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Characterizing Peripheral Cellular and Humoral Immune Responses to *Haemonchus contortus* in Sheep

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Characterizing Peripheral Cellular and Humoral Immune
Responses to *Haemonchus contortus* in Sheep

Jesica Rae Jacobs

Thesis submitted to the Davis College of Agriculture, Natural
Resources and Design at West Virginia University in partial
fulfillment of the requirements for the degree of

Master of Science in Animal Physiology

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2013

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Abstract

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Jesica Rae Jacobs

Gastrointestinal nematode parasitism is the most widely reported health concern among sheep producers worldwide. The most concerning of which is *Haemonchus contortus*; a blood-sucking nematode found in the abomasum of sheep. Worldwide anthelmintic resistance has prompted exploration of alternative methods of parasite control. Many studies have shown St. Croix hair sheep possess an immunologically mediated resistance to *H. contortus* infection, however no studies have utilized a true parasite naïve animal for immune characterization. In this study, development of peripheral immune response was characterized in parasite resistant St. Croix lambs and parasite susceptible Suffolk crossbred Lambs. Lambs were separated into three treatment groups: naïve, primary and challenge infection and experimentally infected with *H. contortus* larvae. St. Croix lambs had drastically reduced fecal egg count (FEC) during primary infection and were able to reduce FEC to 0. No FEC was observed in challenged St. Croix lambs. Suffolk crossbred lambs had significantly higher FEC in primary and challenge infections, which persisted to the end of the experimental period. St. Croix lambs maintained significantly higher packed cell volumes (PCV) and challenged St. Croix lambs generated significantly higher numbers of circulating eosinophils and monocytes. Additionally, St. Croix lambs had significantly higher levels of circulating IgA and *H. contortus* antigen specific IgG while Suffolk crossbred lambs had declining levels of antibody. These data indicate St. Croix lambs generate and maintain a more robust immune response to *H. contortus* infection.

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Chapter 1 : Literature Review

Introduction

Overuse of anthelmintic drugs has caused widespread resistance among gastrointestinal trichostrongylid (GIT) worm populations affecting the management of parasitism in commercial sheep. *Haemonchus contortus* in particular has been reported to be resistant to multiple anthelmintics for more than 30 years (Waller, 1999). With no new drugs available for use in the U.S., producers are in search of new methods to manage parasitism. Alternative methods of parasite control include pasture rotation, grazing of natural anthelmintics such as *Sericea lespedeza*, feed additives such as garlic and copper oxide wire particles, breeding for reduced fecal egg count and incorporation of parasite resistant breeds. Some of these methods have had promising results, but not enough to recover the billions of dollars lost due to *H. contortus* and other GIT parasitism (Roeber et al., 2013). However, an alternative method that has resulted in the greatest reduction of parasitism is incorporation of parasite resistant breeds.

St. Croix hair sheep are a parasite resistant breed originating on the St. Croix Island. This breed of hair sheep are slower growing with low carcass weights and are less acceptable for commercial production. The fact that St Croix sheep are highly resistant to *H. contortus* may permit their use as a model for in-depth analysis of immune responses to *H. contortus*. Multiple studies have shown that resistance found in St. Croix sheep lies in immune function and the genetic upregulation of immunity related genes (Gamble and Zajac, 1992; MacKinnon et al., 2009). The ability of St. Croix hair sheep to resist *H. contortus* is due, in large part, to the production of a potent T-helper

type 2 immune response (Gamble and Zajac, 1992). The more robust immune response observed in these sheep results in greater infiltration of innate immune cells (Alba-Hurtado and Muñoz-Guzmán, 2013) as well as development of adaptive immunity (Gamble and Zajac, 1992). A major deficiency of many studies that evaluate immune responses is the lack of naïve sheep as a component of the experimental design. Additionally, most use breeds that have developed GIT-resistance as a result of artificial selection. Subsequently there is a need for an accurate characterization of early cellular and humoral immune responses that occur during a primary and challenge infection in comparison to naïve St. Croix sheep.

Thus, experiments conducted in this thesis aimed to characterize peripheral cellular and humoral immune responses to *H. contortus* in both resistant St. Croix sheep and susceptible Suffolk crossbred sheep. Use of parasite naïve sheep provided an ability to observe the precise development of protective immunity immediately following infection. Knowledge of early immune responses found in parasite resistant sheep may lead to future discoveries of potential parasite management strategies, reducing the impact of GIT parasitism on sheep production in the United States.

Gastrointestinal Nematodes in Sheep

Parasitism in Small Ruminants

Gastrointestinal trichostrongylid parasitism (GIT) is the most frequent reported health issue by U.S. sheep producers (USDA, 2001). In this survey, 74% of sheep producers reported gastrointestinal nematodes present in their flocks. The next highest reported disease was sore mouth at 40% prevalence. The effects of parasitism can result in devastating economic loss. Annual reported costs to the Australian sheep

industry is \$222 million (McLeod, 1995). Economic losses are incurred as a result of lack in gain, poor body scores, reduced wool quality and death; all of which are symptoms common to GIT infection.

Parasites of the superfamily Trichostrongyloidea can cause a variety of problems to ruminant species. This family contains the genera *Trichostrongylus*, *Ostertagia*, *Teladorsagia*, *Haemonchus*, *Mecistocirrus*, *Cooperia*, *Nematodirus*, *Hyostrongylus*, and *Dictyocalulus*. With the exception of *Dictyocalulus*, these parasites dwell in the abomasum and small intestine of ruminants. Of the species in these genera, *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* cause particular concern to small ruminant producers.

Teladorsagia circumcincta is the primary cause of parasitic gastroenteritis observed in sheep raised in temperate regions (McNeilly et al. 2009). Once infected, larvae travel to the abomasum where they infiltrate gastric glands of the abomasum and mature before passing to the lumen. Through this migration, *Teladorsagia* disrupt gastric pit conversion of pepsin to pepsinogen, reducing abomasal chemical digestion of feedstuffs. Pathologies associated with this parasite include weight loss, dehydration, diarrhea and death (Greer et al. 2008). Effects of *Teladorsagia circumcincta* are typically seen in lambs rather than mature adults.

Trichostrongylus colubriformis is found in the small intestine of ruminants. This parasite inhibits small intestinal nutrient absorption resulting in weight loss, lack of weight gain, diarrhea and death (Bowman, 2009). *Trichostrongylus colubriformis* is primarily found in areas of mass sheep production such as New Zealand and Australia, but is not limited to these countries.

The GIT of most concern to sheep producers is *Haemonchus contortus* as it is a blood-feeding parasite and can cause rapid death in young, grazing lambs.

Haemonchus affects sheep worldwide and has been seen to comprise 75% - 100% of GIN populations infecting sheep (Kaplan, 2004). This parasite has a simple life cycle, starting with eggs produced by adult females being released in fecal matter onto pastures where sheep graze. Eggs develop in the environment by going through two larval stages and become infective upon reaching the third stage of their larval development (L₃), after a week in optimal conditions. Larvae of *H. contortus* are particularly sensitive to desiccation; whereby development of larval stages is dependent on a warm, humid environment. Thus, lack of optimal conditions delay progression to the L₃ stage. When consumed by grazing ruminant animals, L₃ infective larvae migrate to the abomasum, begin to feed on blood and will further develop to larval stage 4 (L₄) if the host internal environment is conducive to development.

Larvae begin to deplete the host of blood within seven days of being consumed. Lack of optimal external or internal environmental conditions may induce larvae to become hypobiotic inside the host (Bowman, 2009). During this time, worms are dormant and metabolically inactive. Favorable environmental conditions typically do not exist during winter months in the northern hemisphere leaving eggs and larvae less likely to survive on pastures during these times. Hypobiotic parasites are maintained as L₄ or adult stages in the host. Adult worms may also survive in the abomasum while L₄ larvae remain undeveloped in the alimentary mucosa until environmental cues trigger their development (Herd et al., 1983). These triggers are not completely understood but may include photoperiod and hormone changes associated with pregnancy in the host

(Fleming and Gamble, 1993). Evidence of *H. contortus* emergence from hypobiosis is typically observed as an increase in fecal egg count. Elevated fecal egg counts occurring at the onset of spring is a phenomenon known as “spring rise.” Increased fecal egg count is also found to occur starting two weeks prior to parturition and lasting for 8 weeks following parturition, resulting in a phenomenon known as “periparturient rise” (Crofton, 1954). Periparturient rise has been also hypothesized to be associated with preparturient relaxation of immunity (Beasley et al., 2012).

Under normal conditions L₄ larvae develop to adult worms and feed on blood by piercing the abomasum. Adult *H. contortus* worms are capable of depleting one fifth of total circulating erythrocyte volume daily in a lamb and adult sheep may lose up to one tenth of their erythrocyte volume in a day (Georgi and Whitlock, 1967). Symptoms of haemonchosis are weakness, lack of growth and poor body and wool conditions. Blood feeding by *H. contortus* leads to host anemia and is quantified by low hematocrit and lack of plasma proteins in the blood. Evidence of anemia results in lack of redness in the ocular mucous membrane and is used as an “on-farm” diagnostic of anemia; useful for detecting *H. contortus* infection. Lack of plasma proteins in blood manifests itself as submaxillary edema, commonly known as “bottle jaw.” Animals most affected by *H. contortus* are those already suffering from poor body nutrition, physical stress, malnutrition, heavy infection or aged and weaned animals. If left untreated, host hematopoietic regeneration may be surpassed by blood loss due to parasitism, resulting in death.

Adult stage *H. contortus* worms are capable of sexual reproduction, which marks the patent stage of infection. Adult female worms produce between 5,000 and 10,000

fertilized eggs each day (Bowman, 2009). The eggs are passed through the feces onto pastures and begin development into L₃ stage larvae, which are consumed by the host animal. The entire *H. contortus* life cycle can be completed in 21 days under optimal conditions.

Anthelmintic Resistance

Three classes of drugs are approved for treatment of *H. contortus* which include macrocyclic lactones, imidazothiazoles and benzimidazoles (Bowman, 2009). As a result of overuse, some drugs in each of these classes are no longer effective due to adaptation of drug resistance in GIT (Howell et al., 2008). Commercial sheep operations have relied on chemotherapeutics to manage parasitism and tend to medicate in frequent intervals, often monthly, hastening the development of drug resistance by GIT (Waller, 1999). In 1957, *H. contortus* was the first GIT to be described as drug resistant (Drudge et al., 1957). In response to increasing anthelmintic resistance by GIT, thiabendazole was made available to producers in 1961. Use of thiabendazole was widespread and abundant, which lead to *H. contortus* resistance to thiabendazole (Conway, 1964). Resistance to the next class of drugs released, imidazothiazoles, was reported in the 1980's with *H. contortus* again being the first GIT to become resistant (Prichard et al., 1980). Finally, in the 1990's ruminant GIT populations were described to be resistant to macrocyclic lactones which indicates resistance to all three classes of drugs, worldwide (Waller, 1999).

It has become common to treat an entire flock based on timing of grazing or if any single animal were to exhibit symptoms associated with GIT infection. To combat overuse of anthelmintic drugs researchers have promoted and producers have

adopted the FAMACHA scoring system. Faffa Malan's Chart (FAMACHA) is an on-farm method to determine anemia. The FAMACHA system was developed as a method to selectively deworm sheep based on the concept of maintaining parasitic refugia. Refugia are a population of parasites not exposed to anthelmintic treatment, whereby, these parasites maintain susceptibility to anthelmintics. This system allows sheep with moderate to low levels of *H. contortus* infection to remain parasitized while heavily burdened animals are dewormed; thus maintaining drug-susceptible worms among the worm population. The FAMACHA system scores the level of host anemia and uses a scale of 1 – 5 with the following values scoring palor of the ocular mucous membrane: 1 = red, non-anemic; 2 = red-pink, non-anemic; 3 = pink, mildly-anemic; 4 = pink-white, anemic; 5 = white, severely anemic (Kaplan et al., 2004). Animals with FAMACHA scores of 4 - 5 are treated with anthelmintics, while those receiving scores of 1 and 2 are left untreated. Individual producers determine treatment of animals with FAMACHA scores of 3. Widespread use of the FAMACHA system should permit current and future anthelmintics to retain their efficacy for a longer period of time and as a result managing refugia of GIT populations ensures future anthelmintic efficacy when treatment is required (Kaplan et al., 2004).

Alternative Methods of Control

Prevalence of anthelmintic resistance has prompted an exploration of alternative control methods. Methods of alternative control include various grazing techniques such as pasture rotation (Gujja et al., 2012), grazing *Sericea lespedeza* (Burke et al., 2012) and other high tannin grasses (Oliveira et al., 2013), selecting replacements with low FEC (Bishop, 2012a), the use of copper oxide wire particles

(Vatta et al., 2012), breed specific resistance (Bishop, 2012b) and several food additives such as orange oil (Squires et al., 2010), garlic and papaya (Burke et al., 2009b). These methods may be used on their own or in conjunction with another method. It is common to implement a grazing rotation in addition to one or more of the alternate methods to better manage GIT parasitism in small ruminants.

Sheep become infected with *H. contortus* only when they consume larvae while grazing. Therefore, one method of controlling parasitism is breaking the parasite lifecycle by rotating pastures, thus removing animals from the source of infection. Burke et al. (2009) conducted a grazing study in which lambs were not rotated, rotated by time or rotated by length of grass. They observed decreased FEC and packed cell volume (PCV) in the lambs and increased body weights in all rotational grazing treatments as compared to control lambs. However, there was not a difference in the economic value of any treatment group due to supplementation required due to the poor condition of control animals (Burke et al., 2009a).

In a 1975 study, pasture rotation and *S. lespedeza* grazing were combined in a study in which sheep were rotated between alfalfa or *S. lespedeza* pastures every 3-4 days or every 2 days. In both cases, rotated ewes and lambs had higher nematode burdens and lower weight gains than non-rotated animals (Levine et al., 1975). These studies highlight the difficulties of pasture rotation yet fail to discuss the fact that pasture rotation requires an increase in labor for farmers and producers and may cause increased stress on the animals. Therefore, pasture rotation alone may not be a suitable method of GIT control.

Copper oxide wire particles (COWP) have been heavily studied recently as an alternative anthelmintic in light of failing drugs. Copper oxide wire particles have been studied in multiple breeds of sheep and goats, naturally and experimentally infected animals and their lasting effects studied. Results from these studies have shown that COWP has the ability to reduce the burden of *H. contortus* by approximately 60% in sheep (Bang, 1990; Burke et al., 2004; Knox, 2002; Soli et al., 2010). As sheep are especially sensitive to copper supplementation, care must be taken when administering copper oxide. Therefore treatment using COWP is limited by dose (Burke et al., 2004). Considering COWP only reduced FEC by approximately 60%, the capacity for GIT to build resistance is hastened by COWP administration alone. Thus, COWP is best used in combination with at least some form of rotational grazing, to prevent rapid development of resistance in GIT parasites.

Breeding for parasite resistance is a popular technique employed by sheep producers, however, selection for this trait using FEC measurement can be difficult. Studies in selection for FEC also vary greatly due to animals' exposure to pasture, the level of parasitism of pasture and the resistance status of parasites found in each pasture. There are several factors contributing to variability observed in FEC. As fecal samples are generally taken at weaning, and as a result of varied GIT exposure, a single FEC measurement may not be sufficient to predict lifetime performance of parasite resistance. As few of these factors are equal on a single pasture from year to year and FEC studies are conducted in many places worldwide, these variables are impossible to control for leading to variability in reported data. Calculated heritability of selection for reduced FEC has been reported to range between 0.2 - 0.6 (Vanimisetti et

al., 2004a). As a result of low and often variable FEC heritability, selection for parasite resistance using FEC does not consistently result in resistant offspring (Laurenson et al., 2012) and improvement has been shown to require generations to occur.

Several commercial breeds of sheep have been studied to determine FEC heritability values. Data from these studies indicated that selecting animals for low FEC requires several generations to preserve meaningful improvement (Waller, 1999). Hematocrit or packed cell volume (PCV) has also been studied for use of resistance breeding (Vanimisetti et al., 2004a), however heritability for PCV is lower than FEC as a result of greater environmental impact (Vanimisetti et al., 2004a). Breeds that have been artificially selected for resistance may have developed different genetic mechanisms of parasite resistance whereas natural selection for parasite resistance may have more useful implications. Therefore, it is worthwhile to study breeds of sheep with natural resistance to *H. contortus* to characterize their mechanism of parasite resistance.

Several breeds of sheep have been documented to exhibit natural resistance to *H. contortus*. Resistant breeds typically originate from tropical environments in which animals are exposed to *H. contortus* on a year-round basis. Gulf Coast Native sheep are a breed originating from that region of the United States and have been heavily studied for *H. contortus* resistance and often compared to Suffolk sheep. Gulf Coast Natives have lower FEC and higher PCV upon infection with *H. contortus* (Miller et al., 1998). During this infection, Suffolk sheep suffered from lower PCV indicating increased susceptibility to *H. contortus*, while PCV stayed normal in Gulf Coast native sheep. Breed differences were also found in the periparturient FEC rise, as this rise was less

profound in Gulf Coast Native sheep (Miller et al., 1998). Barbados Blackbelly sheep are a hair breed developed in the Caribbean and been studied to determine their resistance to *H. contortus*. When compared to Dorset and Suffolk sheep, Barbados Blackbelly sheep had lower FEC and higher PCV values. Crossbreeding of Barbados Blackbelly sheep to Dorset or Suffolk sheep also caused higher white cell and hemoglobin counts in all animals during *H. contortus* infection (Yazwinski et al., 1980). Katahdin sheep are a composite breed established in the United States during the 1950's using commercial wool breeds and parasite resistant hair breeds in an attempt to create a breed without the requirement of shearing. Katahdin sheep retain viable production traits while acquiring a greater resistance to *H. contortus* infection than traditional wool counterparts, though not to the extent of their progenitor hair breeds (Vanimisetti et al., 2004b). Of all parasite-resistant breeds of sheep, Katahdins represent the breed with the most promise as a result of greater carcass size and growth rate (Wildeus, 1997). The Katahdin breed is currently the fourth largest breed registry in the United States and the only breed of sheep participating in genetic evaluation for parasitism in the United States (NSIP, 2013).

The most notable breed with naturally-selected resistance to *H. contortus* is the St. Croix. Multiple studies have examined the ability of St. Croix sheep to successfully resist *H. contortus* infection compared to commercial breeds and other parasite resistant breeds (Bradley et al., 1973; Burke and Miller, 2002; Courtney et al., 1985; Gamble and Zajac, 1992). St. Croix sheep consistently have higher PCV, faster clearance of *H. contortus*, as measured by repeated FEC, and resistance upon challenge infection. These studies have resulted in the acceptance of St. Croix hair

sheep as a model of GIT resistance. The mechanism of St. Croix's resistance has been shown to be largely immune mediated (Bowdridge et al., 2013; MacKinnon et al., 2010) and therefore a greater evaluation and characterization of precise immune events resulting in parasite tolerance is warranted.

Immune Response to *Haemonchus contortus*

Cellular Immunity

The response to *H. contortus* begins on an innate, cellular level. The inflammatory environment can be seen fluctuating with the life cycle and migration of parasitic larvae. As larva migrate, the host produces a cellular response involving the infiltration of mast cells, neutrophils, eosinophils, dendritic cells (DC) and macrophages to the site of infection. Upon recognition by toll-like receptors (TLR), possibly 2, 4 and 9 (Ingham et al., 2008), these cells together help to mount an initial response to the parasite (Balic et al., 2006; Lacroux et al., 2006; Maizels et al., 2012). Innate immune cells may attack the larvae as they migrate to the abomasum, however, the larvae shed their outer cuticle during development which aids immune evasion (Lacroux et al., 2006).

When larvae reach the abomasum, approximately four days after ingestion, they begin feeding on blood (Albers and Le Jambre, 1983), which causes damage that signals an immune response. As *H. contortus* develops in the abomasum, antigen presenting cells (APC) sample antigen and migrate to abomasal lymph nodes (ALN) and present antigen to CD4⁺ T helper (Th) cells initiating the development of adaptive immunity. This specificity is indicated by the cellular profile of CD4⁺ Th2 cells in the ALN expressing MHC class II, but not expressing CD25 typical of a regulatory T cell

phenotype (Balic et al., 2000; Murphy, 2012). Activation of CD4⁺ T cells is required for the development of antigen specific B cells (Murphy, 2012) capable of producing immunoglobulins (Ig) G, A and E which have been shown to increase during *H. contortus* and other helminth infections (Bendixsen et al., 2004; Lacroux et al., 2006; Pernthaner et al., 2005; Schallig, 2000; Shakya et al., 2011). An increase in antibody response typically does not occur until later stages of *H. contortus* infection (Lacroux et al., 2006; Pernthaner et al., 2005) suggesting antibody production targets adult parasite and egg antigen.

Upon pathogen invasion, the body responds to infection using both branches of immunity: innate and adaptive. Studies elucidating the importance of both innate and adaptive immunity in resistance to *H. contortus* larvae and adult parasites indicate that both innate (Adams, 1982) and acquired immunity (Meeusen et al., 2005) are crucial in parasite expulsion. Dexamethasone is a glucocorticoid agonist and a common anti-inflammatory steroidal drug which prevents migration of leukocytes (Wang et al., 2007). Dexamethasone was used to suppress immunity in a study using Gulf Coast Native lambs. Neonates (4 weeks of age) and weaned lambs (19 weeks of age) were treated intramuscularly with dexamethasone three times a week for 10 or 15 weeks, respectively. Neonatal lambs were allowed to maintain a natural infection while weaned lambs were dewormed and given an experimental trickle infection of *H. contortus*. Lambs of each age receiving dexamethasone had significantly higher FEC and parasite burdens than controls at necropsy: 2325 ± 335 vs. 238 ± 156 for neonates and 1389 ± 526 vs. 886 ± 329 for weaned lambs (Peña et al., 2004) indicating the immunity plays a critical role in the reduction of worm burden.

Immune responses to challenge helminth infections of sheep have been well studied, yet few reports characterize transient changes in immune response resulting in parasite elimination. Most studies utilize challenged animals that have had prior exposure to helminth infection and thereby are unable to describe the development of immunity from naïve to primary and from primary to challenge infections, particularly in St. Croix sheep. While studies with dexamethasone indicate a role for immunity in parasite reduction, these studies fail to determine the precise mechanism responsible for parasite clearance. To better understand transient changes in immune responses to *H. contortus* it is important to recognize the contribution of individual immune cells, antibodies, cytokines and interactions each together in parasite expulsion.

Innate Cellular Immunity

Mast cells and eosinophils have been implicated in the mechanism of parasite expulsion, and their recruitment is dependent on the cytokine environment elicited by parasite infections. Parasite infections, including *H. contortus*, elicit T helper type 2 (Th2) responses in their hosts (Urban et al., 1996). This response is characterized by the production of interleukin (IL) -4, 5, 9, 10 and 13. Interleukin-5, a Th2 cytokine, functions as an eosinophil chemotactic factor and increases in IL-5 induced by parasitic infections result in the influx of eosinophils (Gleich et al., 1979). Eosinophils are granulated cells including substances such as histamine, eosinophil peroxidase, ribonuclease, deoxyribonuclease, lipase and plasminogen; all of which are released during degranulation in the presence of parasites to assist in expulsion (Reinhardt et al., 2011). Eosinophil degranulation around the parasite damages the cuticle of the worm, which can result in death. Parasites themselves may be responsible for producing some

of the eosinophil chemotactic factor which recruits eosinophils to the area of infection (Reinhardt et al., 2011). While Th2 and mast cells also secrete IL-5 necessary for eosinophil recruitment, upon administration of anti-IL-5, rodents infected with *Nippostrongylus brasiliensis* were unable to recruit eosinophils to the area of infection (Coffman et al., 1989). Lack of eosinophil recruitment led to delayed expulsion of *N. brasiliensis*. Balic et al. (2006) found that sheep immunized 9 weeks prior had elevated numbers of eosinophils 24 and 48 hours after experimental *H. contortus* infection. Granulomas formed at the site of abomasal infection revealed large amounts of eosinophils and apparent death and damage to the larvae (Balic et al., 2006). These eosinophilic granulomas were mostly resolved by 48 hours after infection (Balic et al., 2006). In addition, blood and abomasal eosinophils were found to be higher in Gulf Coast Native sheep compared to Suffolk sheep in experimental *H. contortus* infection, indicating eosinophils are at least involved in differing immune responses of parasite resistant breeds of sheep (Shakya et al., 2011). While eosinophils are important in parasitic infections, they are not the sole mediators of parasite expulsion. Upon administration of anti-IL-5, rodents infected with *N. brasiliensis* were unable to recruit eosinophils to the area of infection, yet infected mice receiving anti-IL-5 were still able to expel the parasitic infection (Coffman et al., 1989). Thus, eosinophils are not explicitly required for helminth clearance.

Mast cells have also been implicated in parasite infections, as they are a type of granulated cell containing histamine and heparin. Mast cells are resident cells in many tissues and generation of mucosal mast cells (MMC) is IL-3 dependent, requiring T cell activation (Koketsu et al., 2013). In this case, T cells must have been presented antigen

from parasites and stimulated to begin producing IL-3. An increased mast cell response did not occur during a *Trichinella spiralis* infection in thymectomized mice (Coffman et al., 1989), implicating the role of antigen specific T cells in the activation of mast cells. Thought to arise from mast cell progenitors, globule leukocytes prevent the establishment of larvae in the crypts of the abomasum (Kemp et al., 2009). Globule leukocytes are found to increase early during *H. contortus* infection (Kemp et al., 2009).

Neutrophils are typically the first cell to respond to pathogens in the body. These innate cells phagocytose pathogens such as viruses and bacteria and rid the body of cellular debris caused by pathogenic invasion. Infiltration of neutrophils to the site of helminth infection has been shown to be influenced by signaling through IL-4 receptor α (IL4R α) (Chen et al., 2012) and most abundant during a patent infection of *H. contortus* (Adams, 1993). Prevention of signaling through IL-4R α has been shown to lead to decreases of neutrophil accumulation and increases in pathology during *N. brasiliensis* infection in mice (Chen et al., 2012). Shakya et al. (2011) demonstrated that neutrophils in the abomasal mucosa increased to day 14 and returned to normal levels by day 21, indicating that early accumulation of neutrophils is associated with larval stage of the *H. contortus* life cycle.

Recently, helminth immunological research has focused on the role of macrophages during GIT infection. Macrophages exhibit plasticity in their ability to shift from a classical (M1) to an alternative (M2) phenotype. Classically activated macrophages are characterized by nitric oxide (iNOS) production, whereas M2 cells produce arginase-1 (Arg1) in mouse models (Eligini et al., 2012). Classically activated macrophages are implicated in inflammatory responses and function to kill pathogens at

sites of infection, whereas M2 macrophages are anti-inflammatory and have functions including wound healing, tissue repair and tumor suppression (Mosser and Edwards, 2008). Enzymes produced by M1 and M2 macrophages, iNOS and Arg1 respectively, compete for the same substrate (arginine) to generate products consistent with each type of polarization. When products of either macrophage phenotype are present in the cell, they will actively downregulate production of the opposing macrophage phenotype. Thus, production of Arg1 is involved in conversion of arginine to ornithine which downregulates M1 type polarization. The Th2 cytokine environment induced by helminth infection, specifically IL-4 signaling, polarizes macrophages to a M2 phenotype (Sato et al., 2010). Alternatively activated macrophages express the mannose receptor (CD206) which may be activated by factors found on helminth parasites (Schallig and van Leeuwen, 1996).

Alternatively activated macrophages have been found at the host-parasite interface during *Heligmosmoides polygyrus* infection in mice and neutrophils, eosinophils, B cells and CD4⁺ T cells make up the remainder of cellular infiltrate (Anthony et al., 2006). Alternatively activated macrophage phenotype was further verified by using the markers secretory protein Ym1, found in inflammatory zone protein (FIZZ1), and acidic mammalian chitinase (AMCase). The abundance of M2 cells were markedly reduced in IL-4 deficient mice indicating that Th2 cytokines drive M2 differentiation, however deficient IL-4 signaling does not hinder the infiltration of neutrophils (Anthony et al., 2006). To determine the importance of M2 macrophages during helminth infections, Anthony et al. (2006) depleted macrophages *in vivo* in C57BL/6 and BALB/c mice using clodronated liposomes transferred to mice infected

with *H. polygyrus*. Clodronate-loaded liposomes are engulfed by macrophages, releasing clodronate, thereby killing the target host macrophage. As a result of macrophage depletion, egg counts remained low yet adults survived in the host as evidenced by worm counts. To be certain these results were the effect of M2 macrophages, the authors treated challenge infected mice with an arginase inhibitor and found similar results. In both macrophage depletion and Arg1 blocking, all other leukocytes remained normal (Anthony et al., 2006). Alternatively activated macrophages have been cultured using the ovine cell line MOCL7 in the presence of crude parasite extracts and recombinant enzymes of *Fasciola hepatica* (Flynn et al., 2007), a helminth causing a strong Th2 response in murine and ovine models. Results of this study indicate that naïve ovine macrophages can be polarized towards an M2 phenotype as a result of exposure to helminth antigen.

T Cells

Generating an appropriate cytokine environment is reliant on the presence of T helper cells. T helper (Th) cells produce a significant levels of IL-4, IL-5, IL-9, IL-10 and IL-13 needed for parasite expulsion (Alba-Hurtado and Muñoz-Guzmán, 2013). Differentiation of naïve Th cells to a Th2 phenotype is dependent upon many factors including; pathogen, MHC signals, co-stimulatory molecules and specific signaling through the interleukin-4 receptor α (IL-4R α) (Ansel et al., 2006). As a result of ligand binding, IL-4R α activates the transcription factor (STAT6), which in turn upregulates expression of GATA3 promoting Th2 related gene expression in the nucleus (Ansel et al., 2006). In the absence of IL-4R α signaling, mice were unable to clear a *Schistosoma mansoni* infection (Dewals et al., 2009).

Adams (1982) demonstrated the importance of immunity during parasite infections by depleting leukocytes using dexamethasone in sheep immunized to *H. contortus*. The time in which dexamethasone was administered severely affected worm loads, whereby, persistent dexamethasone treatment resulted in no difference in worm load but transient dexamethasone treatment during early stages of infection resulted in marked differences in worm loads between the groups (Adams, 1982). These data support the hypothesis that early cellular responses are driving T cell responses that occur shortly thereafter. To establish a role of CD4⁺Tcells during stages of helminth infection, these cells were depleted during a challenge infection at three time points: early, mid and late infection of *H. polygyrus*. Depletion of CD4⁺ T cells early in challenge infection (day 0) using anti-CD4 antibody, lead to significantly increased parasite burdens. Depletion during mid-infection (day 7) had marginal effects on parasite burden and depletion of CD4⁺ T cells at the end of infection had no effect on parasite burdens, indicating that CD4⁺ T cells are critical during early time points (Anthony et al., 2006). These data indicate that T cell activity during early stages of helminth infection is critical for parasite expulsion.

Differentiation of CD4⁺ T cells to a Th2 phenotype occurs through IL-4R α signaling. The two main cytokines that signal through IL-4R α include IL-4 and IL-13. For most effective parasite clearance, Th2 differentiation must occur early in infection for cytokine-mediated recruitment of eosinophils (Alba-Hurtado and Muñoz-Guzmán, 2013). Early sources of IL-4 crucial for Th2 differentiation have been shown to originate from eosinophils, basophils, mast cells and alternatively activated macrophages in at least mouse models of helminth infection (La Flamme et al., 2012; Murphy, 2012). In

sheep infected with *H. contortus*, CD4⁺ T cell percentage was increased in ALN by 3 days and continued to increase to 5 days after infection. Additionally, lymph node weight increased two-fold indicating an influx in antigen presentation in response to infection (Balic et al., 2000). Secondary lymphoid organs are the location of antigen presentation to T and B cells and their proliferation at this site results in lymph node hypertrophy.

CD4⁺ T lymphocytes also function to activate B cells, which in turn create specific antibodies to antigen. In the absence of CD4⁺ T cells, adaptive immunity does not develop, as there is no formation of memory T cells or presentation to B cells, thus no antigen-specific antibody. To test the importance of CD4⁺ T cells in the ability of parasite resistant Gulf Coast Native sheep to resist *H. contortus* infection, Pena et al. (2006) depleted sheep of these cells using anti-ovine CD4 monoclonal antibody for three weeks to deplete CD4⁺ T lymphocytes. Fecal egg counts were significantly higher in CD4-depleted sheep on days 21 and 28 after *H. contortus* infection. Upon adult worm enumeration in the abomasum, animals treated with anti-CD4 antibody had greater worm load than controls (1743 ± 356 vs. 698 ± 308 , respectively) (Peña et al., 2006). These data indicate that T cells, specifically CD4⁺ T cells are critical for parasite expulsion not only by their presence but also signaling of effector mechanisms.

B cells

The role of B cells in parasite expulsion is related to the life cycle of the parasite. Studies show B cell presence is critical in expulsion of nematodes with strictly enteric life cycles such as *H. polygyrus* and *H. contortus*. However, nematodes with hepato-tracheal migration patterns prior to enteric establishment, such as *N. brasiliensis*, are

not B cell-dependent. The requirement for B cells in strictly enteric parasite infections implicates the role of Peyer's patches in the gut (Liu et al., 2010). Peyer's patches are organized Gut Associated Lymphoid Tissues (GALT) and contain specialized microfold cells (M cells) that sample and present antigen to T cells in formed follicles within the Peyer's patch. Follicular T cells surround a large amount of B cells found in the germinal center of the Peyer's patch and subsequently present antigen to them (Murphy, 2012). Mice deficient of B cells were unable to clear a challenge infection of *H. polygyrus*, a strictly enteric helminth parasite (Liu et al., 2010). Typically, *H. polygyrus* primary inoculation results in a chronic infection, however if treated with anthelmintics and given a challenge dose of *H. polygyrus*, mice are able to expel the parasite within 2 weeks of challenge. Mice deficient in B cells were not able to expel adult worms in a challenge infection, however FEC was similar between B-cell deficient and wild-type mice (Liu et al., 2010).

Due to the specialization of Peyer's patches in the gut, B cell presence is of utmost importance during enteric helminth infection. Conversely, clearance of *N. brasiliensis*, is largely T cell mediated in the lung. Primary infections of *N. brasiliensis* in mice show the parasite traveling from the lung to the intestines. However, upon challenge infection, parasites never reach the intestine, indicating they are killed in the lung mediated by T cell activity (Liu et al., 2010). The presence and accumulation of B cells both locally and in the draining lymph node during helminth infection indicate a role for these cells and consequently the antibody that B cells generate.

Humoral responses to H. contortus

The primary function of B cells in any immune response is antibody production. Immunoglobulin (Ig) G, E and A are increased in response to *H. contortus* in parasite resistant sheep (Schallig, 2000). Antibodies can be produced during primary and challenge infections, with IgA typically produced in response to larvae and specific IgG1 and IgG2 in response to adults (Schallig et al., 1994). The authors found that an IgA response during primary infection developed slowly, but was far more rapid upon challenge infection, indicating an anamnestic response being made to *H. contortus* in Texel sheep. Furthermore, it was determined that serum IgA levels against L₃ larvae and serum IgG1 and IgG2 levels against adult worms increased slowly during primary infections and quickly during early challenge infection. Overall serum antibody levels were doubled during challenge compared to primary infection (Schallig et al., 1994).

Antibody production has been shown to play a crucial role in defense against *H. contortus*. Immunoglobulin (Ig) A, IgE and antigen specific IgG have been shown to be elevated in *H. contortus* infections (Kooyman et al., 1997; Lacroux et al., 2006; Meeusen et al., 2005). In order for antibodies to be made, B cells must be present and activated by T cells. As previously mentioned, mice deficient in B cells were infected with *H. polygyrus*. As expected, B cell deficient mice were not able to produce antibody in response to a primary or secondary infection leading to inability to clear a challenge infection, as described above (Liu et al., 2010). These data indicate a role for antibody produced by B cells as an effector molecule contributing to parasite expulsion.

There is a clear role for humoral immunity in parasite resistant breeds of sheep. To illustrate this dexamethasone was used to suppress immunity. Lymphocyte count in

dexamethasone treated sheep were significantly lower than control sheep and furthermore, antibodies to whole worm antigen were decreased in both dexamethasone treated age groups compared to controls (Peña et al., 2004). Reduction in lymphocyte and antibody responses resulted in higher worm burden of dexamethasone treated groups (Peña et al., 2004).

Resistant breeds of sheep have been shown to generate greater antibody responses upon primary and challenge infection with *H. contortus*, as well as having more circulating IgA in non-infected animals (MacKinnon et al., 2010). No differences in circulating IgE levels were observed between parasite susceptible and parasite resistant breeds, however higher concentrations of IgE in lymph node extract was observed 27 days after infection (MacKinnon et al., 2010). Infected, resistant sheep had higher levels of IgA as early as 3 days and as late as 21 days after infection when compared to parasite susceptible sheep. These data indicate a role for antibodies in parasite clearance and the development of full protective immunity in parasite resistant sheep (MacKinnon et al., 2010).

Collectively, these studies demonstrate that the mechanism of parasite resistance of some breeds of sheep lies in an early, robust cellular immune response and the ability to generate lasting memory responses in the form of antibodies. Nonetheless, no “smoking gun” immune mechanism has been observed in parasite resistant sheep, which would empirically differentiate resistant and susceptible sheep. It has been documented that the response of parasite resistant breeds is simply more robust than that of parasite susceptible sheep, but all branches of the immune system are needed in concert for adequate defense against *H. contortus*. However, very few

published studies have characterized the development of protective immunity from naïve to primary infection with *H. contortus* in sheep and further between the primary and challenge infection. Moreover, characterization of these immune events has not been well-described in St. Croix sheep. Discovering immune events associated with the early stages of *H. contortus* infection will be critical in the discovery of future treatment methods within economically-important breeds of sheep.

Chapter 2: Experimentation

Materials and Methods

Experimental Design

This study was conducted in the fall of 2011. Pregnant St. Croix ewes (provided by Virginia Tech) and pregnant Suffolk-crossbred ewes lambed in an elevated floor barn where lambs were kept through the duration of the experiment. Four weeks after weaning, lambs of each breed were randomly assigned to one of three treatment groups: naïve, primary or challenge infection. Five lambs were assigned to the primary and challenge infection treatments of each breed, 5 lambs were assigned to the naïve St. Croix treatment and 3 lambs were assigned to the naïve Suffolk-crossbred treatment due to limited availability of stock. A naïve Suffolk-crossbred lamb died due to natural causes between experimental days 21 and 28 resulting in a treatment group of 2 lambs for the remainder of the experiment. Fecal Egg Counts (FEC) were performed on all lambs to assure an FEC of 0 using the Modified McMaster's Technique (Whitlock, 1948). Lambs assigned to the challenge infection group were infected by oral inoculation with 10,000 larval stage 3 (L₃) infective *Haemonchus contortus* larvae.

Primary infection of challenge lambs persisted for 5 weeks after which lambs were orally administered moxidectin (Boehringer Ingelheim, Ridgefield, CT) at a dosage of 0.2 mg/kg of body weight orally. Two weeks later, FEC was performed to determine anthelmintic efficacy. At this point, Suffolk-crossbred lambs were still shedding eggs, thus, all primary animals were administered an oral dose of levamisole hydrochloride (AgriLabs, St. Joseph, MO) at a dosage of 8 mg/kg of body weight. Fecal egg counts were performed again one week after Levamisole administration to assure primary

lambs were no longer shedding eggs. As all FEC were zero, challenge lambs were allowed a five-week rest period before the start of the experiment.

Naïve Model

Pregnant ewes on loan from Virginia Tech were transported to the West Virginia University Animal and Veterinary Science Farm. Animals were housed in the Sheep Research Barn constructed with a raised, expanded metal floor. Feces from sheep fall through the floor, separating possibly infected feces from the animals' food source. Pregnant ewes birthed experimental lambs on the raised floor and lambs were kept in the research barn from birth through the duration of the experiment, leaving them unexposed to *H. contortus*. Naïve lambs were sampled prior to experimentation to further prove lack of *H. contortus* exposure.

Sample collection

On day 0 of the experiment, primary and challenge lambs were each given an oral inoculum of 10,000 L₃ *H. contortus* larvae. Blood and fecal samples were taken from all experimental animals. Blood samples were obtained from each animal via jugular venipuncture and 10ml of whole blood was collected into untreated vacutainer tubes (Tyco, Mansfield, MA) and 4ml of blood was collected in vacutainer tubes treated with ethylenediaminetetraacetic acid (EDTA) (Tyco, Mansfield, MA) to prevent coagulation. Blood and fecal samples were collected from each lamb daily from days 0-14 and weekly for an additional 5 weeks. On day 49, primary and challenge lambs were de-wormed using Levamisole at 8 mg/kg.

Parasitological Evaluation

***H. contortus* larval culture**

Larvae were obtained by fecal culture followed by the Baerrman Technique (Zajac and Conboy. 2012). Feces containing *H. contortus* eggs were collected from chronically infected adult sheep housed in the elevated floor barn. Feces were mixed with sterile peat moss and activated charcoal and allowed to incubate for one week to allow eggs to develop to L₃ larvae. Larvae were then collected by the Baermann Technique and diluted to 1,000 *H. contortus* larvae per ml in PBS (pH 7.4). Each lamb was given an oral inoculum of 10ml of diluted *H. contortus* larvae.

Fecal Egg Count

Fecal egg counts (FEC) were performed on each animal using the Modified McMaster's technique (Whitlock, 1948). Briefly, 56ml McMaster's Salt Solution (SPG 1.2) was added to 4g of feces and homogenized then strained through a double layer of cheese cloth. Strained solution was charged into both chambers of a McMaster's slide and eggs were counted in each grid. Total egg count from both grids was multiplied by 50 to yield eggs/gram of feces.

Blood and Serum Analysis

Blood and Serum Analysis

Blood for serum analysis was collected into non-treated blood vacutainer tubes. Tubes containing blood were then chilled at 4°C for 20 minutes and centrifuged at 751 x g for 20 minutes at 4°C. One ml of serum was retained from each sample in a 1.7ml microcentrifuge tube and stored at -80°C.

White blood cell differentials

Blood smears were made for each animal using 5µl of whole, EDTA-treated blood on glass slides. Slides were allowed to air dry at room temperature. Dried slides were stained in Camco Quik Stain (Cambridge Diagnostic Products, Ft. Lauderdale, FL) for 30 seconds and rinsed three times with deionized water and allowed to dry before being counted. One hundred cells were counted on each slide at 40x magnification. Percentages of lymphocytes, monocytes, neutrophils, eosinophils and basophils were multiplied by white blood cell count to determine specific cells/ml of whole blood.

Lymphocyte separation, staining and counting

One ml of whole blood was removed from EDTA treated blood tubes and moved into a sterile 15 ml centrifuge tube. One ml of PBS was added and the solution was mixed. Then, 2 ml of ACK lysis buffer (Lonza, Walkersville, MD) was added, mixed and allowed to incubate at room temperature for 5 minutes. After which, 4ml of PBS was added and the solution was centrifuged at 751 x g for 5 minutes at 4°C. The supernatant was removed and discarded. Re-suspension and centrifugation steps were repeated 3 times. Finally, the washed pellet was re-suspended in 1ml of PBS and kept on ice. In a new microcentrifuge tube, 25 µl of sample, 375 µl of PBS and 100 µl of trypan blue (Thermo Scientific, Waltham, MA) were gently mixed. Each side of a hemacytometer (Thermo Scientific, Waltham, MA) was charged with 12 µl of solution and live cells were counted. Total count of 8 squares was multiplied by 10,000 and again by dilution factor to determine cells/ml.

Eosinophil staining and counting

Using a modification of Becton-Dickinson unopette method, eosinophils were counted in whole blood. Briefly, 25 μ L of whole blood from EDTA was added to conical 1.5 ml microtubes pre-filled with 375 μ L of Phyloxin B solution. The solution was mixed well and allowed to incubate for 10 minutes at room temperature. Each side of a hemacytometer was charged with 12 μ L of the solution and stained eosinophils were counted (Costello, 1970; MacFarlane and Cecil, 1951).

Packed cell volume

Whole blood from EDTA treated tubes was added to heparin treated microhematocrit tubes (StatSpin, Westwood MA). Tubes were placed into a microcentrifuge (StatSpin, Westwood MA) and centrifuged for 3 minutes. Red blood cell percentage was determined by digital hematocrit reading (StatSpin, Westwood MA).

Total IgG ELISA Assay

Serum samples were stored at -80°C until assays were performed. Frozen serum samples were thawed and diluted (1:7200) in PBS (pH 7.4) with 0.1% bovine serum albumin (PBSB). A 96-well high-binding plate (Dynex, Chantilly, VA) was coated with 100 μ L of rabbit anti-sheep IgG (Sigma-Aldrich St. Louis, MO) diluted to 5 μ g/ml in 0.05M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C . The plate was then allowed to reach room temperature (RT) and was washed 5 times with 300 μ L of PBS (pH 7.4) with 0.05% tween-20 (PBST). After washing, the plate was blocked using 200 μ L of PBSB and incubated at RT for 30min then washed 5 times with PBS-T. Serum samples diluted 1:7200 were added to the plate in duplicate, in addition to standards (sheep IgG) and blanks (PBSB); all at a volume of 100 μ L. The plate with

samples, standard and blanks was incubated on a plate shaker at RT for 2hr then was washed with PBST 5 times before adding 100µl of rabbit anti-sheep IgG conjugated with HRP (Sigma-Aldrich St. Louis, MO) diluted 1:10,000 in PBSB. The plate was incubated at RT for 1hr then washed 10 times with PBST. To measure optical density, 100µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Pierce, Rockford, IL) was added and allowed to incubate at room temperature for 20min then 100µl of 2M H₂SO₄ was added to stop the reaction. Optical density of each well was measured using a Bio-Tek ELx800 (Winooski, VT) spectrophotometer at 450nm. All raw values (samples and standards) were adjusted for blank absorbance then concentration of IgG was calculated using a standard curve with a detection range of 1000 – 15.6ng/ml. Coefficient of variation between sample duplicates was < 10% and inter-plate CV was < 10% for all plates analyzed. If duplicate samples had a CV >10%, the duplicates were analyzed again to reach an acceptable CV. Final concentration values were multiplied by the serum dilution factor to yield mg/ml of IgG.

Crude antigen specific IgG ELISA assay

To determine the appropriate concentration of crude worm antigen (CWA) and detection antibody a checkerboard titration was performed. Thus, whole worm antigen was diluted to 4µg/ml in 0.05M carbonate-bicarbonate buffer and added to a 96 well high-binding microtiter plate (Dynex, Chantilly, VA) where 100µl were added per well. Plate was allowed to incubate overnight at 4°C before washing 3 times with PBST. Non-specific binding was prevented by blocking using 200µl of PBSB per well. The plate was incubated at RT for 30 min then washed 5 times with PBST. Serum samples were diluted 1:600 in PBSB and 100µl of serum dilution was added to the plate in

duplicate in addition to blanks and negative control. Negative control serum samples were collected from *H. contortus* naïve sheep located at WVU. After the addition of serum samples, blanks and negative controls the plate was incubated at RT for 2 hr followed by washing 5 times with PBST. To detect antigen-specific IgG, rabbit anti-sheep IgG, conjugated with HRP, was diluted to 1:1500 and 100µl were added to all wells. Plate was incubated at RT for 1 hr then washed 10 times with PBST. Detection substrate (TMB) (Pierce, Rockford, IL) was added to all wells (100µl/well) and incubated at RT for 20 min. The reaction was stopped using 100µl of H₂SO₄ and optical density was measured using a Bio-Tek ELx-800 microplate spectrophotometer at 450nm. All data were adjusted by subtracting the absorbance value of the blanks. Inter-sample CV was less than 10% for all samples, however, if inter-sample CV exceeded 10% samples were analyzed again to reach an acceptable CV. Inter-plate CV was determined using absorbance of negative control samples and did not exceed 10%. Data reported are blank-adjusted optical density (OD) values at 450nm.

Antigen Preparation

Adult *H. contortus* worms extracted from abomasum were pulverized using a dounce homogenizer on ice. Crude worm antigen (CWA) was prepared as described in Bowdridge et al., (2013). Lysate centrifuged at 15,000 x *g* at 4°C for 1hr. Supernatant was removed and sterile filtered through 0.22µm syringe filter. Sterile-filtered supernatant protein concentration was determined using micro-BCA assay (Pierce, Rockford, IL). After protein quantification, protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) was added at a rate of 1µl of cocktail per µg of protein. Crude worm antigen was aliquoted into 1.5ml microcentrifuge tubes and stored at -80°C until further use.

Total IgA ELISA Assay

Serum samples were stored at -80°C until assays were performed. Frozen serum samples were thawed and diluted (1:16000) in PBS (pH7.4) with 0.1% bovine serum albumin (PBSB). A 96-well high-binding plate (Dynex, Chantilly, VA) was coated with 100µl of rabbit anti-sheep IgA (Bethyl Laboratories Montgomery, TX) diluted to 5µg/ml in 0.05M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Plate was then allowed to reach room temperature (RT) and was washed 5 times with 300µl of PBS (pH 7.4) with 0.05% tween-20 (PBST). After washing, the plate was blocked using 200µl of PBSB and incubated at RT for 30min then washed 5 times with PBS-T. Serum samples diluted 1:16,000 were added to the plate in duplicate, in addition to standards (sheep IgA) and blanks (PBSB); all at a volume of 100µl. Plate with samples, standard and blanks was incubated on a plate shaker at RT for 2hr then was washed with PBST 5 times before adding 100µl of rabbit anti-sheep IgA conjugated with HRP (Bethyl Laboratories Montgomery, TX) diluted 1:4,000 in PBSB. The plate was incubated at RT for 1hr then washed 10 times with PBST. To measure optical density, 100µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Pierce, Rockford, IL) was added and allowed to incubate at room temperature for 20min then 100µl of 2M H₂SO₄ was added to stop the reaction. Optical density of each well was measured using a Bio-Tek ELx800 (Winooski, VT) spectrophotometer at 450nm. All raw values (samples and standards) were adjusted for blank absorbance then concentration of IgG was calculated using a standard curve with a detection range of 250 – 1.95 ng/ml. Coefficient of variation between sample duplicates was < 10% and inter-plate CV was < 10% for all plates analyzed. If duplicate samples had a CV >10%, the duplicates were

analyzed again to reach an acceptable CV. Final concentration values were multiplied by the serum dilution factor to yield mg/ml of IgA.

Statistical Analysis

Fecal egg count data were normalized by using the formula $\text{Log}_{10}(\text{FEC}+25)$ and back transformed for means reporting. Data were analyzed using the General Linear Model (GLM) in SAS with fixed effects of treatment, breed and time. All two way and three way interactions were analyzed. Means comparisons for main effects were performed using the Bonferroni procedure. Means comparisons of interactions were analyzed using LS means where differences were detected using Bonferroni's procedure. Significance was accepted at $P < 0.05$. Data from priming and experimental infections were analyzed separately.

Chapter 3: Results

Parasitological Results

Fecal Egg Count

During the priming infection, Suffolk crossbred (SX) lambs generated an FEC twice that of St. Croix (STC) lambs ($P < 0.0001$) (Figure 1A). The highest FEC of Suffolk crossbred (SX) lambs (840 eggs/g) occurred on day 28 (Figure 1A) while FEC of St. Croix lambs peaked on day 14 (250 eggs/g), followed by a reduction to 0 by day 35 (Figure 1A). All lambs were then treated with an anthelmintic (Levamisole, 8 mg/kg) and rested for 5 weeks.

During the main experiment, FEC of primary infected SX lambs peaked at 6950 eggs/g on day 28 whereas challenge infected SX lambs peaked at 3270 eggs/gram feces on day 35 (Figure 1D). There was no statistical difference in FEC between primary and challenge infected SX lambs. Naïve lambs of each breed remained at 0 for the duration of the experiment (Figure 1B). Primary infected STC lambs generated a peak FEC of 3210 eggs/gram feces on day 28 before reducing to 0 eggs/gram feces by day 35, similar to data observed during the priming infection (Figure 1C). However, challenge infected STC lambs never generated an FEC (Figure 1B, 1C). Furthermore, FEC of primary infected differed from FEC of challenge infected STC lambs ($P < 0.0001$). Primary SX lambs had higher FEC compared to STC lambs (3403.75 vs. 758 eggs/g, respectively, $P < 0.0001$, Figure 1B).

When breed and infection status were compared, primary SX lambs had a significantly higher FEC compared to STC lambs (3403.75 vs. 758 eggs/g, respectively,

$P < 0.0001$, Figure 1B) and the peak in STC FEC during primary infection was 46% of that in SX. Challenged SX lambs generated an average FEC of 1520 eggs/g while challenged STC lambs never had a measureable FEC throughout the course of the experiment (Figure 1B).

Packed Cell Volume

Packed cell volume (PCV) remained similar between breeds during the first two weeks of the priming infection, but began to deviate between groups by day 21 (Figure 2A). Statistical analysis revealed that STC lambs had higher average PCV than SX lambs during the priming phase (Figure 2B).

A similar trend in PCV was seen during the main experiment as significant differences in PCV were not observed between breeds, however, PCV in SX lambs steadily declined as time progressed, though there was no effect of time (Figure 2C). Average PCV for SX naïve animals during the main experiment was 30.94% with primary and challenge animals were very similar at 29.15% and 29.7%, respectively (Figure 2D). St. Croix lambs had slightly higher PCV with naïve lambs averaging 36.35%, primary lambs at 35.73% and challenge lambs at 34.01% (Figure 2D).

Cellular Results

Total White Blood Cell Count

Averaged across all time points, SX lambs had higher circulating WBC counts than STC lambs; 3.37×10^6 vs. 3.01×10^6 WBC/ml, respectively ($P < 0.05$, Figure 3A). However, STC primary and challenge infected lambs tended to maintain higher average circulating blood cells above the naïve baseline throughout the course of the

experiment, particularly from day 35 to the conclusion of experiment (Figure 3B). Challenge SX WBC count stayed below the naïve average throughout the experiment while WBC count of primary lamb SX lambs surpassed the naïve average at few time points (Figure 3C).

Average naïve SX circulating WBC count across all time points was 3.54×10^7 cells/ml while the primary SX average was 3.31×10^7 cells/ml whole blood and the challenge average was 3.34×10^7 cells/ml (Figure 3D). Circulating WBC count of STC lambs demonstrated a statistical increase from naïve to challenge infection status. Naïve STC lambs had an average circulating WBC of 2.69×10^6 cells/ml while primary and challenge STC lambs had 2.96×10^6 and 3.32×10^6 cells/ml, respectively (Figure 3D).

Lymphocytes

Analysis of circulating lymphocyte counts revealed that SX lambs across all treatments had greater lymphocyte counts than STC lambs ($P < 0.05$, Figure 4A). St. Croix primary and challenge lymphocyte counts were consistently above the naïve average from day 28 to day 49 (Figure 4B), however no statistical differences were observed. Challenge SX lymphocytes fell below the naïve average on day 28, but otherwise remained above. No type by treatment interaction was found, though a similar trend of elevated lymphocytes was observed in STC lambs (Figure 4C).

Challenge SX lymphocytes fell below the naïve average on day 28, but otherwise remained above whereas primary SX lymphocytes fluctuated around the naïve average (Figure 4C). Primary and challenge lymphocyte count of SX lambs decline steadily beginning at day 35 and continuing to day 49 (Figure 4C).

Neutrophils

Averaged across all time points, SX lambs had greater circulating neutrophil counts than STC lambs; 1.38×10^6 vs. 1.22×10^6 neutrophils/ml, respectively ($P < 0.05$, Figure 5A). However, similar to circulating WBC, STC neutrophils increased numerically from naïve to primary and challenge infection status (Figure 5B) while SX primary and challenge infected lambs had numerically fewer circulating neutrophils than their naïve counterparts (Figure 5C).

Again, while no type by treatment interactions were observed, STC neutrophils exhibited a similar trend to WBC and lymphocyte data with challenged lambs having numerically greater neutrophils than STC naïve lambs (Figure 5D) while SX primary and challenge lambs were numerically lower than naïve lambs (Figure 5D).

Monocytes

Averaged across all time points, SX lambs had greater circulating monocyte counts than STC lambs; 1.02×10^6 monocytes/ml vs. 8.95×10^5 neutrophils/ml, respectively ($P < 0.05$, Figure 6A). Additionally, breed by time effects were observed in STC lambs. No statistical differences in circulating monocytes were seen in SX lambs of any treatment (Figure 6C). Additionally, challenge infected STC lambs generated a greater average circulating monocytes than SX lambs (9.81×10^5 vs. 8.05×10^5 , respectively) (Figure 6D).

Averaged across all time points, SX monocytes numerically increased from naïve to primary infection and numerically decreased from primary to challenge infection. Naïve SX lambs had an average of 1.89×10^4 monocytes/ml which increased to 2.19×10^4 monocytes/ml during the primary infection and then decreased to 2.05×10^4

monocytes/ml during the challenge (Figure 6D). Conversely, STC lambs had a step-wise increase in monocytes from naïve to challenge infection. Naïve STC lambs generated an average of 2.12×10^4 monocytes/ml, which increased to 2.48×10^4 monocytes/ml during the primary infection and was the highest during the challenge infection with 2.90×10^4 monocytes/ml (Figure 6D).

Eosinophils

Though eosinophils were counted both via blood smear and direct phyloxin staining, only data from phyloxin staining are represented. Eosinophil counts from blood smears are often skewed as a result of their rare nature, thus eosinophil stain counts were used to more accurately document changes of eosinophils in blood.

During the first 7 days of *H. contortus* infection, STC lambs generated significantly more circulating eosinophils than STC naïve and primary lambs ($P < 0.05$, Figure 7A). In SX lambs, primary infected lambs generated greater eosinophils than challenge SX lambs ($P = 0.0020$, Figure 7B). Analysis of days 7-49 showed a similar trend in that challenge STC lambs generated greater concentrations of eosinophils than STC naïve lambs ($P = 0.0006$, Figure 7C) and, again, primary SX lambs generated greater eosinophils than SX challenge lambs ($P < 0.0001$, Figure 7D).

In addition to generating more circulating eosinophils than STC naïve and primary lambs in the first 7 days, STC challenge lambs also had greater eosinophil counts than SX challenge lambs ($P < 0.0001$, Figure 7E). Naïve and primary animals of each breed were not significantly different from each other.

During experimental days 7-49, challenged STC lambs again had greater eosinophil counts than STC naïve lambs ($P = 0.0006$, Figure 7F). In addition, STC

challenge lambs had greater eosinophil concentrations than SX challenge lambs ($P = 0.0064$, Figure 7F). Again, SX primary lambs had greater circulating eosinophils than SX naïve and challenge lambs on days 7-49 ($P < 0.0001$, Figure 7F) in as well as greater eosinophil counts than STC primary lambs ($P < 0.0001$, Figure 7F). There were no differences between naïve STC and SX lambs during days 7-49.

Basophils

No significant differences were observed in circulating basophils between breeds or treatments. However, when evaluated by time, basophil counts of infected STC lambs were consistent (Figure 8A) increasing towards the later stages of infection. Primary and challenge SX lambs had reduced numbers of basophils beginning on day 21 (Figure 8B).

Averaged across all time points, SX lambs had similar numbers of circulating basophils while STC lambs, again, displayed an increase in basophil count from naïve to challenge infection. St. Croix naïve lambs generated 2.12×10^4 basophils/ml, which increased to 2.48×10^4 basophils/ml during primary infection and increased again to 2.90×10^4 basophils/ml during the challenge infection (Figure 8C). This trend was not observed in SX lambs whereby naïve SX lambs averaged 1.89×10^4 basophils/ml, which increased to 2.91×10^4 basophils/ml during the primary infection and reduced to 2.05×10^4 basophils/ml during challenge infection (Figure 8C).

Antibody Results

Total IgG

Total circulating IgG of STC lambs stayed above or at naïve baseline throughout the experimental time beginning on day 7 (Figure 9A). Suffolk crossbred total IgG stayed above the naïve average to day 35, yet was significantly reduced on days 42 and 49 ($P < 0.001$, Figure 9B). Total circulating IgG analysis revealed SX lambs have significantly higher circulating IgG than STC lambs; 32.2 mg/ml serum vs. 15.7 mg/ml serum, $P < 0.0001$ (Figure 9C). There were no differences in total-IgG between treatments within breeds. On average, SX naïve lambs had an average circulating IgG of 31.79 mg/ml, while primary lambs had a very similar amount of 31.42 mg/ml, which slightly increased during challenge infection to 33.40 mg/ml (Figure 9C). St. Croix naïve lambs had an average of 15.31 mg/ml IgG, primary lambs generated 18.18 mg/ml IgG and challenge lambs had 15.41 mg/ml IgG (Figure 9C).

Crude Worm Antigen Specific IgG

Crude worm antigen (CWA) IgG increased in STC lambs from day 0 to day 35 before beginning to decline on day 42 through day 49 (Figure 10A). Within the STC breed, challenged lambs had significantly higher CWA-IgG than naïve lambs ($P < 0.0001$, Figure 10C). Infected SX lambs stayed largely below the naïve average (Figure 10B) and there were no effects of treatment. Analysis of CWA-IgG showed STC lambs had significantly higher CWA-IgG than SX lambs ($P < 0.001$, Figure 10C). There were no differences between CWA-IgG levels in SX treatments, however challenge infected SX lambs had the lowest numerically observed CWA-IgG of all breeds and treatments (Figure 10C).

Naïve SX lambs had an average absorbance of 0.54, which decreased to 0.51 in the primary infection and further decreased to 0.44 in the challenge infection (Figure 10C). Conversely, in St. Croix lambs generated greater CWA-IgG as infection status progressed. Naïve STC lambs had an absorbance of 0.63, which increased to 0.80 in the primary infection and peaked during the challenge infection at 0.97 (Figure 10C).

IgA

Averaged across all time points, STC lambs generated greater circulating IgA than SX lambs ($P < 0.0001$, Figure 11A). A breed by time effect was observed with STC lambs having statistically higher total circulating IgA than SX lambs from day 14 to day 49 (Figure 11B). Circulating IgA of STC lambs increases through day 28 at which point it gradually declines ($P < 0.05$, Figure 11B) whereas SX total IgA concentration steadily decreases through the course of most of the experiment (Figure 11B). Naïve STC lambs generated greater circulating IgA than SX naïve lambs ($P = 0.0049$, Figure 11C) and STC challenge lambs had greater circulating IgA than STX challenge lambs ($P < 0.0001$, Figure 11C).

Chapter 4: Discussion

Naïve lambs have been extensively used as a control in previous research studies (Heckendorn et al., 2007; Martínez-Ortíz-de-Montellano et al., 2010; McNeilly et al., 2013; Pemberton et al., 2012), however in most cases, lambs were not truly naïve or generation of naïve lambs was not detailed. Often, “naïve” is used as a term to describe uninfected lambs, resulting in control animals that have had prior exposure to parasites, yet remain uninfected during the course of experimentation. Use of previously infected animals as “naïve” control animals is not accurate as previously infected animals have had the opportunity to create anamnestic immune responses with unknown durations. Our experimental design makes use of animals born in an environment free of *Haemonchus contortus* and allows those lambs to remain there until the end of their experimental use. Lambs used in these experiments were confirmed naïve to *H. contortus* by repeated FEC that consistently remained at 0. As a result, our experiments have a true naïve control for comparison. A study using naïve lambs infected with a single bolus dose of 1×10^6 L3 *H. contortus* larvae reported that primary lambs generated an FEC immediately after infection that continued to rise through 10 days of experimentation (Christie et al., 1975). Our experiment showed a minimum of one week was required for the appearance of eggs in feces upon first infection of naïve lambs, however lambs in this study were given a smaller initial dose of *H. contortus* larvae than in the Christie study (1.0×10^4 vs. 1.0×10^6 L3 larvae). Our data also show peaks in FEC followed by gradual decreases in FEC as animals are not being re-infected, though discrepancy between our and Christie’s data could be due to breed and larval dosage difference.

Through use of naïve sheep in this experiment, the response of St. Croix and Suffolk Crossbred sheep to *H. contortus* was able to be observed during a primary infection. Data measuring primary responses to *H. contortus* infection are scarce, especially in the St. Croix breed. Though the self-cure phenomenon is well documented, such studies were conducted with animals chronically exposed to *H. contortus* (Luffau et al., 1981). Our model of naïve to primary or challenge exposure allowed us to observe transient peripheral innate and humoral immune responses to a single group of parasites, similar to a vaccine and booster. Interestingly, while not statistically different, challenged SX lambs had numerically lower FEC than primary SX lambs, indicating a possible role for vaccine / booster type treatment in production lambs prior to pasture exposure.

Thus, using a naïve model, we have found that STC lambs are capable of reducing FEC to 0 upon first exposure to infection. Typically, female *H. contortus* worms would be producing eggs by 21 days after infection (Bowman, 2009) which would be seen in the feces, however neither primary or challenge STC lambs were shedding eggs at this time, which could mean no adult worms were present, or an effect on fecundity of worms present in these animals or, finally, could be the result of immune attack specific to *H. contortus* eggs.

Additionally, the absence of FEC in challenge STC lambs is particularly compelling data supporting the hypothesis that St. Croix sheep are resistant to *H. contortus* infection. These studies did not evaluate worm load at the site of infection and therefore it can only be speculated that STC lambs have an effect on worm clearance, notably during *H. contortus* challenge. However, these preliminary data

provide a basis for developing future experiments to determine the ability of STC sheep to eliminate parasitic larvae or worms.

Despite being fed a 16% protein diet throughout the experiment, PCV in SX lambs decreased as FEC increased. This is typical as greater numbers of parasites lead to greater blood loss in sheep. Packed cell volume of STC naïve lambs was higher than that of SX lambs, which could possibly be a characteristic evolved as a result of constant *H. contortus* challenge in STC lambs. Previous research has shown sheep with hair breed backgrounds or that are crossed with St. Croix have higher PCV during *H. contortus* infection (Vanimisetti et al., 2004b), though truly naïve St. Croix sheep have not been observed for baseline resting PCV until now.

While differences were not observed in most cell types between naive animals of either breed, differences in challenged animals were common. St. Croix challenged animals had numerically greater cell count than naïves, which was observed in every cell analysis. These data indicate that immunological memory in response to *H. contortus* infection is being developed and utilized by STC lambs. While no single cell type can be indicated as the source of St. Croix's ability to clear *H. contortus* during a primary infection, a clear trend is seen in most circulating cell types. St. Croix lambs generate a step-wise increase in many cell types, including total WBC, lymphocytes, neutrophils, monocytes, eosinophils and basophils from naïve to primary to challenge infection indicating a collaborative and efficient immune response. A study using Awassi sheep, a dairy breed of sheep, showed WBC was low in *H. contortus* infected animals as compared to controls, fewer neutrophils compared to controls, unchanging lymphocytes, and slightly increased monocytes (Ali, 2012), which is consistent with the

lack of immune response observed in SX lambs. Previous research using murine models have shown that elimination of some cell types, including eosinophils, does not leave mice unable to clear infections, though it does delay expulsion (Coffman et al., 1989). These data are consistent with the idea that one cell type is not solely responsible for parasite expulsion, but rather a collaboration of innate immunity.

Cellular responses of SX challenged lambs can best be described as lackluster as challenged SX lambs had cell counts that were numerically lower than or near naïve controls. During the primary infection, SX lambs had decreased cell counts in comparison with their naïve controls as was the case in lymphocyte and neutrophil counts. The only cell type to be statistically increased in SX animals of any treatment was eosinophils during the primary infection, however there was a large amount of variance associated with this statistic with one single animal with high circulating eosinophils being the source of most of the variation found. Eosinophils have shown to be commonly elevated in parasite resistant breeds of sheep (Alba-Hurtado and Muñoz-Guzmán, 2013), which is consistent with data seen in this experiment. As with all other cell types, STC eosinophils showed a step-wise increase from naïve to challenge lambs during early *H. contortus* infection with the same trend appearing in the latter half of the experiment. Conversely, challenge SX lambs showed a dramatic decrease of eosinophils in both early and late infection and primary SX lambs had elevated eosinophils. Thus, while eosinophilia is associated with reduced FEC in STC lambs, SX primary lambs generated greater numbers of eosinophils than STC lambs without a reduction in FEC, indicating eosinophils alone are not sufficient for reduced FEC.

The lack of immune response in Suffolk sheep is well documented, yet the question remains why do these sheep fail to generate protective immunity? Data presented in this thesis indicate lack of responsiveness to *H. contortus* during challenge which may indicate that these lambs require more exposure to *H. contortus* or that they simply lack an ability to develop potent immunological memory to *H. contortus*.

Sampling of antigen by APC and subsequent presentation to T and B cells during priming infection allows for production of antigen specific antibody (Murphy, 2012). Possible coating of *H. contortus* by both general and antigen specific antibody upon challenge infection could contribute to cellular rises seen in STC animals during challenge infection and subsequent lack of FEC. Additionally, the increase of CWA-IgG seen in primary STC infection could contribute to the reduction of FEC to 0 in those animals by the same mechanism.

Suffolk crossbred lambs of each treatment, including naïve, had significantly higher circulating IgG than STC lambs, possibly as general defense mechanism against pathogens in light of non-responding innate cells. High IgG in these animals also may be the result of other pathogens, as the facility the lambs were kept in was not a pathogen-free facility. While the WVU research facility allowed for control of *H. contortus* infection, there was no ability to control any other pathogen the lambs may encounter. Immunoglobulin G (IgG) is the predominant antibody found in serum. This antibody can activate complement, is an early antibody generated in response to pathogenic infections (Murphy, 2012) and increased in response to many common infections of sheep including, but not limited to Foot-and-Mouth disease (Chenwen Xiao, 2007), sore mouth (Haig and McInnes, 2002) and *Clostridium perfringens* toxins (Griner,

1961). Therefore, any variation in total IgG of animals of either breed cannot be solely attributed to *H. contortus* infection. Though, IgG has been reported to be elevated in *H. contortus* infection, a consistent correlation has yet to be made as data are variable. (Amarante et al., 1999; Jacobs et al., 1995)

Despite high total circulating IgG, SX lambs had significantly lower CWA-IgG. In fact, CWA-IgG is lower in challenge SX animals. In STC lambs CWA-IgG is increased during primary infection compared to naïve lambs and increased again in challenged lambs compared to both primary and naïve lambs. The increase in primary STC CWA-IgG indicates a timely response to *H. contortus* and may play a crucial role in the ability of STC sheep to expel *H. contortus* during a primary infection. Antigen specific IgG absorbance can be seen increasing from 0.58 on day 14 to 0.81 on day 21, which is the largest increase between two time points in STC primary animals and is followed by a reduction of STC primary FEC reduction to 0 by the following week. Furthermore, challenged STC animals had higher CWA-IgG and when paired with heightened cellular immune response may prevent of establishment of *H. contortus* during a challenge infection.

Increased CWA-IgG in STC lambs could possibly be related to heritability of major histocompatibility complex (MHC) genes. Major histocompatibility complex proteins bind antigen on the surface of APCs (Murphy, 2012). Due to their role in antigen presentation, MHC genes are thought to play a role in parasite resistance (Buitkamp, 1996). As MHC diversity increases, so does antibody specificity (Xu, 2000). Suffolk sheep have been found to have decreased MHC alleles (DRB1) associated with

reduction in FEC count compared to Texel sheep (Sayers, 2005). Therefore, increased CWA-IgG in STC lambs may be associated with greater diversity of MHC genes.

In addition to having increased CWA specific IgG, all STC lambs had increased IgA. Increased IgA has been universally seen in parasitic infections of both ovine and murine models (Charley-Poulain et al., 1984; Siński and Holmes, 1977). Previous research has shown IgA and IgE are increased in Caribbean hair sheep typically thought to be parasite resistant during *H. contortus* infection (MacKinnon et al., 2010). Because anti-sheep IgE is not commercially available, we did not analyze total circulating serum IgE, however, our findings were consistent with increased IgA during experimental infection.

These data further support claims that enhanced immunity is the mechanism of resistance to *H. contortus* in St. Croix sheep and substantiate the failure of Suffolk crossbred lambs to generate and maintain enhanced immune responses contributing to pathogenicity of *H. contortus* in this breed. Our analysis of peripheral cellular and humoral immunity indicate activation of systemic immunity thus prompting further investigation at the local site of infection. Knowledge of cellular profiles at the site of the host-parasite interface in the abomasum of infected and naïve lambs of each breed would help elucidate the importance of specific cell types at the site of infection.

Data in this thesis have validated the generation and use of *H. contortus* naïve animals in the sheep research facility at West Virginia University, which will serve as a basis for future *H. contortus* research. Additionally, it has been shown that successful peripheral monitoring of immune responses to *H. contortus* can be accomplished in sheep, which has the potential to be utilized as a clinical diagnostic for sheep helminth

infection. Additionally, knowledge of the lack of sustained innate and humoral immune responses in Suffolk crossbred sheep may be critical in the development of future therapeutics.

Figure 1. FEC after priming and experimental infections. A. STC and SX FEC during priming infection. B. Average FEC of naïve, primary and challenge infected STC and SX lambs for the duration of the main experiment. C. Weekly FEC of STC primary and challenge lambs. D. Weekly FEC of SX primary and challenge lambs. Error bars represent SEM. * $P < 0.05$, *** $P < 0.0001$.

Figure 2. Packed Cell Volume after priming and experimental infections. A. Weekly PCV of STC and SX lambs during priming infection. B. Average PCV of STC and SX lambs for the duration of the priming infection. C. Weekly STC and SX primary and challenge PCV during main experiment. D. Average PCV of naïve, primary and challenge STC and SX lambs for the duration of the main experiment. Error bars represent SEM. *** $P < 0.0001$; ** $P = 0.014$.

Figure 3 WBC count after experimental infection. A. Average circulating WBC of STC and SX lambs averaged across all time points. B. Weekly STC primary and challenge WBC count. STC naïve WBC count has been averaged for the entire experimental time period. C. Weekly SX primary and challenge WBC count. SX naïve WBC count has been averaged for the entire experimental time period. D. Average naïve, primary and challenge STC and SX WBC count for entire experimental time period. Error bars represent SEM. * $P < 0.05$.

Figure 4. Lymphocyte count after experimental infection. A. Weekly STC primary and challenge lymphocyte count. STC naïve lymphocyte count has been averaged for the entire experimental time period. B. Weekly SX primary and challenge lymphocyte count. SX naïve lymphocyte count has been averaged for the entire experimental time period. C. Average naïve, primary and challenge STC and SX lymphocyte count for entire experimental time period. Error bars represent SEM.

Figure 5. Neutrophil count after experimental infection. A. Average circulating neutrophils of STC and SX lambs averaged across all time points. B. Weekly STC primary and challenge neutrophil count. STC naïve neutrophil count has been averaged for the entire experimental time period. C. Weekly SX primary and challenge neutrophil count. SX naïve neutrophil count has been averaged for the entire experimental time period. D. Average naïve, primary and challenge STC and SX neutrophil count for entire experimental time period. Error bars represent SEM. * $P < 0.05$.

Figure 6. Monocyte count after experimental infection. A. Average circulating monocytes of STC and SX lambs averaged across all time points. B. Daily STC primary and challenge monocyte count. STC naïve monocyte count has been averaged for the entire experimental time period. C. Daily SX primary and challenge monocyte count. SX naïve monocyte count has been averaged for the entire experimental time period. D. Average naïve, primary and challenge STC and SX monocyte count for entire experimental time period. Error bars represent SEM. * $P < 0.05$.

Figure 7. Eosinophil count after experimental infection. A. Weekly STC primary and challenge eosinophil count from d0-7. STC naïve eosinophil count has been averaged for the entire experimental time period. B. Weekly SX primary and challenge eosinophil count from d0-7. SX naïve eosinophil count has been averaged for the entire experimental time period. C. Weekly STC primary and challenge eosinophil count from d7-49. STC naïve eosinophil count has been averaged for the entire experimental time period. D. Weekly SX primary and challenge eosinophil count from d0-7. SX naïve eosinophil count has been averaged for the entire experimental time period. E. Average eosinophil count of d0-7. F. Average eosinophil count of d7-49. Error bars represent SEM. Means with different letter are sig. $P < 0.05$.

Figure 8. Basophil count after experimental infection. A. Weekly STC primary and challenge basophil count. STC naïve basophil count has been averaged for the entire experimental time period. B. Weekly SX primary and challenge basophil count. SX naïve basophil count has been averaged for the entire experimental time period. C. Average naïve, primary and challenge STC and SX basophil count for entire experimental time period. Error bars represents SEM.

Figure 9. Circulating IgG after experimental infection. A. Weekly STC primary and challenge circulating IgG. STC naïve circulating IgG has been averaged for the entire experimental time period. B. Daily SX primary and challenge circulating IgG. SX naïve circulating IgG has been averaged for the entire experimental time period. C. Average naïve, primary and challenge STC and SX circulating IgG for entire experimental time period. Error bars represent SEM. * $P < 0.05$

Figure 10. Absorbance of CWA-IgG after experimental infection. A. Weekly STC primary and challenge CWA specific IgG. STC naïve CWA-IgG has be averaged for the entire experimental time period. B. Daily SX primary and challenge CWA-IgG. SX naïve CWA-IgG has been averaged for the entire experimental time period. C. Average naïve, primary and challenge STC and SX CWA-IgG for entire experimental time period. Error bars represents SEM. * $P < 0.05$.

Figure 11. Total circulating IgA after experimental infection. A. Total IgA of STC and SX lambs averaged across all time points. B. Weekly circulating IgA of STC and SX lambs. C. Total average IgA of ST and SX lambs across all time points. Error bars represent SEM. * $P < 0.05$, ** $P = 0.0049$, *** $P < 0.0001$.

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