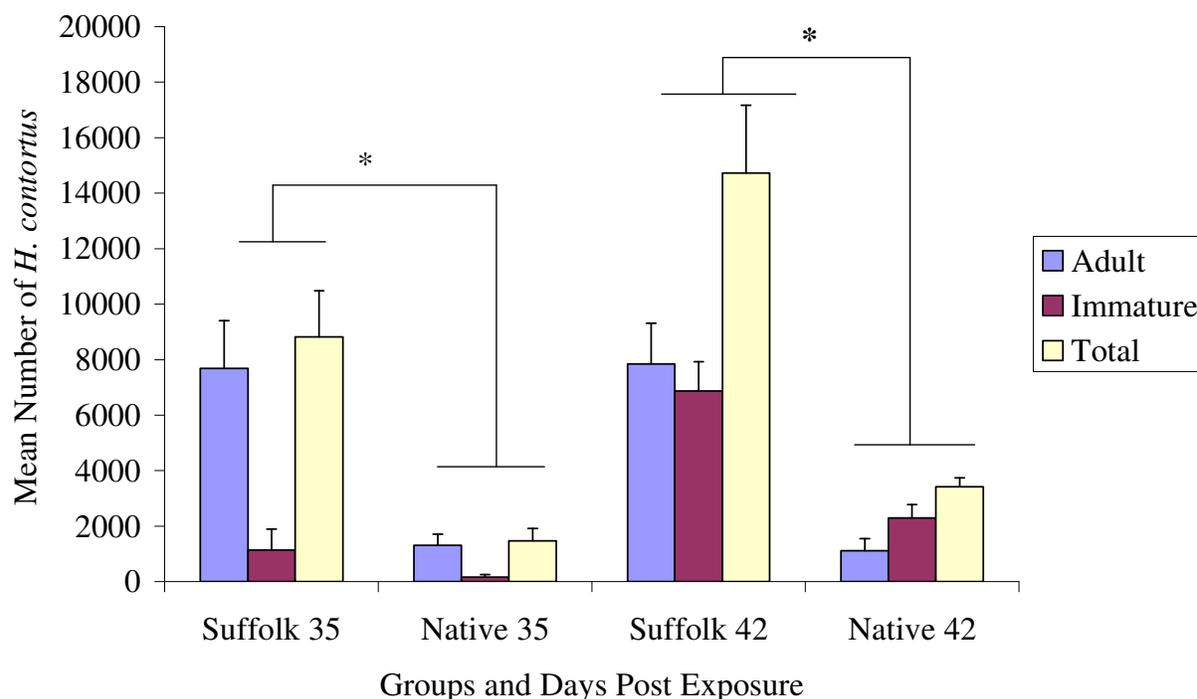


Figure 3.10. Mean number of *Haemonchus contortus* recovered from naturally infected Native and Suffolk lambs on days 35, and 42 post exposures. Significant differences ( $p<0.05$ ) are indicated by asterisk marks. ( $n=5$ ).

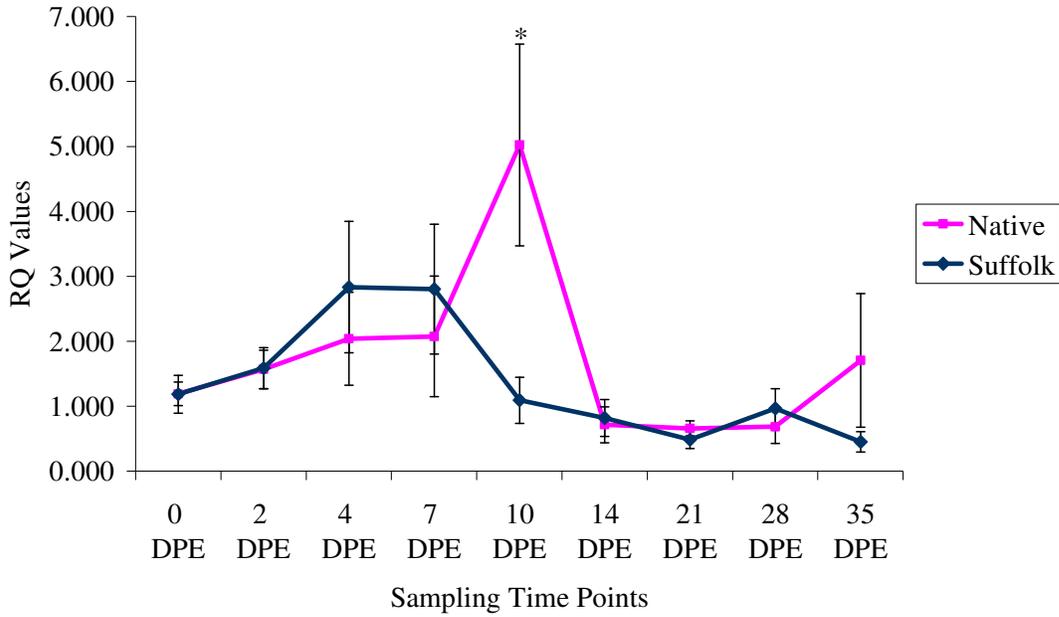
#### 3.3.9. IL-4 mRNA Expression

Native lambs demonstrated a significantly ( $p<0.05$ ) greater expression of IL-4 mRNA on day 10 post exposure (Figure 3.11.A). After day 14 post exposure, the expression remained similar for both the groups. However, there was a slight increase for Native lambs after day 28 post exposure while the Suffolk lambs decreased over the same period.

#### 3.3.10. IL-10 mRNA Expression

Suffolk lambs demonstrated a significantly ( $p<0.05$ ) greater expression of IL-10 on days 7, 10 and 14 post exposure compared with Native lambs and a tendency to remain higher throughout the experiment (Fig. 3.11.B.).

A. IL-4 mRNA Expression Level



B. IL-10 mRNA Expression Level

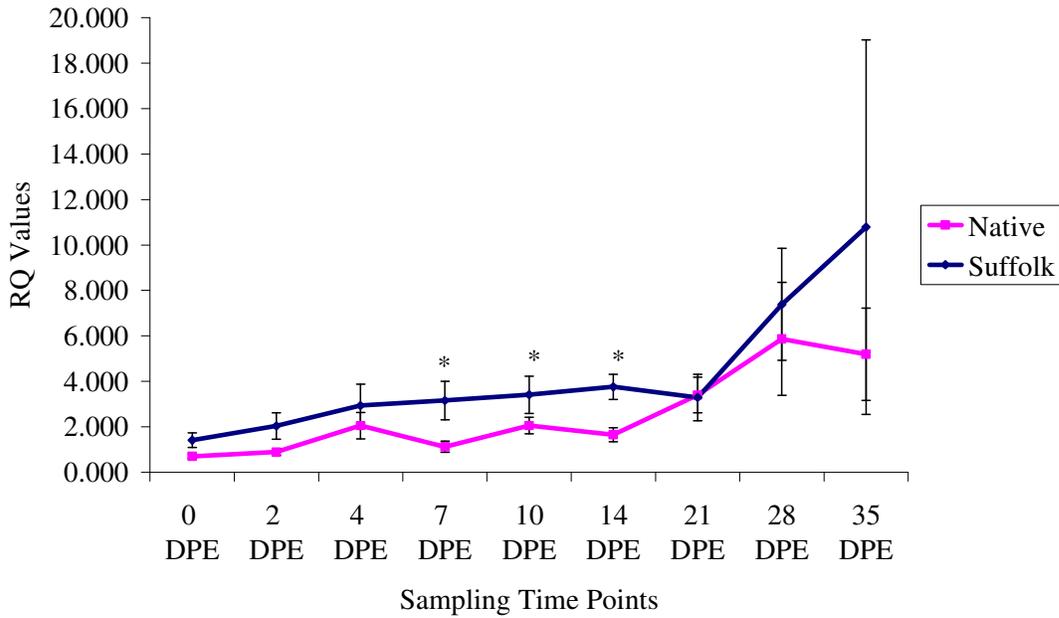


Figure 3.11. Mean relative quantitation (RQ) value and standard error of the mean in naturally infected Native and Suffolk lambs. A) IL-4 mRNA, and B) IL-10 mRNA. Significant differences are indicated by asterisk marks. (n=10).

### 3.3.11. IFN- $\gamma$ mRNA Expression

Suffolk lambs demonstrated a trend for greater expression of IFN- $\gamma$  mRNA compared with Native lambs however, there was no significant ( $p>0.05$ ) difference observed between the breeds (Figure 3.12). The expression level was highest on day 4 post exposure in both the breeds and decreased there after.

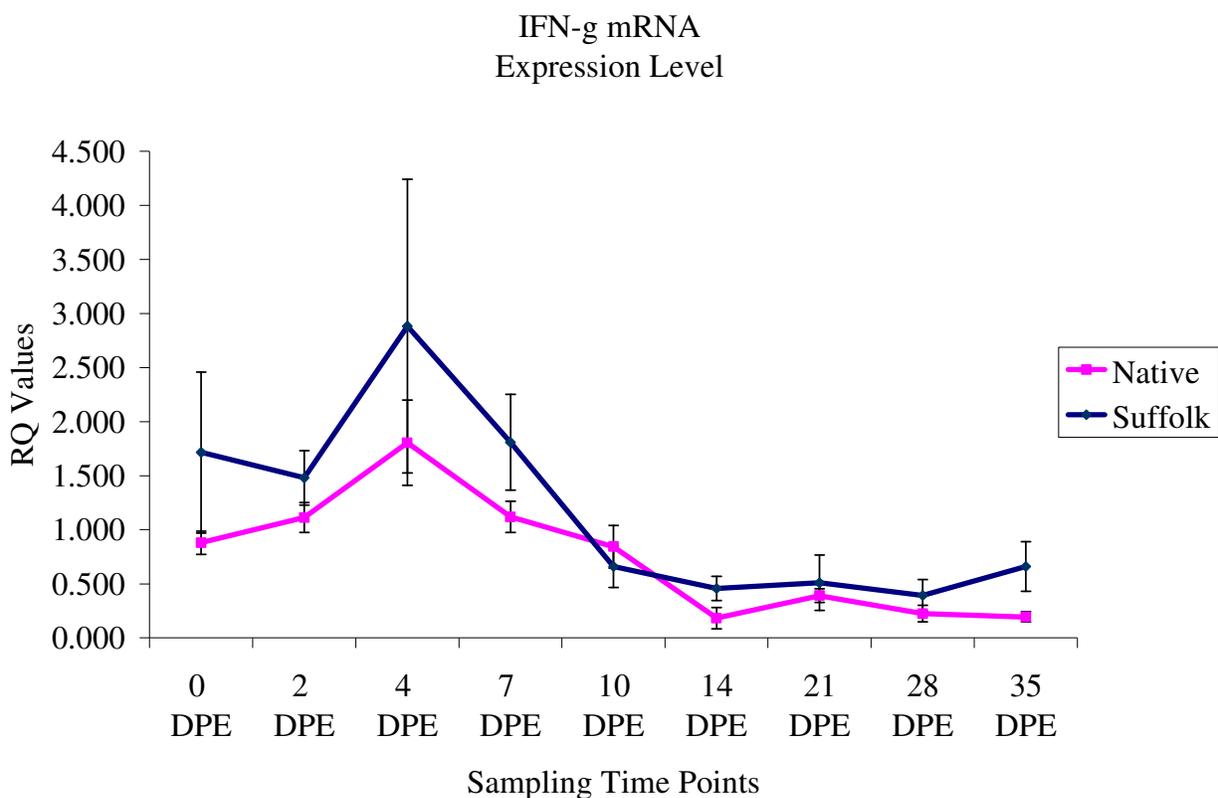


Figure 3.12. Mean relative quantitation (RQ) value for IFN- $\gamma$  mRNA expression and standard error of the mean in naturally infected Native and Suffolk lambs. (n=5).

### 3.3.12. Serum IgM Level

No significant difference ( $p>0.05$ ) in serum level of immunoglobulin isotype IgM was detected between the two breeds of lambs tested at any sampling time points post exposure (Figure 3.13). Neither was there any discernible trend observed.

## IgM ELISA

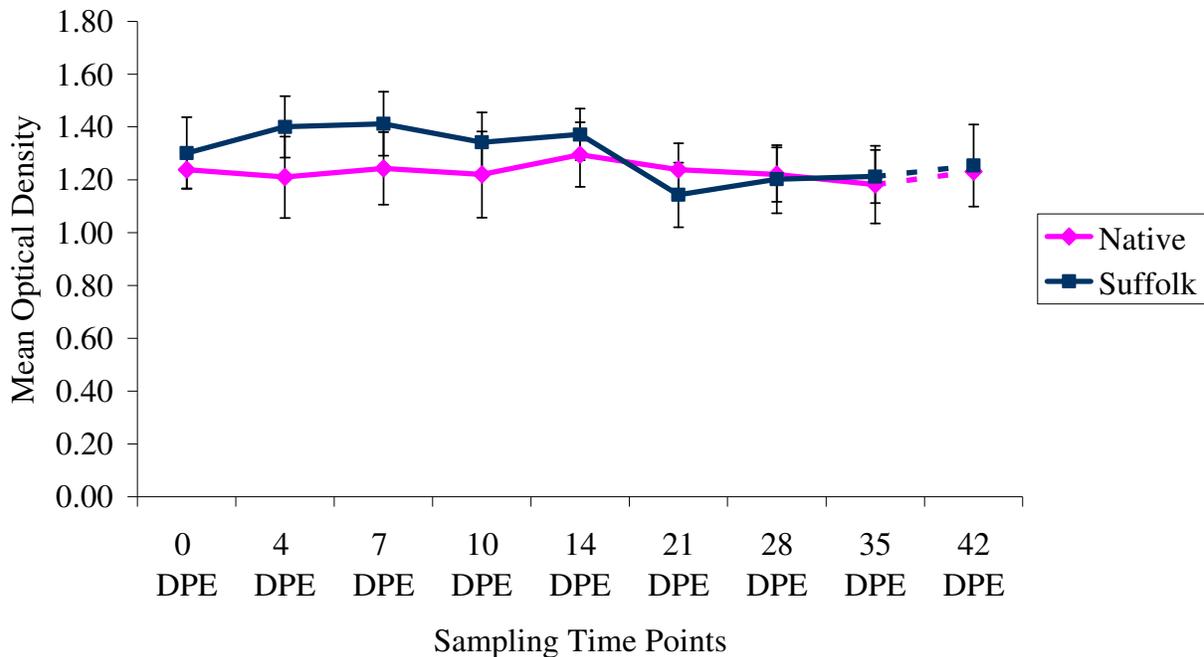


Figure 3.13. Mean optical densities representing the serum level of IgM against *H. contortus* whole worm antigen in naturally infected Native and Suffolk lambs. Solid line indicates 10 lambs and dashed line indicates 5 lambs. (DPE=Days post exposure).

### 3.3.13. Serum IgG1 Level

Serum levels of immunoglobulin isotype IgG1 are presented in Figure 3.14.A. Levels were similar ( $p>0.05$ ) through day 35 and on day 42 the level in Native lambs was significantly greater than Suffolk lambs. There was a trend for IgG1 to continually increase in native lambs and remain unchanged in Suffolk lambs.

### 3.3.14. Serum IgG2 Level

Serum levels of immunoglobulin isotype IgG2 are presented in Figure 3.14.B. There was no significant ( $p>0.05$ ) difference between breeds throughout the experiment. However, there was a trend for Native lambs to be greater than Suffolk lambs except after day 35.

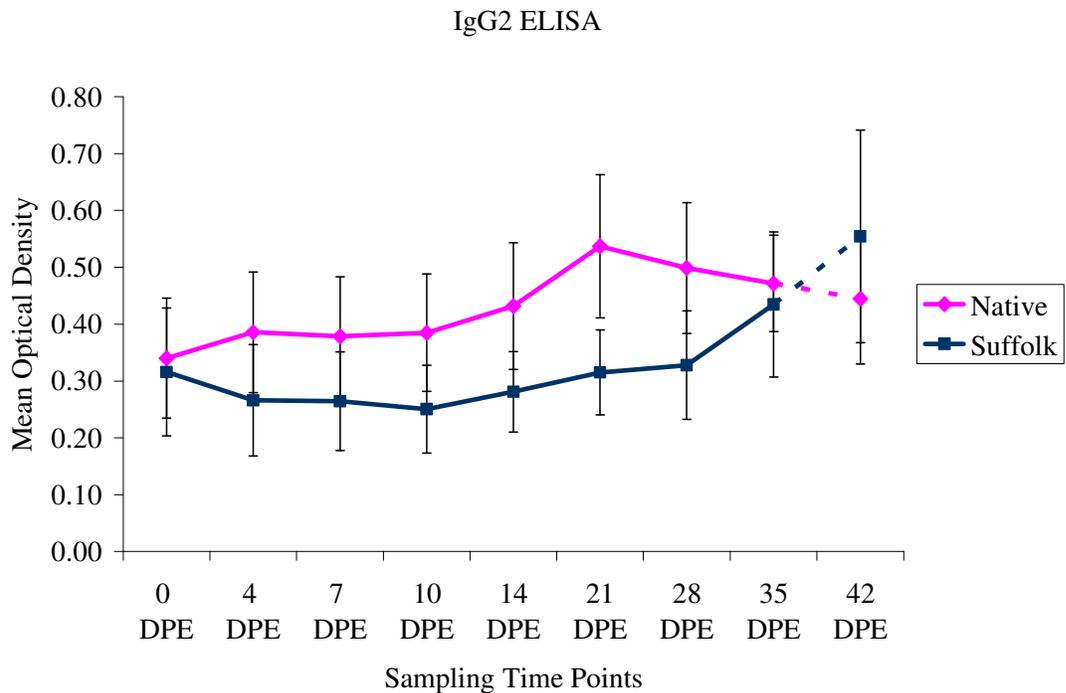
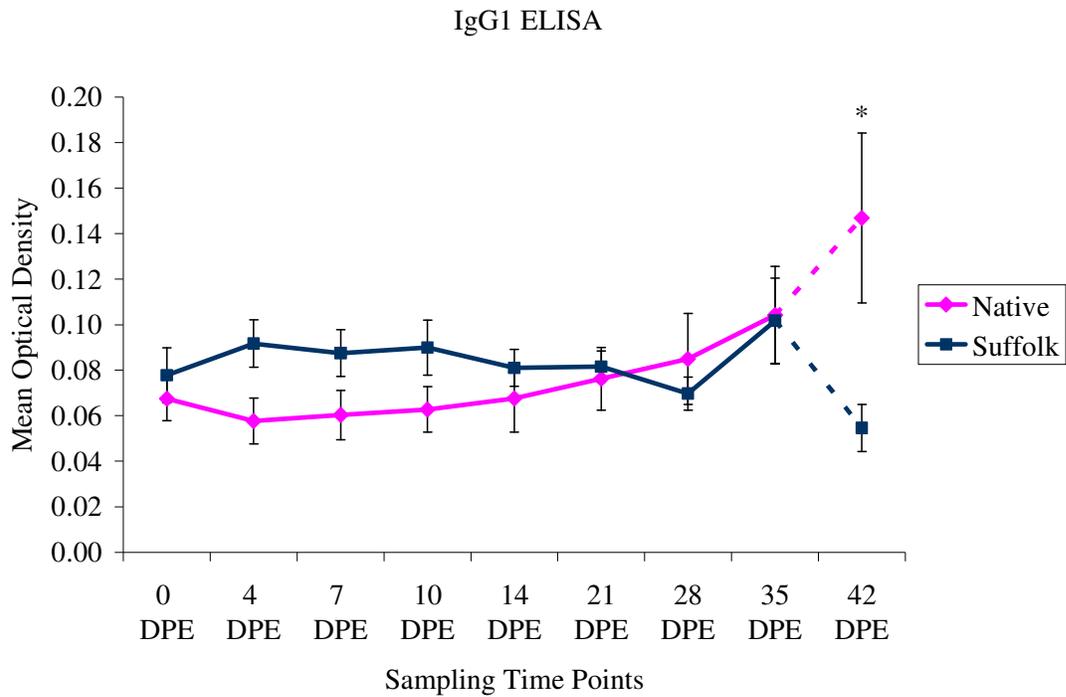


Figure 3.14. Mean optical densities representing the serum level A) IgG1 and B) IgG2 against *H. contortus* whole worm antigen in naturally infected Native and Suffolk lambs. Significant difference ( $p < 0.05$ ) is indicated by an asterisk mark. Solid line indicates 10 lambs while dashed line indicates 5 lambs. (DPE=Days post exposure).

### 3.3.15. Serum IgE Level

Serum level of immunoglobulin isotype IgE was similar ( $p>0.05$ ) through day 10 (Figure 3.15). Subsequently, the level was significantly ( $p<0.05$ ) greater in Native lambs. IgE level in Suffolk lambs remained around the base level through out the experiment.

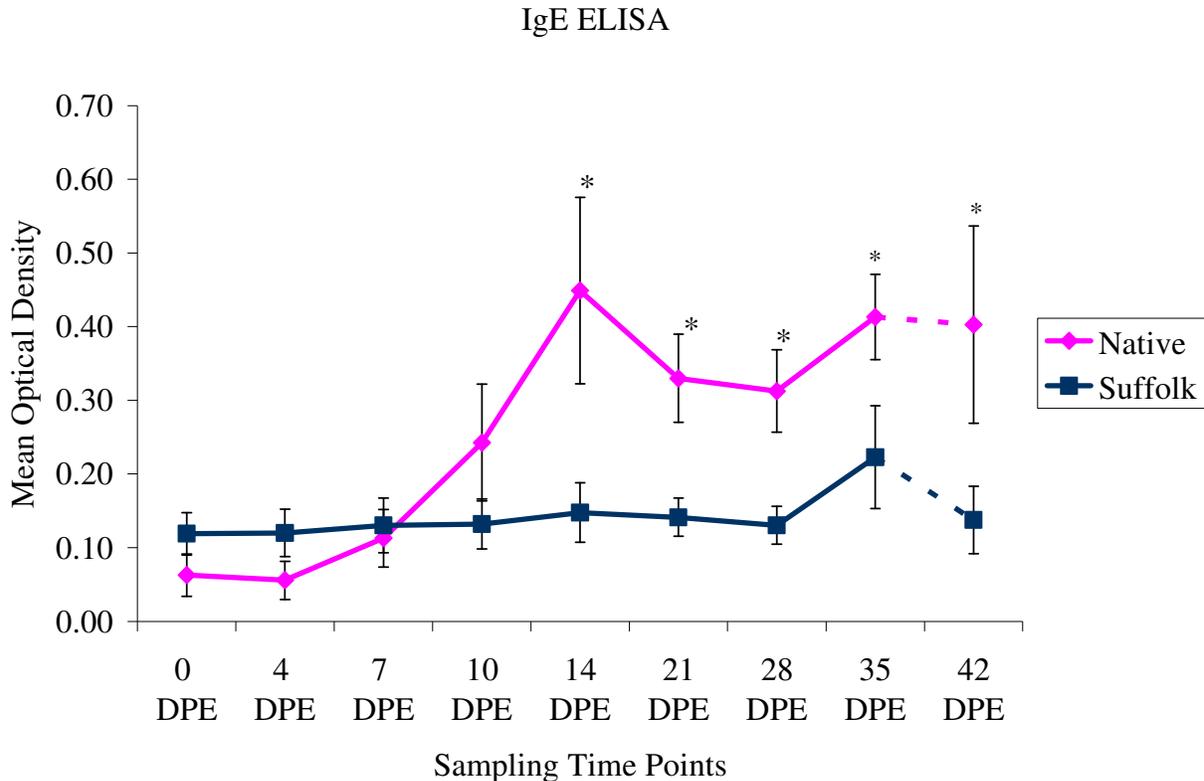


Figure 3.15. Mean optical density representing serum level of immunoglobulin isotype IgE in naturally infected Native and Suffolk lambs. Significant ( $p<0.05$ ) differences are indicated by an asterisk mark. Solid line indicates 10 lambs while broken line indicates 5 lambs. (DPE=Days post exposure).

### 3.4. Discussion

Trichostrongyle type eggs were observed in the fecal samples of both the breeds starting 3 weeks after the lambs were put out to pasture. The FEC increased rapidly with the progression of the infection in Suffolk lambs and peaked on the 5<sup>th</sup> week. In Native lambs, the maximum FEC was recorded on the 4<sup>th</sup> week. At the peak count, the Suffolk lambs demonstrated over three fold greater FEC compared with Native lambs. This is in agreement with previously results

comparing Native and Suffolk sheep (Bahirathan et al., 1996; Miller et al., 1998). Similar to FEC, the PCV reduction percentage was not different for the first 3 weeks, after which Suffolk lambs demonstrated a significantly higher PCV reduction percentage. The decrease in PCV percentage reduction coincided with the decrease in FEC of Native lambs after week 4 and reached near normal range. This is an indication that Native lambs infected with *H. contortus* were able to recover rather quickly from the anemia induced by the worms that were expelled during the course of infection. On the other hand, Suffolk lamb PCV reduction percentage remained high. It appears that the PCV reduction is directly associated with number of *H. contortus* parasites present in the abomasum. *H. contortus* start feeding on blood with the L4 stage and it is estimated that each parasite consumes about 0.5 ml of blood every day. Therefore, greater numbers of parasites in Suffolk lambs resulted in a greater amount of blood loss in comparison to Native lambs. The fewer worms in the Native lambs whether due to reduced establishment or elimination of established worms is not clear. However, the equivalent blood PCV reduction percentages in both the groups during initial 2 weeks of the exposure may be an indication of the equivalent level of initial infection in these two breeds. The difference after week 3 period may be attributable to the difference in establishment or elimination of established parasite resulting in the difference in parasite burden observed between the two groups.

The peripheral eosinophil count increased in Native lambs where as Suffolk lambs remained around the baseline. This characteristic has been considered to be a potential marker for resistant individuals in Australian lines of Merino and Romney sheep for *H. contortus* and *Trichostrongylus colubriformis*, however high variability among the individuals makes it less preferential (Douch et al., 1996). Likewise, tissue eosinophilia has been reported in immunized lambs as well as in the resistant lines or breeds of sheep (Gill, 1991; Gill et al., 2000; Woolaston et al., 1996; Hohenhaus et al., 1998; Hooda et al., 1999). The results of these experiments,

demonstrated a consistent tissue eosinophilia associated with infection. Both breeds demonstrated abomasal eosinophilia with the Native lambs having greater numbers than Suffolk lambs. Balic et al., (2002) reported tissue eosinophilia to occur as early as 3 days post exposure in secondary infections. In another study, eosinophil density was found to be significantly higher in abomasal mucosa from resistant lambs when compared with random bred lambs on day 28 post infection (Gill et al., 2000). The experimental design of the present study did not permit the observation and enumeration of eosinophils in abomasal mucosa during infection, however, the results suggest tissue eosinophilia ensued after the exposure and continued through day 42 post exposure. Furthermore, eosinophils have been reported surrounding gastrointestinal larvae in the tissue (Balic et al., 2006; Rainbird et al., 1998). In another *in vitro* study, eosinophils extracted from mammary washes of repeatedly infected sheep were shown to hinder mobility and kill *H. contortus* L3s in the presence of anti-L3 surface antibody (Rainbird et al., 1998). Eosinophils possess FcεRI which is a high affinity receptor for IgE. IgE is known to be involved in antibody dependent cell-mediated cytotoxicity (Abbas and Lichtman, 2005a). Eosinophils carry major basic protein, eosinophil cationic protein, eosinophil peroxidase, lysosomal hydrolases, and lysophospholipase in their cytoplasmic granules. These proteins are capable of inflicting damage to larval surface and are toxic to helminth parasites (Abbas and Lichtman, 2005b). Sensitized eosinophils, upon exposure to *H. contortus* antigen, degranulate and inflict damage through the proteins and mediators released. Therefore, greater number of eosinophils and increased IgE may be two of the components involved with greater anti-*Haemonchus* resistance in Native lambs.

Enumeration of mucosal mast cells (MMC) in all three regions of abomasal mucosa demonstrated a consistently greater number in Native compared with Suffolk lambs. Variation in numbers among the different regions may be due to the preferential attachment of the parasites as well as responsiveness of that particular region. *H. contortus* worms have been observed to

attach mostly in the pyloric region (Dr. Miller, personal communication). Why *H. contortus* parasites prefer this particular region for attachment is not understood. Gill et al., (2000) reported greater MMC density 28 days post infection in resistant compared with random bred lambs. Similarly, Lacroux et al. (2006) found an earlier presence of MMC in secondarily infected lambs compared with naïve lambs infected with *H. contortus*. MMC count in the abomasal mucosa was found to be negatively correlated with *H. contortus* (Amarante et al., 1999a). The results from resistant sheep conform to the results obtained from Native lambs in this study. Globule leukocytes were recorded only in the cardiac and fundic regions of abomasa of infected lambs of both breeds with Native lambs having demonstrated significantly greater numbers. This is in agreement with the findings of other work involving *H. contortus* (Amarante et al., 1999a; Lacroux et al., 2006) and in *Oesophagostomum columbianum* (Dobson, 1966) infection in sheep. However, specific location of these cells was not reported in any particular region of abomasum. Huntley et al., (1992) described globule leukocytes associated with resistance to *H. contortus*. Furthermore, it was also reported that sheep MMC and globule leukocytes contained sheep mast cell protease (SMCP). Tissue concentration of SMCP is directly correlated with the number of MMC present and inversely related to the *Trichostrongylus vitrinus* and *Teladorsagia circumcincta* population present (Huntley et al., 1995). MMCs carry FCεRI, a high affinity receptor for IgE and most of the IgE produced is cell bound. Sensitized MMC when exposed to antigen degranulate and release the mediators including SMCP in the mucosal tissue and abomasal mucus of the infected sheep (Huntley et al., 1992). MMC are also regarded as responsible for the leakiness of the epithelium by causing degradation of occludin leading to increased epithelial permeability (McDermott et al., 2003). Increased permeability may lead to increased dissemination of the cells and cellular products in the abomasal lumen resulting in an altered abomasal environment which in turn may be unsuitable for parasite survival.

Additionally, the substances released from MMC and globule leukocytes that come in contact with the parasites may cause damage to the parasites and eventually lead to their expulsion.

Neutrophils are known to be present in late phase reaction of immediate hypersensitivity (Abbas and Lichtman, 2005c) although their function is not well understood. They were present in the abomasal mucosa on 35 and 42 day post exposure.

In this experiment, serum IgA could not be determined due to very high nonspecific reactivity of anti-sheep IgA monoclonal antibodies. Results indicated that there was no overall difference in serum IgM, and IgG2 tended to be greater in Suffolk lambs through day 28 post exposure. IgG1 was only significantly greater on day 42 post exposure in Native lambs. The most evident immunoglobulin response was IgE where Native lambs demonstrated a clear superior response compared with Suffolk lambs. This concurs with that of Kooyman et al. (1997) where increased level of IgE, specific to *H. contortus*, was reported. Increased level of IgG1 was reported to be followed by IgG2, IgM and IgA for *H. contortus* infection in sheep (Schallig et al., 1995). Similarly, resistant lines of sheep were found to produce greater levels of IgE and IgG1 when compared with random bred lambs (Gill et al., 2000). On the contrary, these isotypes have been reported to be not significantly correlated with resistance against *H. contortus* (Gomez-Munoz et al., 1999). Lacroux et al., (2006) reported induction of IgA production in secondarily challenged animals by day 7 and 15 in mucus while serum IgG and IgA were detected on 15 and 28 days in response to *H. contortus* ES products. While in *T. colubriformis* infection in resistant lambs, a significantly higher level of IgG1 and IgM were found (Bisset et al., 1996). The effector functions of MMC can be brought about in the presence of IgE (Gurish et al., 2004).

In the present experiment, expression of three cytokine mRNAs, IFN- $\gamma$ , IL-4 and IL-10 represented Th1, Th2 regulatory T cell response. IL-4 is the signature cytokine for the Th2 response and it is mainly responsible for isotype switching (Matsuda et al., 1995). Up-regulation

of this cytokine in *H. contortus* infections in sheep has been attributed to increased IgE specific to *H. contortus* (Kooyman et al., 1997). The relative IL-4 mRNA expression suggested an early induction in Suffolk lambs that diminish after day 35 of the experiment. On the other hand, Native lambs had a significantly greater production at day 10 post exposure which also diminished but increased again towards the end of the experiment. The initial IL-4 expression preceded the first peak of peripheral eosinophils in Native lambs and there is an increasing tendency preceding the second peak. In addition, peak serum IgE occurred after the peak production of IL-4 mRNA. Since IL-4 is a signature cytokine for a Th2 type response (Matsuda et al., 1995) and is associated with isotype switching, upregulation of IgE production may be attributed to IL-4 (Kooyman et al., 1997).

There was no difference in the Th1 type cytokine IFN- $\gamma$  mRNA expression; however Suffolk lambs demonstrated greater expression during the first few days. By day 10, expression decreased and remained around the base level through the rest of the experiment. The expression of mRNA encoding IL-10, a regulatory cytokine, consistently increased from the beginning of the experiment and at times it was significantly greater in Suffolk lambs. Expression increased substantially in Suffolk lambs after day 21 of the infection. The peak in IL-4 mRNA expression in the Native lambs coincided with the decreased level of IFN- $\gamma$  mRNA on day 10. Similarly, when IL-4 mRNA expression in Suffolk lambs was lowest, IL-10 expression had increased substantially.

It has been reported that abomasal and mesenteric lymph nodes from *H. contortus* infected lambs drained cells that had lower expression of IFN- $\gamma$  compared to uninfected lambs regardless of resistance status while the same line of cells induced an increased expression of IL-5 (Gill et al., 2000). In another study, IL-4, IL-13 and IL-5 expression increased three days after challenge, but each with a different pattern. IL-4 expression peaked 5 days post exposure and

remained elevated throughout the challenge period which indicated a role in early or rapid expulsion, while IL-5 and IL-13 expression increased later which indicated a role in the later stages of infection (Meeusen et al., 2005). In *T. spiralis* and *N. brasiliensis* infections in mice, IFN- $\gamma$  expression switched to a Th2 type response later in infection (Ishikawa et al., 1998). Similarly, a *T. colubriformis* resistant line of sheep also expressed IL-5 and IL-13 at greater levels compared to a susceptible line (Pernthaner et al., 2005). However, IFN- $\gamma$ , IL-4 and IL-10 expression was not different between the two lines.

The low expression of IL-4 in both the groups of lambs during third and fourth week of infection may be due to down regulation by the regulatory cytokine IL-10 as it is known to suppress both Th1 and Th2 mediated inflammatory reactions (Helmbj and Grecis, 2003a). Down regulation might have resulted in establishment of new incoming larvae that were recovered in greater number on day 42. The depression in expression of IL-4 in both groups indicated that resistant animals are also vulnerable, but they may have some other mechanism to overcome the down regulatory effect. It may be the result of immune modulatory components that are secreted by this parasite. Calreticulin was found to enhance coagulation time and modulate the immune response by binding with C1q (Suchitra and Joshi, 2005) and another 66 kDa adult *H. contortus* ES product which is known to inhibit monocytes (Rathore et al., 2006). The 66 kDa antigen was not found in L3 which indicated adult worms capable of modulate the immune response. The greater expression of IFN- $\gamma$  during early infection, especially the peak on day 4 may have been in response to the invasion of larvae in the tissue and the subsequent reduction may have been a result of the shift towards induction of a Th2 type response accompanied by increased IL-4 expression. The constant greater expression IL-10 may account for the reduced expression of IL-4 and IFN- $\gamma$  and failure to induce production of IgE in Suffolk lambs. The increased expression of IL-10 around day 28 in Suffolk lambs may have accounted

for the decreasing trend observed in IgG1 expression and reduced numbers of eosinophils, mast cells, eosinophils and globule leukocytes in the abomasa and peripheral eosinophilia. On the contrary, the reduced expression of IL-10 in Native lambs may not have been sufficient to down regulate the production of immunoglobulins and cellular response.

*H. contortus* recovery was about 6 and 4 times greater in Suffolk lambs compared with Native lambs on days 35 and 42 post exposure respectively. The immunological responses observed may account for this difference at least in part. The number of immature worms that increased after 35 day may have been an indication of breach in immunity as the peripheral eosinophilia declined during this period.

### **3.5. Conclusions**

Taken together, the induction of a Th2 type of immune response characterized by significantly greater eosinophil, mast cell and globule leukocyte numbers in abomasal mucosa accompanied by increased production of IgE in Native lambs may be the components of the immune response responsible for the reduced establishment and or survival of established *H. contortus* in Native lambs. The relative lack of immune response components, especially IgE in Suffolk lambs is not clearly understood as whether these animals constitutively lack the ability to mount a response or if a response may be temporarily suppressed due to parasite products.

The peripheral eosinophilia and increased serum IgE were associated with the reduced infection in Native lambs. Hence, these might be considered for use as markers while selecting individuals for greater resistance to *H. contortus*.

### **3.6. References**

Abbas, A.K., Lichtman, A.H., 2005a, Cellular and Molecular Immunology, Fifth Edition, Elsevier Saunders, 443 p.

Abbas, A.K., Lichtman, A.H., 2005b, Cellular and Molecular Immunology, Fifth Edition. Elsevier Saunders, 447 p.

- Abbas, A.K., Lichtman, A.H., 2005c, Cellular and Molecular Immunology, Fifth Edition. Elsevier Saunders, 432-447 pp.
- Amarante, A.F., Craig, T.M., Ramsey, W.S., Davis, S.K., Bazer, F.W., 1999a, Nematode burdens and cellular responses in the abomasal mucosa and blood of Florida Native, Rambouillet and crossbreed lambs. *Vet Parasitol* 80, 311-324.
- Bahirathan, M., Miller, J.E., Barras, S.R., Kearney, M.T., 1996, Susceptibility of Suffolk and Gulf Coast Native suckling lambs to naturally acquired strongylate nematode infection. *Vet Parasitol* 65, 259-268.
- Balic, A., Bowles, V.M., Meeusen, E.N., 2002, Mechanisms of immunity to *Haemonchus contortus* infection in sheep. *Parasite Immunol* 24, 39-46.
- Balic, A., Cunningham, C.P., Meeusen, E.N., 2006, Eosinophil interactions with *Haemonchus contortus* larvae in the ovine gastrointestinal tract. *Parasite Immunol* 28, 107-115.
- Bisset, S.A., Vlassoff, A., Douch, P.G., Jonas, W.E., West, C.J., Green, R.S., 1996, Nematode burdens and immunological responses following natural challenge in Romney lambs selectively bred for low or high faecal worm egg count. *Vet Parasitol* 61, 249-263.
- Budhia, S., Haring, L.F., McConnell, I., Blacklaws, B.A., 2006, Quantitation of ovine cytokine mRNA by real-time RT-PCR. *J Immunol Methods* 309, 160-172.
- Cliffe, L.J., Humphreys, N.E., Lane, T.E., Potten, C.S., Booth, C., Grecis, R.K., 2005, Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. *Science* 308, 1463-1465.
- Davidson, W.R., McGhee, M.B., Nettles, V.F., Chappell, L.C., 1980, Haemonchosis in white-tailed deer in the southeastern United States. *J Wildl Dis* 16, 499-508.
- Dobson, C., 1966, Studies on the immunity of sheep to *Oesophagostomum columbianum*: the nature and fate of the globule leukocyte. *Aust. J. Agric. Res* 17, 955-966.
- Doligalska, M., Rzepecka, J., Drela, N., Donskow, K., Gerwel-Wronka, M., 2006, The role of TGF-beta in mice infected with *Heligmosomoides polygyrus*. *Parasite Immunol* 28, 387-395.
- Douch, P.G., Green, R.S., Morris, C.A., McEewan, J.C., Windon, R.G., 1996, Phenotypic markers for selection of nematode-resistant sheep. *Int J Parasitol* 26, 899-911.
- Finkelman, F.D., Shea-Donohue, T., Morris, S.C., Gildea, L., Strait, R., Madden, K.B., Schopf, L., Urban, J.F., Jr., 2004, Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunol Rev* 201, 139-155.
- Garside, P., Kennedy, M.W., Wakelin, D., Lawrence, C.E., 2000, Immunopathology of intestinal helminth infection. *Parasite Immunol* 22, 605-612.

- Gill, H.S., 1991, Genetic control of acquired resistance to haemonchosis in Merino lambs. *Parasite Immunol* 13, 617-628.
- Gill, H.S., Altmann, K., Cross, M.L., Husband, A.J., 2000, Induction of T helper 1- and T helper 2-type immune responses during *Haemonchus contortus* infection in sheep. *Immunology* 99, 458-463.
- Gomez-Munoz, M.T., Cuquerella, M., Gomez-Iglesias, L.A., Mendez, S., Fernandez-Perez, F.J., de la Fuente, C., Alunda, J.M., 1999, Serum antibody response of Castellana sheep to *Haemonchus contortus* infection and challenge: relationship to abomasal worm burdens. *Vet Parasitol* 81, 281-293.
- Gurish, M.F., Bryce, P.J., Tao, H., Kisselgof, A.B., Thornton, E.M., Miller, H.R., Friend, D.S., Oettgen, H.C., 2004, IgE enhances parasite clearance and regulates mast cell responses in mice infected with *Trichinella spiralis*. *J Immunol* 172, 1139-1145.
- Hein, W.R., Barber, T., Cole, S.A., Morrison, L., Pernthaner, A., 2004, Long-term collection and characterization of afferent lymph from the ovine small intestine. *J Immunol Methods* 293, 153-168.
- Helmbly, H., Grencis, R.K., 2003a, Contrasting roles for IL-10 in protective immunity to different life cycle stages of intestinal nematode parasites. *Eur J Immunol* 33, 2382-2390.
- Helmbly, H., Grencis, R.K., 2003b, IFN-gamma-independent effects of IL-12 during intestinal nematode infection. *J Immunol* 171, 3691-3696.
- Hohenhaus, M.A., Josey, M.J., Dobson, C., Outteridge, P.M., 1998, The eosinophil leucocyte, a phenotypic marker of resistance to nematode parasites, is associated with calm behaviour in sheep. *Immunol Cell Biol* 76, 153-158.
- Hooda, V., Yadav, C.L., Chaudhri, S.S., Rajpurohit, B.S., 1999, Variation in resistance to haemonchosis: selection of female sheep resistant to *Haemonchus contortus*. *J Helminthol* 73, 137-142.
- Horohov, D.W., Beadle, R.E., Mouch, S., Pourciau, S.S., 2005, Temporal regulation of cytokine mRNA expression in equine recurrent airway obstruction. *Vet Immunol Immunopathol* 108, 237-245.
- Huntley, J.F., Newlands, G.F., Jackson, F., Miller, H.R., 1992, The influence of challenge dose, duration of immunity, or steroid treatment on mucosal mast cells and on the distribution of sheep mast cell proteinase in *Haemonchus*-infected sheep. *Parasite Immunol* 14, 429-440.
- Huntley, J.F., Patterson, M., Mackellar, A., Jackson, F., Stevenson, L.M., Coop, R.L., 1995, A comparison of the mast cell and eosinophil responses of sheep and goats to gastrointestinal nematode infections. *Res Vet Sci* 58, 5-10.

Ishikawa, N., Goyal, P.K., Mahida, Y.R., Li, K.F., Wakelin, D., 1998, Early cytokine responses during intestinal parasitic infections. *Immunology* 93, 257-263.

Kabagambe, E.K., 1997. Field evaluation of quil A-based vaccines against *Haemonchus contortus* in sheep. MS Thesis. LSU, Baton Rouge.

Kooyman, F.N., Van Kooten, P.J., Huntley, J.F., MacKellar, A., Cornelissen, A.W., Schallig, H.D., 1997, Production of a monoclonal antibody specific for ovine immunoglobulin E and its application to monitor serum IgE responses to *Haemonchus contortus* infection. *Parasitology* 114 (Pt 4), 395-406.

Koyama, K., Tamauchi, H., Tomita, M., Kitajima, T., Ito, Y., 1999, B-cell activation in the mesenteric lymph nodes of resistant BALB/c mice infected with the murine nematode parasite *Trichuris muris*. *Parasitol Res* 85, 194-199.

Lacroux, C., Nguyen, T.H., Andreoletti, O., Prevot, F., Grisez, C., Bergeaud, J.P., Gruner, L., Brunel, J.C., Francois, D., Dorchies, P., Jacquet, P., 2006, *Haemonchus contortus* (Nematoda: Trichostrongylidae) infection in lambs elicits an unequivocal Th2 immune response. *Vet Res* 37, 607-622.

Li, R.W., Sonstegard, T.S., Van Tassel, C.P., Gasbarre, L.C., 2006, Local inflammation as a possible mechanism of resistance to gastrointestinal nematodes in Angus heifers. *Vet Parasitol* 18, 18.

Mahida, Y.R., 2003, Host-parasite interactions in rodent nematode infections. *J Helminthol* 77, 125-131.

Matsuda, S., Uchikawa, R., Yamada, M., Arizono, N., 1995, Cytokine mRNA expression profiles in rats infected with the intestinal nematode *Nippostrongylus brasiliensis*. *Infect Immun* 63, 4653-4660.

McDermott, J.R., Bartram, R.E., Knight, P.A., Miller, H.R., Garrod, D.R., Grecis, R.K., 2003, Mast cells disrupt epithelial barrier function during enteric nematode infection. *Proc Natl Acad Sci USA* 100, 7761-7766.

Meeusen, E.N., Balic, A., Bowles, V., 2005, Cells, cytokines and other molecules associated with rejection of gastrointestinal nematode parasites. *Vet Immunol Immunopathol* 108, 121-125.

Miller, J.E., Bahirathan, M., Lemarie, S.L., Hembry, F.G., Kearney, M.T., Barras, S.R., 1998, Epidemiology of gastrointestinal nematode parasitism in Suffolk and Gulf Coast Native sheep with special emphasis on relative susceptibility to *Haemonchus contortus* infection. *Vet Parasitol* 74, 55-74.

Miller, J.E., Bishop, S.C., Cockett, N.E., McGraw, R.A., 2006, Segregation of natural and experimental gastrointestinal nematode infection in F2 progeny of susceptible Suffolk and resistant Gulf Coast Native sheep and its usefulness in assessment of genetic variation. *Vet Parasitol* 140, 83-89.

Ogilvie, B.M., Askenase, P.W., Rose, M.E., 1980, Basophils and eosinophils in three strains of rats and in athymic (nude) rats following infection with the nematodes *Nippostrongylus brasiliensis* or *Trichinella spiralis*. *Immunology* 39, 385-389.

Pena, M.T., Miller, J.E., Horohov, D.W., 2004, Effect of dexamethasone treatment on the immune response of Gulf Coast Native lambs to *Haemonchus contortus* infection. *Vet Parasitol* 119, 223-235.

Pena, M.T., Miller, J.E., Horohov, D.W., 2006, Effect of CD4+ T lymphocyte depletion on resistance of Gulf Coast Native lambs to *Haemonchus contortus* infection. *Vet Parasitol* 138, 240-246.

Pernthaner, A., Cole, S.A., Morrison, L., Hein, W.R., 2005, Increased expression of interleukin-5 (IL-5), IL-13, and tumor necrosis factor alpha genes in intestinal lymph cells of sheep selected for enhanced resistance to nematodes during infection with *Trichostrongylus colubriformis*. *Infect Immun* 73, 2175-2183.

Pfeffer, A., Douch, P.G., Shaw, R.J., Gatehouse, T.K., Rabel, B., Green, R.S., Shirer, C.L., Jonas, W.E., Bisset, S., 1996, Sequential cellular and humoral responses in the abomasal mucosa and blood of Romney sheep dosed with *Trichostrongylus axei*. *Int J Parasitol* 26, 765-773.

Rainbird, M.A., Macmillan, D., Meeusen, E.N., 1998, Eosinophil-mediated killing of *Haemonchus contortus* larvae: effect of eosinophil activation and role of antibody, complement and interleukin-5. *Parasite Immunol* 20, 93-103.

Rathore, D.K., Suchitra, S., Saini, M., Singh, B.P., Joshi, P., 2006, Identification of a 66kDa *Haemonchus contortus* excretory/secretory antigen that inhibits host monocytes. *Vet Parasitol* 138, 291-300.

Schallig, H.D., van Leeuwen, M.A., Bernadina, W.E., Hendrikx, W.M., 1994, Serum antibody responses of Texel sheep experimentally infected with *Haemonchus contortus*. *Res Vet Sci* 57, 63-68.

Schallig, H.D., van Leeuwen, M.A., Hendrikx, W.M., 1995, Isotype-specific serum antibody responses of sheep to *Haemonchus contortus* antigens. *Vet Parasitol* 56, 149-162.

Stankiewicz, M., Pernthaner, A., Cabaj, W., Jonas, W.E., Douch, P.G., Bisset, S.A., Rabel, B., Pfeffer, A., Green, R.S., 1995, Immunization of sheep against parasitic nematodes leads to elevated levels of globule leukocytes in the small intestine lumen. *Int J Parasitol* 25, 389-394.

Suchitra, S., Joshi, P., 2005, Characterization of *Haemonchus contortus* calreticulin suggests its role in feeding and immune evasion by the parasite. *Biochim Biophys Acta* 1722, 293-303.

Urban, J.F., Jr., Katona, I.M., Paul, W.E., Finkelman, F.D., 1991, Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. *Proc Natl Acad Sci USA* 88, 5513-5517.

Urban, J.F., Jr., Noben-Trauth, N., Schopf, L., Madden, K.B., Finkelman, F.D., 2001, Cutting edge: IL-4 receptor expression by non-bone marrow-derived cells is required to expel gastrointestinal nematode parasites. *J Immunol* 167, 6078-6081.

Urquhart, G.M., Armour, J., Duncan, J.L., Dunn, A.M., Jennings, F.W., 1996, *Veterinary Parasitology*, Second Edition. Blackwell Science, 1-22 pp.

Waller, P.J., 2003, Global perspectives on nematode parasite control in ruminant livestock: the need to adopt alternatives to chemotherapy, with emphasis on biological control. *Anim Health Res Rev* 4, 35-43.

Woolaston, R.R., Manuelli, P., Eady, S.J., Barger, I.A., Le Jambre, L.F., Banks, D.J., Windon, R.G., 1996, The value of circulating eosinophil count as a selection criteria for resistance of sheep to trichostrongyle parasites. *Int J Parasitol* 26, 123-126.

## CHAPTER 4

### GENERAL DISCUSSION

#### 4.1. Discussion of Results

The main objective of these experiments was to determine components of the immune response that are associated with the higher degree of resistance in Native breed of sheep to *Haemonchus contortus* compared to a known susceptible breed of sheep, Suffolk. In order to meet the objective, the dynamics and magnitude of the components of acquired immunity involved in these responses were evaluated under one time bolus and repeated trickle infections (Experiment 1, Chapter 2) in lambs reared in concrete floored pens as well as in those kept in semi enclosed pens and infected naturally on pasture (Experiment 2, Chapter 3). Experiment 1 was designed such that responses on day 14 and 21 of infection could be observed. The parameters evaluated included infection level (based in FEC, PCV and worm numbers) and alterations in peripheral as well as tissue eosinophilia, tissue mastocytosis, neutrophil density in abomasal mucosa and serum levels of immunoglobulin isotypes IgG1, IgG2, IgA, IgM and IgE. In addition to the parameters evaluated in Experiment 1, globule leukocytosis and quantitation of mRNA transcripts for cytokines IL-4, IL-10 and IFN- $\gamma$  in peripheral leukocytes were included in Experiment 2. The pathological effects of parasite, such as development of anemia, unthriftiness, and loss of body conditions were found more pronounced in Suffolk lambs when compared with the Native lambs. These effects were more intense in Experiment 1 artificially infected animals kept in concrete floored pens. At the time of necropsy the Native lambs showed a reasonable quantity of body fat and were in relatively better condition while it was almost absent in most of the Suffolk lambs.

In Experiment 1, the FEC results indicated a lower egg excretion in Native lambs compared with Suffolk lambs regardless of the infection regimen followed. Differences in the

FEC may be a result of inhibition or delayed growth of the parasite, suppression of female worm fecundity or low proportion of adult female parasites in the abomasum. The lower FEC in the bolus infected Native lambs may be attributed to the lower adult immature ratio when compared with Suffolk lambs 21 days post infection. This inhibition or delay in growth of parasites in the Native lambs appears to have resulted in reduced numbers of females capable of producing eggs. Whether these inhibited parasites would have been eliminated later is unknown. In the trickle infected lambs, the FEC were not significantly different between the breeds however that was due to one Native lamb that had a greater FEC than four Suffolk lambs in their corresponding group. This lamb was one of the two Native lambs in the group from a different source.

In Experiment 2, there was a significantly lower total number of adult worms in Native lambs resulting in lower FEC. These results concur with the results observed in previous studies involving Native lambs (Bahirathan et al., 1996; Miller et al., 1998). Similar results with lower FEC have been reported for several other breeds or lines within breeds known to be resistant to *H. contortus* (Emery et al., 2000; Bricarello et al., 2002; Bricarello et al., 2004). FEC and parasite burden results concur with other studies involving comparison between resistant and susceptible breeds of sheep. In one study, Blackbelly sheep known to have higher resistance against *H. contortus* demonstrated lower FEC as well as worm burden compared with susceptible INRA 401 sheep after 35 days of artificial infection (Aumont et al., 2003). In another study, Florida Native lambs showed a lower FEC and lower worm burden when compared with Rambouillet and F1 generation lambs of these breeds (Amarante et al., 1999). Similarly St. Croix lambs, another resistant breed of sheep demonstrated low FEC and worm burden at 5 weeks after infection when compared with Dorset lambs (Gamble and Zajac, 1992). These results indicate that resistance against *H. contortus* not only results in decreased FEC but also reduction in parasite burden.

In Experiment 2, both Suffolk and Native lambs demonstrated an increased number of immature *H. contortus* on day 42 of the experiment. The Suffolk lambs showed higher number of immature worms when compared with their corresponding Native lambs. That may be an indication of a lapse in protective immunity on the part of the host and or increased availability of L3 from pasture due to rain during the preceding week leading to ingestion and establishment in a greater number. Both the groups demonstrated a trend for increased IL-10 from day 21 onwards. The increase was continuous in Suffolk lambs while there was an indication of reduction in Native lambs after 28 days of exposure. Increased level of IL-10 may be responsible for the lapse in resistance and would have given an opportunity for establishment of the incoming larvae in greater numbers which were released in the pasture after the rain.

In Experiment 1, the high PCV reduction percentages among all groups regardless of breed and infection regimens may be attributed to high establishment of *H. contortus* larvae. The L3 used for those infections were freshly harvested and motility was vigorous. Hence, worm recovery was substantially higher in these lambs than expected. Since both L4 and adult *H. contortus* feed on blood loss may have been aided by continued bleeding from the ulcers caused even after the parasites leave the site. Calreticulin, a calcium binding protein present in excretory secretory product of *H. contortus*, increases the plasma coagulation time (Suchitra and Joshi, 2005) leading to enhanced bleeding time. Therefore, the higher establishment of worms coupled with more calreticulin probably accounted for the PCV reduction percentage observed. In Experiment 2, Suffolk lambs showed a significantly higher PCV reduction percentage compared with their corresponding Native lambs. Native lambs indicated that they recovered from the blood loss, which was evident after week 4 and continuous there after. Whether the recovery was due to erythropoietic processes or lowered establishment of larvae or elimination of already established worms is not clear. It may be one or combination of these factors. The presence of

greater numbers of worms in Suffolk lambs appeared to have caused a continuous blood loss leading to high PCV reduction percentage. In addition, partial or complete absence of effective compensatory mechanisms for blood loss in Suffolk lambs may be another reason for increased anemia. These results are in line with previous studies that involved these two breeds of lambs (Bahirathan et al., 1996 Miller et al., 1998).

In Experiment 1, the peripheral eosinophil percentage increased 7 days after the infection in all the Native groups regardless of the infection regimen where as Suffolk lambs remained around the baseline similar to the untreated control groups. Eosinophilia is a consistent feature in nematode infections in small ruminants and other species of animals including laboratory animals like mice and rats. This characteristic has been considered as a marker for resistant individuals in Australian lines of Merino and Romney sheep infected with *H. contortus* and *Trichostrongylus colubriformis*; however, high variability among the individuals observed makes it less preferential (Douch et al., 1996). Therefore, combining this with other consistent characteristics may be a suitable strategy for selecting for resistance (Buddle et al., 1992; Douch et al., 1996).

Tissue eosinophilia has been reported in immunized lambs as well as in resistant lines or breeds of sheep (Gill, 1991; Woolaston et al., 1996; Hohenhaus et al., 1998; Hooda et al., 1999; Gill et al., 2000). The results of those experiments, demonstrated that tissue eosinophilia was consistently associated with infection whether it was natural or artificial. In Experiment 1, although both breeds showed abomasal eosinophilia, the Native groups of lambs had greater numbers at both time points. Balic et al., (2002) have reported tissue eosinophilia to occur as early as 3 days post infection in secondary infections. In another study, eosinophil density was found to be significantly higher in abomasal mucosa from resistant lambs when compared with random bred lambs on day 28 post infection (Gill et al., 2000). Furthermore, eosinophils are

reported to congregate around the tissue bound gastrointestinal larvae inflicting structural damage (Balic et al., 2006; Rainbird et al., 1998). In an *in vitro* study, the eosinophils extracted from mammary washes of repeatedly infected sheep were shown to immobilize and kill *H. contortus* L3s in the presence of anti-L3 surface antibody (Rainbird et al., 1998). Eosinophils have FcεRI which is a high affinity IgE receptor and mediates IgE directed antibody dependent cell-mediated cytotoxicity (Abbas and Lichtman, 2005a). Eosinophils carry major basic protein, eosinophil cationic protein, eosinophil peroxidase, lysosomal hydrolases, and lysophospholipase in cytoplasmic granules that are capable of inflicting damage to larval surface and are toxic to helminth parasites (Abbas and Lichtman, 2005b). The sensitized eosinophils upon contact with *H. contortus* antigens degranulate releasing the enzymes and proteins and inflict damage to the worm. These findings indicate that association of increased eosinophils and increased IgE may be among the components responsible for anti-*Haemonchus* activity in Native lambs.

Mucosal mast cell hyperplasia is another prominent feature of gastrointestinal parasitism. Enumeration of mucosal mast cells (MMC) in different regions of the abomasum demonstrated a consistently greater number in infected Native lambs when compared with Suffolk lambs in Experiment 2. The density of MMC however was more variable among the Native lambs in Experiment 1. Variation in density in different regions of the abomasum may be due to preferential attachment of the parasites as well as responsiveness of that particular region. *H. contortus* attach mostly in the pyloric region of the abomasum (Dr. Miller, personal communication). Why *H. contortus* parasites prefer this particular region is not understood. The variation in the density of MMC within a breed may be due to the difference in sampling time points and the number of parasites they were exposed to. However, the uninfected control groups demonstrated significantly fewer number of MMC compared to the infected groups. These data conform to the results reported by Schallig et al., (1997). In another study, MMC were recorded

to be significantly higher in abomasal mucosa on day 28 post infection with *H. contortus* in sheep (Gill et al., 2000).

Globule leukocytes were recorded in the cardiac and fundic regions of abomasa in Experiment 2 lambs but not in pyloric region and the Native lambs demonstrated significantly greater numbers when compared with Suffolk lambs. Similar results have been reported for *H. contortus* (Amarante et al., 1999; Lacroux et al., 2006) and *Oesophagostomum columbianum* (Dobson, 1966) infections. Globule leukocytes have been associated with resistance against these parasites (Huntley et al., 1992). The sheep mucosal mast cells and globule leukocytes are known to contain sheep mast cell protease (SMCP) (Huntley et al., 1992). Tissue concentration of SMCP is directly correlated with number of mast cells present and inversely related to *Trichostrongylus vitrinus* and *Teladorsagia circumcincta* populations (Huntley et al., 1995). MMC carry FCεRI, a high affinity receptor for IgE and most of the IgE produced is cell bound. Sensitized mast cells when exposed to the antigen degranulate and release the contents including SMCP in the mucosal tissue and abomasal mucus of the infected sheep (Huntley et al., 1992). MMC are also considered responsible for the leakiness of the epithelium by causing degradation of occludin, which keeps the intercellular junctions sealed, leading to increased epithelial permeability (McDermott et al., 2003). Increased permeability may lead to the increased dissemination of cells and cellular products into the abomasal lumen leading to an altered abomasal environment that may be unsuitable for the parasites. Additionally, anti-parasitic substances released from MMC and globule leukocytes that come in contact with the parasites in the abomasal lumen may cause damage that eventually may lead to their expulsion. Neutrophils are known to be present in late phase reaction of immediate hypersensitivity (Abbas and Lichtman, 2005c) although their functions are not well understood. In these studies, neutrophils

were found to be present in greater number in Experiment 1 early infection groups. However, they were also present in the abomasal mucosa on 35 and 42 day post exposure in Experiment 2.

Lymph containing antigens from abomasal events drain into the abomasal lymph nodes where immunological events like antigen presentation, T and B cell activation, clonal expansion of B cells take place. These events usually result in a change in weight of the affected lymph nodes. Balic et al., (2002) found that there was no change in abomasal lymph node weights among uninfected control and naïve lambs infected with *H. contortus*; however, in adult sheep abomasal lymph nodes weighed significantly higher on 5 day post infection. In both Experiments 1 and 2, the abomasal lymph nodes from infected lambs were significantly heavier compared with uninfected controls of the respective breeds. However, there was no difference found between infected groups between breeds. The lambs used were previously exposed to the parasites and were cleaned before they were used; therefore, the response measured was secondary which concur with the results of Balic et al. (2002). The lack of difference between the two breeds may be due to the sampling time points. The weights were measured on the day of necropsy in both the experiments and it may be possible that any changes had already occurred and were returned to their normal size and weight by the time they were sampled.

The ovine immunoglobulin isotypes are classified as IgG1, IgG2, IgM, IgA and IgE. Among them, IgG1, IgA and IgE are considered to have an important role in protection against nematode infections (Schallig, 2000). Kooyman et al., (1997) found increased serum levels of IgE between 2-4 weeks after infection with *H. contortus*. In repeatedly infected sheep there was a negative correlation between parasite burden and level of IgE. In Experiment 1, IgE determination was performed on pooled serum samples; therefore, the data could not be statistically analyzed. However, there was a tendency for increased serum IgE level in the infected Native lambs when compared with the uninfected control and infected Suffolk lambs. In

Experiment 2, the level of IgE was significantly higher in Native lambs by day 14 post exposure which continued until the end of the experiment on day 42. In contrast to the findings of Kooyman et al. (1997), results of the present studies indicate that the production of IgE continues beyond week four post infection. Huntley et al. (1998) reported a four times greater concentration of IgE in gastric lymph than serum with *T. circumcincta* infection in sheep and concluded that the majority of IgE is produced locally in the regional lymph nodes. In an *in vitro* study, abomasal lymph node cells from *H. contortus* infected lambs produced a significantly higher amount of IgE following stimulation with parasite antigen (Gill et al., 2000). It was also indicated that repeatedly exposed animals responded with early induction of IgE production rather than increased level. Those results concur with the results from the present studies.

In Experiment 1, there was as an increased serum level of total IgG in Native lambs infected with the bolus dose which was not observed in infected Suffolk and uninfected control lambs. Similarly, IgG1 level showed an increased level in infected Native lambs on 14 and 21 of infection. However, in Experiment 2, naturally infected Native sheep only showed a significantly increased level of serum IgG1 on day 42 after the exposure in Native lambs when compared with Suffolk lambs. Similar results have been reported by others. Serum IgG levels were found increased in secondary infections but after the FEC had already fallen (Charley-Poulain et al., 1984). IgG1 containing cells in the abomasum of the repeatedly infected lambs increased by 6 fold in 7 days with no change in uninfected control lambs (Gill et al., 1992). Furthermore, a resistant line of sheep had increased IgG1 production between 24 and 31 day of infection which was negatively correlated to the worm burden (Gill et al., 1993). Spleen and abomasal lymph node cells from resistant lambs produced a significantly higher amount of IgG1 on day 28 (Gill et al., 2000). However, the results from the present experiments indicated that production of IgG1 in Native lambs occurred during the later part of the infections.

Immunoglobulin A is reported to play a vital role in resistance against nematode infections (Strain et al., 2002; Stear et al., 2004). In Experiment 1, IgA increased in bolus infected Native lambs after 7 days post infection but it did not increase in trickle infected lambs. Smith et al. (1984) reported an increased level of cellular and gastric lymph IgA that peaked on day 3 and 6 days post infection respectively. A secondary response of serum IgA was reported to have increased and a temporal relation with a self cure reaction was observed (Charley-Poulain et al., 1984). Similarly, IgA producing cells were the most abundant cell type in abomasal submucosa in a study involving cannulation of the abomasum in sheep infected with *H. contortus* (Gill et al., 1992). These cells continued to increase until day 28. Similarly, in another study, serum IgA specific to *H. contortus* in resistant sheep were high between day 10 and 31 of infection (Gill et al., 1993). Those results concur with the temporal increase of serum IgA observed in Experiment 1.

Mechanism of action of IgG and IgA are not completely clear on resistance against nematode parasites. However, various workers have suggested different mechanisms. Gill et al., (1993) speculated that parasite specific IgA and IgG neutralize or inactivate enzymes that are vital for the survival of the parasite. Similarly, Smith, (1988) observed a negative correlation between concentration of IgA in gastric lymph and *Teladorsagia circumcincta* length suggesting its interference with parasite feeding. Furthermore, *Trichostrongylus colubriformis* feeding was hindered by the presence of anti-*T. colubriformis* IgG1 *in vitro* (Bottjer, et al., 1985). Immunoglobulin was also bound to the excretory pores, stoma and cuticle of the parasite.

Cytokines are important mediators of immune response. Cytokines involved in protective responses to nematode infection are classified under the Th2 subset (Schallig, 2000). These cytokines are IL-4, IL-5, IL-9, and IL-13 among others (Gill et al., 2000; Schallig, 2000). The immunity mediated by Th1 type of cytokines like IL-2 and IFN- $\gamma$  is not protective against

*Ostertagia ostertagi* infection in cattle (Canals et al., 1997). Cytokine IL-10 although previously classified under Th2 type of cytokines, is known to play a regulatory role during the immune response (Specht et al., 2004).

Schallig, (2000) found that there was a Th1 type of response to primary infection with *H. contortus* and upon repeated infections there was a switch to the Th2 type. In Experiment 2, expression of IL-4, IL-10 and IFN- $\gamma$  were quantified by real time RT-PCR. The results indicated that IL-4 was upregulated and peaked on day 10 after exposure. However, before and after day 10, IL-4 was not significantly different between the two breeds. This peak preceded the increased mastocytosis recorded in artificially infected lambs. In contrast, Meeusen et al., (2005) reported the peak of IL-4 production on day 5 which remained at a higher level through out the period of their experiment in sheep infected with *H. contortus*. In contrast, Lacroux et al., (2006) reported IL-4 and IL-13 was over expressed in repeatedly infected lambs with *H. contortus*. IL-5 was reported to have been produced by abomasal lymph node cells from resistant lambs in greater amounts than random bred lambs in response to parasite antigen at days 5 and 28 post infection (Gill et al., 2000). Since IL-5 is involved in stimulation and maturation of eosinophils, this cytokine may account for the induction of eosinophilia in the Native lambs. However, mRNA expression level for IL-5 was not determined in this study.

IFN- $\gamma$  levels were not significantly different at any sampling time points in this experiment. However, during the first few days of the exposure, IFN- $\gamma$  increased and then decreased and remained at a low level. Schallig (2000) reported that primary infection in lambs induced a Th1 type response with increased levels of IL-2 and IFN- $\gamma$ . This may be one reason behind increased susceptibility of lambs that are exposed to the *H. contortus* infection for the first time. The lambs in this experiment were all previously exposed and none of them was naïve at the time of experiment. The high level of IL-10 in Suffolk lambs may have a role in the lower

level of IFN- $\gamma$  in Suffolk lambs as IL-10 is reported to have a suppressive role on both types of cytokines (Helmbly and Grecnis, 2003; Specht et al., 2004). Similarly, IFN- $\gamma$  production by abomasal lymph node cells was lower than the uninfected control lambs in response to parasite antigen on day 5 and 28 which indicated down regulation of IFN- $\gamma$  after the infection (Gill et al., 2000). However, in this experiment, there was no significant difference in expression level of IFN- $\gamma$  between the two breeds, and without uninfected control animals it is not possible to determine whether there was any down regulation of this cytokine.

Taken together, Native lambs appeared to respond with peripheral and abomasal mucosal eosinophilia, mastocytosis, globule leukocytosis and increased level of serum IgE to *H. contortus* infection. These components seem to have an adverse effect on *H. contortus* establishment and may account for the higher degree of resistance in Native lambs. Where as absence or lower levels of these components in Suffolk lambs appeared to be associated with increased susceptibility to the parasite. Furthermore, whether increased susceptibility in Suffolk lambs is due to lack of their ability to mount a protective response constitutively or is induced by the parasite, is not clearly understood.

#### **4.2. Recommendations for Future Studies**

Other components of the immune response like the cytokines IL-5, IL-9, IL-13 and tumor growth factor-beta (TGF- $\beta$ ) and the role of macrophages in the abomasal mucosa need to be evaluated. Changes in the abomasal physiology following infection and its effects on worms in the abomasum and effects exerted by eosinophils, mast cells, globule leukocytes and their products combined with IgA, IgE on worm and T, B and plasma cell profile in abomasum, LN cell profiling are other areas that are important for further studies. Possible down regulation of immune responses by *H. contortus* and any mechanism by which it is overcome by Native lambs would further enhance the knowledge in this field and help formulate parasite control strategies.

Furthermore, studies to determine genetic markers associated with resistance should be performed so that marker assisted selection of individuals can be made for resistance against *H. contortus*.

#### **4.3. Conclusions**

The findings of these studies have contributed to the understanding of protective immune responses against *H. contortus* in sheep in general and Native sheep in particular under the circumstances in these experiments. These findings will certainly be useful in designing the essential immune response to be induced by a successful vaccine against this worm. These findings also will contribute in selection of individuals with higher degree of resistance in order to breed for resistance and will help to have better producing animals that are resistance to this parasite and help produce animal products that are free of chemical residues.

#### **4.4. References**

- Abbas, A.K., Lichtman, A.H., 2005a, Cellular and Molecular Immunology Fifth Edition. Elsevier Saunders, 324-325 pp.
- Abbas, A.K., Lichtman, A.H., 2005b, Cellular and Molecular Immunology, Fifth Edition. Elsevier Saunders, 443 p.
- Abbas, A.K., Lichtman, A.H., 2005c, Cellular and Molecular Immunology, Fifth Edition. Elsevier Saunders, 447 p.
- Amarante, A.F., Craig, T.M., Ramsey, W.S., Davis, S.K., Bazer, F.W., 1999, Nematode burdens and cellular responses in the abomasal mucosa and blood of Florida Native, Rambouillet and crossbreed lambs. *Vet Parasitol* 80, 311-324.
- Aumont, G., Gruner, L., Hostache, G., 2003, Comparison of the resistance to sympatric and allopatric isolates of *Haemonchus contortus* of Black Belly sheep in Guadeloupe (FWI) and of INRA 401 sheep in France. *Vet Parasitol* 116, 139-150.
- Bahirathan, M., Miller, J.E., Barras, S.R., Kearney, M.T., 1996, Susceptibility of Suffolk and Gulf Coast Native suckling lambs to naturally acquired strongylate nematode infection. *Vet Parasitol* 65, 259-268.
- Balic, A., Bowles, V.M., Meeusen, E.N., 2002, Mechanisms of immunity to *Haemonchus contortus* infection in sheep. *Parasite Immunol* 24, 39-46.

- Balic, A., Cunningham, C.P., Meeusen, E.N., 2006, Eosinophil interactions with *Haemonchus contortus* larvae in the ovine gastrointestinal tract. *Parasite Immunol* 28, 107-115.
- Bottjer, K.P., Klesius, P.H., Bone, L.W. 1985, Effects of host serum on feeding by *Trichostrongylus colubriformis* (Nematoda). *Parasite Immunol* 7, 1-7.
- Bricarello, P.A., Gennari, S.M., Oliveira-Sequeira, T.C., Vaz, C.M., Goncalves de Goncalves, I., Echevarria, F.A., 2002, Response of Corriedale and Crioula Lanada sheep to artificial primary infection with *Haemonchus contortus*. *Vet Res Commun* 26, 447-457.
- Bricarello, P.A., Gennarib, S.M., Oliveira-Sequeirac, T.C.G., Vazd, C.M.S.L., Gonçaves de Gonçalvesa, I., Echevarriad, F.A.M., 2004, Worm burden and immunological responses in Corriedale and Crioula Lanada sheep following natural infection with *Haemonchus contortus* *Small Ruminant Research* 51, 75-83.
- Buddle, B.M., Jowett, G., Green, R.S., Douch, P.G., Risdon, P.L., 1992, Association of blood eosinophilia with the expression of resistance in Romney lambs to nematodes. *Int J Parasitol* 22, 955-960.
- Canals A. Z., D.S., Almeria S., and Gasbarre, L.C., 1997, Cytokine profile induced by a primary infection with *Ostertagia ostertagi* in cattle. *Vet Immunol Immunopathol* 58, 63-75.
- Charley-Poulain, J., Luffau, G., Pery, P., 1984, Serum and abomasal antibody response of sheep to infections with *Haemonchus contortus*. *Vet Parasitol* 14, 129-141.
- Dobson, C., 1966, Studies on the immunity of sheep to *Oesophagostomum columbianum*: the nature and fate of the globule leukocyte. *Aust. J. Agric. Res* 17, 955-966.
- Douch, P.G., Green, R.S., Morris, C.A., McEewan, J.C., Windon, R.G., 1996, Phenotypic markers for selection of nematode-resistant sheep. *Int J Parasitol* 26, 899-911.
- Emery, D.L., McClure, S.J., Davey, R.J., 2000, Protection of Merino lambs against *Haemonchus contortus* by trickle infection of neonates. *Parasitol Int* 49, 165-170.
- Gamble, H.R., Zajac, A.M., 1992, Resistance of St. Croix lambs to *Haemonchus contortus* in experimentally and naturally acquired infections. *Vet Parasitol* 41, 211-225.
- Gill, H.S., 1991, Genetic control of acquired resistance to haemonchosis in Merino lambs. *Parasite Immunol* 13, 617-628.
- Gill, H.S., Altmann, K., Cross, M.L., Husband, A.J., 2000, Induction of T helper 1- and T helper 2-type immune responses during *Haemonchus contortus* infection in sheep. *Immunology* 99, 458-463.
- Gill, H.S., Gray, G.D., Watson, D.L., Husband, A.J., 1993, Isotype-specific antibody responses to *Haemonchus contortus* in genetically resistant sheep. *Parasite Immunol* 15, 61-67.

- Gill, H.S., Husband, A.J., Watson, D.L., 1992, Localization of immunoglobulin-containing cells in the abomasum of sheep following infection with *Haemonchus contortus*. *Vet Immunol Immunopathol* 31, 179-187.
- Helmby, H., Grecis, R.K., 2003, Contrasting roles for IL-10 in protective immunity to different life cycle stages of intestinal nematode parasites. *Eur J Immunol* 33, 2382-2390.
- Hohenhaus, M.A., Josey, M.J., Dobson, C., Outteridge, P.M., 1998, The eosinophil leucocyte, a phenotypic marker of resistance to nematode parasites, is associated with calm behaviour in sheep. *Immunol Cell Biol* 76, 153-158.
- Hooda, V., Yadav, C.L., Chaudhri, S.S., Rajpurohit, B.S., 1999, Variation in resistance to haemonchosis: selection of female sheep resistant to *Haemonchus contortus*. *J Helminthol* 73, 137-142.
- Huntley, J.F., Newlands, G.F., Jackson, F., Miller, H.R., 1992, The influence of challenge dose, duration of immunity, or steroid treatment on mucosal mast cells and on the distribution of sheep mast cell proteinase in *Haemonchus*-infected sheep. *Parasite Immunol* 14, 429-440.
- Huntley, J.F., Patterson, M., Mackellar, A., Jackson, F., Stevenson, L.M., Coop, R.L., 1995, A comparison of the mast cell and eosinophil responses of sheep and goats to gastrointestinal nematode infections. *Res Vet Sci* 58, 5-10.
- Huntley, J.F., Schallig, H.D., Kooyman, F.N., MacKellar, A., Millership, J., Smith, W.D., 1998, IgE responses in the serum and gastric lymph of sheep infected with *Teladorsagia circumcincta*. *Parasite Immunol* 20, 163-168.
- Kooyman, F.M., Van Kooten, P.J., Huntley, J.F., MacKellar, A., Cornelissen, A.W., Schallig, H.D., 1997, Production of a monoclonal antibody specific for ovine immunoglobulin E and its application to monitor serum IgE responses to *Haemonchus contortus* infection. *Parasitology* 114 (Pt 4), 395-406.
- Lacroux, C., Nguyen, T.H., Andreoletti, O., Prevot, F., Grisez, C., Bergeaud, J.P., Gruner, L., Brunel, J.C., Francois, D., Dorchies, P., Jacquet, P., 2006, *Haemonchus contortus* (Nematoda: Trichostrongylidae) infection in lambs elicits an unequivocal Th2 immune response. *Vet Res* 37, 607-622.
- McDermott, J.R., Bartram, R.E., Knight, P.A., Miller, H.R., Garrod, D.R., Grecis, R.K., 2003, Mast cells disrupt epithelial barrier function during enteric nematode infection. *Proc Natl Acad Sci USA* 100, 7761-7766.
- Meeusen, E.N., Balic, A., Bowles, V., 2005, Cells, cytokines and other molecules associated with rejection of gastrointestinal nematode parasites. *Vet Immunol Immunopathol* 108, 121-125.
- Miller, J.E., Bahirathan, M., Lemarie, S.L., Hembry, F.G., Kearney, M.T., Barras, S.R., 1998, Epidemiology of gastrointestinal nematode parasitism in Suffolk and Gulf Coast Native sheep

with special emphasis on relative susceptibility to *Haemonchus contortus* infection. *Vet Parasitol* 74, 55-74.

Rainbird, M.A., Macmillan, D., Meeusen, E.N., 1998, Eosinophil-mediated killing of *Haemonchus contortus* larvae: effect of eosinophil activation and role of antibody, complement and interleukin-5. *Parasite Immunol* 20, 93-103.

Schallig, H.D., van Leeuwen, M.A., Cornelissen, A.W., 1997, Protective immunity induced by vaccination with two *Haemonchus contortus* excretory secretory proteins in sheep. *Parasite Immunol* 19, 447-453.

Schallig, H.D., 2000, Immunological responses of sheep to *Haemonchus contortus*. *Parasitology* 120 Suppl, S63-72.

Smith, W.D. 1988. Mechanism of immunity to gastrointestinal nematodes of sheep. In *Increasing Small Ruminants productivity in Semi-arid Areas* (ed. Thomson, E.F. & Thomson, F.S.), 275-286 pp.

Smith, W.D., Jackson, F., Jackson, E., Williams, J., Miller, H.R., 1984, Manifestations of resistance to ovine ostertagiasis associated with immunological responses in the gastric lymph. *J Comp Pathol* 94, 591-601.

Specht, S., Volkmann, L., Wynn, T., Hoerauf, A., 2004, Interleukin-10 (IL-10) counter regulates IL-4-dependent effector mechanisms in Murine Filariasis. *Infect Immun* 72, 6287-6293.

Stear, M.J., Bairden, K., Innocent, G.T., Mitchell, S., Strain, S., Bishop, S.C., 2004. The relationship between IgA activity against 4th-stage larvae and density-dependent effects on the number of 4th-stage larvae of *Teladorsagia circumcincta* in naturally infected sheep. *Parasitol* 129, 363-369.

Strain, S.A., Bishop, S.C., Henderson, N.G., Kerr, A., McKellar, Q.A., Mitchell, S., Stear, M.J., 2002. The genetic control of IgA activity against *Teladorsagia circumcincta* and its association with parasite resistance in naturally infected sheep. *Parasitol* 124, 545-552.

Suchitra, S., Joshi, P., 2005, Characterization of *Haemonchus contortus* calreticulin suggests its role in feeding and immune evasion by the parasite. *Biochim Biophys Acta* 1722, 293-303.

Woolaston, R.R., Manuelli, P., Eady, S.J., Barger, I.A., Le Jambre, L.F., Banks, D.J., Windon, R.G., 1996, The value of circulating eosinophil count as a selection criteria for resistance of sheep to trichostrongyle parasites. *Int J Parasitol* 26, 123-126.

## APPENDIX A

### ISOLATION OF RNA FROM WHOLE BLOOD COLLECTED INTO PAXGENE BLOOD RNA TUBES

The PAXgene Blood RNA tubes with blood sample were incubated at room temperature for at least 2 hours and the following procedure was followed as recommended by the manufacturer of the Paxgene blood RNA kit.

#### **PAXgene Blood RNA Kit**

1. Paxgene blood RNA tubes were centrifuged for 10 minutes at 4500xg after the blood samples were incubated for at least two hours at room temperature.
2. The supernatant was removed by decanting without disturbing the pellet and 4 ml of RNase-free water was added after the rim was dried with a clean paper towel to the pellet and the tube was closed with a fresh secondary Homeguard closure.
3. The tube was vortexed until the pellet was visibly dissolved, and centrifuged for 10 minutes at 4500xg. Supernatant was discarded completely.
4. 350µl of Buffer BR1 was added and vortexed until the pellet was visibly dissolved.
5. The sample was pipetted into a 1.5 ml centrifuge tube and 300 µl of Buffer BR2 was and 40 µl proteinase K were added. The mixture was mixed by vortexing for 5 seconds, and incubated for 10 minutes at 55°C.
6. The lysate was pipetted directly into a PAXgene shredder spin column (lilac colored) placed in a 2 ml processing tube, and centrifuged for 3 minutes at 16000xg.
7. The entire supernatant of the flow-throw fraction was carefully transferred to a fresh 1.5 ml microcentrifuge tube without disturbing the pellet in the processing tube.
8. 350 µl absolute ethanol was added and mixed by vortexing, and centrifuged briefly for 1-2 seconds at 1000xg to remove drops from the inside of the tube lid.
9. 700 µl of sample was pipetted into the PAXgene RNA spin column (red colored) placed in a 2 ml processing tube, and centrifuged for one minute at 16000xg. The spin column was placed in a new 2 ml processing tube and the old processing tube was discarded containing flow through.
10. The remaining sample was pipetted into the PAXgene RNA spin column, and centrifuged for one minute at 16000xg. The spin column was placed in a new two ml processing tube and the old processing tube containing flow-through was discarded.
11. 350 µl of Buffer BR3 was pipetted into the PAXgene RNA spin column and centrifuged for one minute at 16000xg. The spin column was placed in a new two ml processing tube and the old processing tube containing flow-through was discarded.
12. 10 µl of DNase-I stock solution was added to 70 µl Buffer RDD in a 1.5ml microcentrifuge tube and was mixed by gently flicking the tube and was centrifuged briefly to collect residual liquid from the sides of the tube. DNase-I stock solution was prepared for a batch of 10 tubes at one time. 80 µl DNase-I incubation mix was pipetted directly onto the PAXgene RNA spin column membrane, and placed on the bench top for 15 minutes.
13. 350 µl of Buffer BR3 was pipetted into the PAXgene RNA spin column and centrifuged for one minute at 16000xg. The spin column was placed in a new two ml processing tube and the old processing tube containing flow-through was discarded.

14. 44 ml of absolute alcohol was added to 11 ml of Buffer BR4 and used. 500  $\mu$ l of Buffer BR4 was pipetted to the PAXgene RNA spin column and centrifuged for one minute at 16000xg. The spin column was placed in a new two ml processing tube and the old processing tube containing flow-through was discarded.
15. Another 500  $\mu$ L BR4 was added to the PAXgene RNA spin column and centrifuged for 3 minutes at 16000xg.
16. The tube containing the flow-through was discarded and the PAXgene RNA spin column was placed in a new twp ml processing tube and centrifuged for one minute at 16000xg.
17. The tube containing flow-through was discarded and the PAXgene RNA spin column was placed in a 1.5 ml microcentrifuge tube. 40  $\mu$ l of Buffer BR5 was placed directly onto the PAXgene RNA spin column membrane making it wet entirely and was centrifuged for one minute at 16000xg to elute the RNA.
18. The elution step was repeated after adding 40  $\mu$ L of Buffer BR5 and centrifuged in the same microcentrifuge tube.
19. The eluate was incubated for 5 minutes at 65°C in the incubator and chilled immediately on ice in order to denature the RNA for downstream applications.
20. The RNA samples were stored at -70°C until used later for synthesis of cDNA.
21. For quantification purpose the RNA samples were diluted in Tris buffer and calculated by using the relationship  $A_{260}=1=44 \mu\text{g/ml}$ .

## APPENDIX B.

### VERSAGENE RNA PURIFICATION SYSTEM

1. The PAXgene<sup>®</sup> Blood RNA tubes (preAnalytiX) with blood samples were centrifuged at 3000xg for 10 minutes.
2. The supernatant was poured off and the rim of the tube was blotted with a clean absorbent paper. A flat brown pellet was observed remaining.
3. Five ml of RNase-free water included in kit was added and the tubes were capped and vortexed for 20 seconds on high speed to re-suspend the pellet. Small black flakes may be visible.
4. The tubes were centrifuged at 3000xg for 10 minutes (centrifuge and brand).
5. Supernatant was poured off and the tubes were inverted on clean absorbent paper until step six was performed.
6. The tubes were blotted with a clean absorbent paper and 150  $\mu$ l of resuspension buffer was added. Steps 6-9 were completed for each tube one sample at a time. Because the RNA is not protected in resuspension buffer and each tube must be brought to step 9 before processing subsequent samples.
7. Tube was vortexed for 20 seconds on high speed and step 8 was carried out immediately.
8. Immediately 310 $\mu$ l lysis solution/protease K was added to sample.
9. The sample was vortexed on high speed for 10 seconds to thoroughly homogenize it. Samples were batch processed now on.
10. Samples were incubated on ice for 15 minutes and were vortexed vigorously to completely mix the sample twice during this incubation for at least 30 seconds each time.
11. The lysate was pipetted up and down three times to shear the DNA and thin the lysate.
12. The entire lysate was pipetted out and transferred to a purification column.
13. The column was centrifuged at 13,000-16,000xg for one minute.
14. The purification column was transferred to a new tube.
15. 400  $\mu$ l of wash-I solution was added to the purification column by washing the sides of the basket during this process.
16. The tubes were centrifuged at 15000xg for two minutes. Additional centrifugation for two minutes was carried out in cases when the wash-I solution was not completely removed.
17. The purification column was transferred to a new tube.
18. Fifty  $\mu$ L of DNase solution was added to the column and incubated at room temperature for 15 minutes.
19. 200  $\mu$ L of DNase wash solution was added to the column.
20. The column was centrifuged at 15000xg for two minutes.
21. Another 200  $\mu$ l of DNase wash solution was added to the purification column and centrifuged at 15000xg for one minute.
22. The purification column was transferred to a new tube.
23. 200  $\mu$ l of wash-II solution was added to the purification column and centrifuged at 15000xg for one minute.
24. 200  $\mu$ l of wash-II solution was added to the purification column and centrifuged at 15000xg for two minutes.
25. The purification column was carefully transferred to a new tube. Care was taken especially to avoid the purification column to come into contact with the waste in the tube prior to or

during the transferring of the column. If that could not be avoided then the tube was centrifuged again for two minutes at 15000xg to remove residue.

26. 50  $\mu$ l of elution solution was added to the purification column and centrifuged at 15000xg for one minute.
27. The purification column was discarded.
28. The tubes containing the purified RNA were lidded immediately and placed in ice and later stored at  $-70^{\circ}\text{C}$  to  $-80^{\circ}\text{C}$  until cDNA was prepared.

## VITA

Krishna P. Shakya was born in Jajarkot, Nepal. He graduated from Veterinary College, University of Agricultural Sciences, Bangalore, India, in 1987. He worked as a Veterinary Officer for Department of Livestock Services, Nepal, before he came for his graduate studies in August, 2002. During this period he was involved in various activities ranging from clinical practice to planning of disease control programs. After graduation he would like to work in the field of parasite host interaction and disease prevention and control. He is married with Asha and has twin daughters Aabha and Aastha.