Differential gene expression and immune regulatory mechanisms in parasite-resistant hair and susceptible wool sheep infected with the parasitic nematode, *Haemonchus contortus*

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Dissertation submitted to the faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Genetics, Bioinformatics, and Computational Biology

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July 23rd, 2007
Blacksburg, Virginia

Keywords: Gene expression, immune response, cytokine, antibody, parasite, sheep

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(ABSTRACT)

Among sheep producers, the parasitic nematode *Haemonchus contortus* is a major animal health concern. Caribbean hair sheep are more resistant than conventional wool breeds to this blood-feeding, abomasal parasite. Our objective was to determine differences in the immune response associated with parasite-resistant hair and susceptible wool lambs infected with 10,000 *H. contortus* and in uninfected controls. Animals were sacrificed and abomasum and lymph node tissues were collected at 3 or 27 days post-infection (PI), and for controls on day 17, 27, or 38 relative to d 0 of infected animals. Blood and fecal samples were collected throughout the study.

Lower fecal egg counts, higher packed cell volumes, and heavier lymph nodes of infected hair compared to wool lambs, suggests hair lambs have increased parasite resistance. Greater tissue infiltration of eosinophils ($P < 0.05$) was observed in hair compared to wool sheep by 3 days PI, with no breed differences in globule leukocytes. Total serum IgA and IgE were greater in control hair versus wool sheep ($P < 0.05$). After 3, 5, and 21 of infection, total serum IgA ($P < 0.05$), total lymph node IgE ($P < 0.01$), but not total serum IgE were greater in hair sheep compared to wool sheep.

Gene expression was measured between hair and wool lambs for abomasal and lymph node tissues using bovine cDNA microarrays and real-time RT-PCR. Microarray analysis revealed cell survival, endosome function, gut motility, and anti-coagulation pathways are important in abomasal and lymph node tissues during *H. contortus* infection. Immune genes, including IL-4, IL-4 Rα, IL-12 Rβ1, and IL-12 Rβ2, are also highly represented in abomasal or lymph node tissue of infected animals. Eleven genes were evaluated using real-time RT-PCR and included TH1 and TH2 cytokines, cytokine receptors, and IgE. Parasite infection leads to increased expression of IL-13 and IgE in both tissues and breeds when compared to control animals. Breed comparison of gene expression shows resistant hair sheep produce a stronger modified TH2-type immune response during infection. Differential cell infiltration, antibody

production, and regulation of TH2 cytokines between breeds may be partially responsible for differences in parasite resistance.

ACKNOWLEDGMENTS

Many people from multiple universities and departments have assisted me in developing various aspects of this research project and guiding me to become a better scientist. I want to thank you all. Most of all I want to thank one of the most intelligent people I had the pleasure to work with, my advisor, Dr. David Notter. You provided me guidance and assistance when I needed it most, but also allowed me to figure things out on my own and work with you to develop this great project. I am very thankful that everything turned out as it has. I have many great memories from my time here at Virginia Tech and I am going miss all the friends I have made here.

To Randy, Bindu, Larry, Phoenix, and Smitha thank you for the great philosophical discussions and for being so helpful whenever I was in need of a hand. It was great to have so many wonderful friends and office mates. Thank you for putting up with all my crazy stories and talk about Jazzercise© (yes, I had to get it in somewhere). A special thanks to my fellow pug owners, Elizabeth and Amy. Thank for all the times you kept the girls. We will miss you and Edy and Bella. I wish you all the best!

I would especially like to thank Dr. Jeannie Burton for her assistance, friendship, and many wonderful personal and professional discussions. I had a great time during my trips to Michigan State University. I want to thank everyone there for making me feel so welcome and for their assistance with the use of microarrays and their analysis. Without you (Dr. Jeannie Burton, Sue Sipkovsky, Dr. Rob Templeman, and Dr. Paul Coussens), the microarray work would not have been possible.

One of the most important people involved in this project was Dr. Anne Zajac, without you the project would not have been possible. You allowed me to use part of your lab, showed me techniques, and gave me many helpful tips and guidance both on this project and for my future. I would like to thank you for everything!

The measurement of IgE was kindly provided by Dr. Frans Kooyman, of which I am grateful. Thank you for your collaboration and patience with all of my questions and concerns.

The last semester of my studies were enlightened by the friendship and guidance of Dr. Isis Mullarky. You have helped me tremendously in this short period of time. I am glad you decided to choose me to "practice" your mentoring skills. Your future students will be very lucky to work with such a great person. Thank you for allowing me to be part of your lab and helping me with all aspects of the real-time RT-PCR. Your encouragement and assistance has made this a better project and me a better scientist.

Most importantly, my sincere appreciation goes out to my family. To my husband Michael, who has never known me to not be in school, thank you for putting up with all of my parasite talk and working long nights and holidays. I am so glad we found each other and I look forward to this new and interesting time to our lives. To my mother, thank you for being there for me, being so interested in my research, taking the time to understand what I have been doing, and why I am so excited about it. I am grateful for the wonderful relationship we have!

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INTRODUCTION

Most sheep are infected with a variety of gastrointestinal (GI) parasites daily. Increased stocking density and a greater incidence of parasites resistant to chemical dewormers have led to more infective larvae on pastures and increased parasite burdens for the host. The parasite of greatest concern in tropical and subtropical regions is the blood-feeding abomasal nematode *Haemonchus contortus*. Heavily infected sheep can lose over 50 mL of blood per day, leading to decreased wool production, reduced carcass merit, anemia, and even death. Haemonchosis has placed an economic constraint on sheep production, leading researchers to search for addition methods of parasite control including evaluation of animals with genetic resistance to parasitism (Gamble and Zajac, 1992; Bisset et al., 1996; Pernthaner et al., 1996; Vanimisetti et al., 2004b). There is an increased interest in Caribbean hair sheep, such as the St. Croix, due to their high level of parasite-resistance, ease of management, and lack of wool (Notter, 1999). However, the immune mechanisms responsible for parasite resistance have not been well documented in hair or wool sheep, and research involving hair sheep, in general, is lacking.

Infection with extracellular GI parasites elicits a T-helper-cell type 2 (TH2) response in sheep (Lacroux et al., 2006) and mice (Finkelman et al., 2004), with tissue infiltration of eosinophils and mast cells, production of TH2 cytokines, and increased IgA and IgE. Comparison of parasite-resistant and susceptible sheep show that resistant animals have greater numbers of mucosal mast cells and globule leukocytes (Gamble and Zajac, 1992; Bisset et al., 1996), circulating (Stear et al., 2002) and mucosal eosinophils (Gill et al., 2000), and circulating and higher levels of local IgA and IgE (Strain et al., 2002; Pernthaner et al., 2005b; Pettit et al., 2005), but these results differed among sheep breeds, ages, and species of infective parasite.

Minimal information is available on gene expression in tissues of parasite-infected sheep. The few studies involving expression of cytokines in the GI tract and surrounding lymph nodes have only assessed the response to infection in wool sheep. These studies show increased expression of TH2 cytokines (interleukin (IL)-4, IL-5, and IL-13), a TH1 cytokine (interferon (IFN)-γ), and a pro-inflammatory cytokine (tumor necrosis factor (TNF)-α) after infection (Gill et al., 2000; Pernthaner et al., 2005a; Balic et al., 2006; Lacroux et al., 2006; Pernthaner et al., 2006; Jasmer et al., 2007). Comparison of cells and tissues from resistant and susceptible lines

of wool sheep suggested animals with the resistant phenotype have greater expression of IL-5, IL-13, IFN-γ, and TNF-α after infection (Gill et al., 2000; Pernthaner et al., 2005a). Gene expression in uninfected hair sheep or parasite-infected hair sheep, to the best of our knowledge, has not been evaluated. An enhanced understanding of gene expression in these animals during infection may help determine immune regulatory mechanisms involved in parasite resistance. Eventually, this information could be used to select animals genetically resistant to GI parasites.

The goals of this dissertation were to assess differences between hair and wool sheep while uninfected and after 3 or 27 days of *H. contortus* infection in 1) immune effector cells and antibody concentration in the abomasum, lymph nodes, and/or circulation, 2) gene expression in the abomasum and lymph node tissues, and 3) expression of selected cytokines, receptors, and IgE, and to determine the association of these parameters with measures of resistance.

CHAPTER 1

LITERATURE REVIEW

Gastrointestinal parasitism in sheep

Increased prevalence of disease due to parasitism. Ruminants and internal parasites have co-existed for thousands of years, but an increase in stocking density has led to a greater nematode parasite burden for the host and decreased revenue for the livestock producer and the industry. Concentration of animals can lead to a greater load of free-living stages of parasites on pasture, which makes it easier for the host to become overwhelmed by infection. Use of anthelmintics can diminish parasite burdens in susceptible animals, but efforts to control and treat gastrointestinal parasitism in sheep leads to extensive economic loss worldwide. The financial burden to the US sheep industry has not been estimated; however, according to the Australian Wool Innovation Limited losses in the Australian sheep industry are estimated at $369 million (Australian) per year (Schröder, 2007). Most of the estimated cost is due to frequent use of anthelmintics and reduced meat and wool production. Production losses are difficult to measure in practice, but Besier and Love (2003) estimated a 10 % loss in wool growth during moderate gastrointestinal parasite infection. In addition to the financial impact on the industry as a whole, 62 % of US sheep producers identified stomach/intestinal worms as their primary animal health concern (NAHMS, 1996). Thus, gastrointestinal parasitism of sheep is detrimental to the sheep industry, the individual producer, and the health of the animal.

Anthelmintic resistance. The three major classes of chemical dewormers were introduced in the 1960's through early 1980's, but within 30 years resistance has emerged to all classes of anthelmintics in nematode parasites of small ruminants. One of the earlier incidences of anthelmintic resistance was reposrted by Ketlle et al. in 1982 in New Zealand. Since that time, nematodes resistant to individual dewormers and multiple classes of dewormers have been reported around the world, including in the US (Zajac and Gipson, 2000), Scotland (Bartley et al., 2003), and South Africa (Schnyder et al., 2005). Parasites resistant to anthelmintics seem to have emerged quickly, but Coles and colleagues (2005) were able to obtain pure cultures of resistant parasites after only three generations of selection. Accumulation of parasites that are resistant to dewormers is becoming a constraint to livestock production, and new classes of

dewormers are not currently being developed. Therefore, alternative mechanisms of parasite control are needed.

Alternative methods of parasite control. Pasture management (Reinecke, 1994), identification and treatment of only anemic animals (Kaplan et al., 2004), use of nematode-trapping fungi (Pena et al., 2002), oral dosing of copper wire (Burke and Miller, 2006) or condensed tannins (Lange et al., 2006), vaccination (Newton et al., 1995), and selection of parasite-resistant animals (Pernthaner et al., 1996) contribute to reductions in parasite burden. All of these methods have drawbacks including copper toxicity, increased labor and product cost, decreased weight gains, and limitations in product availability. The most easily implemented parasite control method, and most advantageous for future generations, would be selection of animals resistant to internal parasites. In addition, animals that are genetically resistant to parasites would benefit producers by facilitating production of a chemical-free product to supply organic markets.

Parasite resistance, as measured by fecal egg count (FEC), is moderately heritable (h^2 = 0.23 to 0.41) and therefore can be improved through selection (Dominik, 2005). Many production systems already have programs for animal recording and genetic evaluation in place, so one additional trait could easily be included. However, the mechanisms involved in resistance to nematode infection are not clear and may vary with the host, parasite species, and location of parasite establishment. Additionally, selection for a particular trait associated with resistance may be antagonistic to favorable wool, growth, and carcass characteristics, leading to an undesirable correlated response (Morris, 2001). The characterization of the response to parasitism in sheep, and in particular sheep resistant to gastrointestinal nematode parasites, will be the focus of this review.

Factors altering the response to parasites

Sheep do not have a standard response to all types of parasites. The species of parasite (Smith et al., 2001), feeding behavior, and stage of parasite development (Balic et al., 2000) can all alter the host's response to infection. Variation and specificity in response to different parasites invading host tissues allows for a more productive mechanism of clearance. Factors that alter the animals' ability to respond to a particular species of parasite include the animals' age (Vanimisetti et al., 2004a), plane of nutrition (Strain and Stear, 2001), immune status (Zajac

et al., 1988; Gamble and Zajac, 1992), and genetic composition (Bisset et al., 1996). Effects of some of the above mentioned factors as they relate to parasite burden and expulsion will be further explored.

Species of extracellular parasitic nematode

There are a number of nematodes that commonly infect sheep. The three most important genera in hot, humid regions like the Southeastern U.S. are *Haemonchus* and *Trichostrongylus* (Bahirathan et al., 1996; Amarante et al., 1999b) and *Teladorsagia* in more temperate climates such as those found in the U.K. (Stear et al., 2006). These parasites reside in the abomasum (*Haemonchus contortus*, *Trichostrongylus axei,* and *Teladorsagia circumcincta*) and small intestine (*Trichostrongylus colubriformis*) of sheep. Although the gastrointestinal nematodes mentioned above are all members of the family Trichostrongyloidea, they vary in feeding behavior and tissue niche. Full development of *Teladorsagia* and *Trichostrongylus* takes 21 days, whereas *Haemonchus* can develop into adults in 14 days (Whittier et al., 2003). All three of these parasites cause damage to the gastrointestinal tract as they develop and feed. A distinctive and particularly detrimental characteristic of *H. contortus* is the requirement for late larval and adult stages to feed on blood. *Teladorsagia* and *Trichostrongylus* cause less damage as adults, since they feed on cellular secretions of the infected animal. Hosts infected with one species of parasite can develop immunity that often offers cross-protection upon infection with other genera of nematodes (Dobson and Barnes, 1995; Colditz et al., 1996; Stankiewicz et al., 2000). These results suggest either similar host response mechanisms and/or the presence of homologous parasite antigens in these species (Stankiewicz et al., 2000). However, one study of sheep vaccinated with proteins derived from one species of parasite found no cross-protection during infection by other nematode parasite species (Smith et al., 2001), pointing out that unique antigens are also present among these parasites. Overall, these parasites share similar antigens and infection strategies, eliciting similar responses from the host. However, variations in the host response are due to unique antigens, feeding behavior, and the tissue niche occupied by the nematode species (Woolaston, 1992). Due to the potentially devastating impact *H. contortus* has on the health of infected animals, and the economic impact on sheep producers, it is one of the most heavily studied internal parasites of sheep and will be the main emphasis of this review.

Life-cycle of Haemonchus contortus. In order to appreciate the host response to *H. contortus*, the life-cycle of the parasite must be understood. The cycle starts with eggs passed out of the sheep with the feces. Once the parasitic nematodes hatch, they develop into free-living larval stage 1 (L1) and then L2 while feeding on bacteria in the feces. The L2 larvae develop into infective L3 and retain the cuticle of the L2 for protection from desiccation and temperature fluctuations. Under optimal conditions (37°C and 100 % humidity), development from egg to L3 larvae can occur in 2.5 days (O'Connor et al., 2006). Resistant hosts produce feces containing eggs with reduced larval development. Adult ewes from Perendale flocks selected for high and low FEC have 49 and 13 % larvae development to the L3 stage, respectively (Jorgensen et al., 1998). Lambs, with increased susceptibility to infection, have 67 and 35 % larvae development in high and low FEC lines, respectively.

If moisture is adequate, surviving L3 larvae migrate out of the feces and up blades of grass. Ingestion of infective L3 larvae by sheep occurs during grazing. The larvae immediately start to exsheath in the rumen, reach the abomasum within 2 to 3 days, and move down into the gastric pits of the abomasal mucosa. If environmental conditions were not optimal, larvae can undergo hypobiosis, or arrested development, at this stage. Hypobiotic larvae can remain in an inactive state until more favorable conditions occur, such as immune suppression during the periparturient period (Courtney et al., 1986). Mechanisms triggering entry into and departure from the hypobiotic state are currently unknown, but are related to environmental temperature and immune responsiveness of the host.

Larvae may be easily damaged by immune mechanisms of the host while in close contact with the mucosa (Balic et al., 2006). Development into the L4 stage initiates migration of larvae out of the gastric pits and into the lumen of the abomasum. The L3 and L4 parasite antigens vary from the antigens produced by adult parasites (Meeusen et al., 2005). Antigenic changes between parasite stages may enable the parasite to evade host recognition. In the lumen, late-L4 develop into L5 and then into adults. Establishment rates of infective larvae through development to adult parasites ranged from around 3 to 50 % in 5-month-old sheep after artificial infection (Aumont et al., 2003). Mating of male and female worms leads to egg production and, after at least 14 days of infection, passage of eggs in the host feces can occur, completing the cycle. Differences in female worm egg output of different strains of *H. contortus*

have been reported (Aumont et al., 2003), and should be recognized if comparing FEC across locations and studies.

Late-L4, L5, and adult *H. contortus* feed on the blood of the host. Blood-letting is achieved by piercing and scraping the mucosa wall with a small lancet located on the anterior end of the parasite (Georgi, 1990). This process can lead to the loss of around 50 mL of blood per day under a moderate infection of 1,000 worms. Extensive blood loss of animals under heavy *H. contortus* infection can lead to decrease packed cell volume (PCV), anorexia, loss of condition, depression, and even death.

Immune Suppression

Lambs: Effects of previous parasite exposure. Young animals are more susceptible to disease and infection than their adult counterparts (Colditz et al., 1996). However, the response to parasitism also varies with previous parasite exposure, and length and severity of previous infections. Exposure of lambs to parasites is required for development of acquired immunity and increased resistance. Gamble and Zajac (1992) infected parasite-naïve 8-week-old lambs of two breeds with *H. contortus* and detected high FEC at 4 weeks post-infection (PI). Upon deworming and reinfection, substantially lower FEC were observed in comparison to the levels measured at the previous infection and in comparison to age-matched controls receiving a primary infection. Similar results were obtained in 5-month-old lambs, where previously infected lambs have one-third of the worms found in lambs receiving an initial challenge (Aumont et al., 2003). Higher FEC and lower PCV ($P < 0.05$) were observed throughout infection in artificially challenged 1-year-old ewes compared to older ewes (Vanimisetti et al., 2004a). However, FEC and PCV of 1-year-old ewes compared to their lambs still suggested substantial development of resistance in young ewes. These results demonstrate that even though these animals have been previously exposed, full development of resistance does not occur by one year of age. The phenomenon is explained as an "immunological hypo-responsiveness" of the lamb during development of its immune system (Colditz et al., 1996). Thus, even though previous parasite exposure can increase resistance to a certain extent, younger animals still exhibit greater susceptibility than older animals.

Adult ewes during the periparturient period. Around the time of lambing, adult ewes are more susceptible to parasitism and experience a phenomenon frequently referred to as the

periparturient rise (PPR) in FEC. Periparturient ewes seem to experience suppression of immune function causing increased egg production by established worms, increased larval establishment, decreased worm expulsion, and development of previously arrested larvae (Courtney et al., 1984). A similar increase in nematode and coccidian (intracellular intestinal parasite) FEC occurs in cows around the time of calving (Faber et al., 2002). Immune suppression also occurs in pregnant mice and is partially attributed to reduced production of antibody-producing cells in these animals (Medina et al., 1993).

Infected ewes experience a rise in FEC beginning 2 to 4 weeks before lambing (Courtney et al., 1984; Courtney et al., 1986; Zajac et al., 1988; Woolaston, 1992). The FEC of lactating ewes peak at approximately 6 weeks post-lambing, potentially suggesting resumption in immune responsiveness. However, high nutritional demands of lactation, especially in ewes nursing triplets, prolong immune suppression (Woolaston, 1992). To determine effects of parturition on development of hypobiotic larvae, ewes may be housed indoors to preclude additional parasitic infection. Under these conditions, susceptible wool ewes still experience a rise in FEC around the time of lambing, which is significantly greater than the marginal increase in FEC observed in St. Croix and Barbados Blackbelly ewes (Courtney et al., 1984; Zajac et al., 1988). The PPR during *H. contortus* infection results from relaxation of the immune response leading to emergence of arrested larvae or increased prolificacy of established worms.

Immune function may be reduced at other times during pregnancy. Ewes evaluated from mating through lambing under continued natural infection experience a peak in FEC approximately 6 weeks after breeding (Wanyangu et al., 1997; Amarante et al., 1999b). These periods of immune suppression are possibly associated with changes in hormone concentration associated with pregnancy. The hormones prolactin and progesterone normally increase during pregnancy and, if administered to ovariectomized ewes, leads to a greater number of worms, larger worms, and increased FEC compared to open intact ewes (Fleming and Conrad, 1989). However the changes in immune response that mediate the PPR effect are still largely unknown.

Resistant versus susceptible host

Variation in parasite resistance exists within breeds of sheep, even for animals of similar age, reproductive status, and type and stage of infection. Much of this variation is associated with differences in the genetic background of the animal. Approximately 10 years of divergent

selection for increased or decreased FEC in Romney sheep led to a 9.2-fold difference in FEC, corresponding to a 65 % reduction in worm burdens (Bisset et al., 1996). Fecal egg counts and worm burdens show a similar decrease in Merino (Woolaston et al., 1990) and INRA401 (Gruner et al., 2002) flocks selected for high and low FEC. These studies demonstrate that selection for resistance is possible and effective, even within initially susceptible breeds. Unfortunately, the selection procedures imposed in Romney sheep led to undesired correlated responses for other traits, including reduced postweaning gains and yearling fleece weights (Morris, 1997, 2001). These flocks have undergone selection for only a few decades, whereas populations that have undergone natural selection for hundreds of years may have higher levels of resistance, potentially involving novel resistance mechanisms.

Wool sheep, such as the Florida Native, Gulf Coast Native, and Louisiana Native, found in hot, humid gulf coast region have increased resistance to nematode parasites. Having undergone many generations without anthelmintic treatment over the past 400 years, they display a high level of resistance compared to other wool breeds (Amarante et al., 1999b). Caribbean hair sheep originated from West African stocks and express an even greater level of parasite resistance than Flordia Native sheep (Zajac et al., 1990), most likely resulting from exposure to high levels of parasite infection for an even longer period of time.

Comparison of hair and wool breeds

Hair Sheep: Origins, Breeds and Characteristics

Hair sheep in the U.S. originated from hot, humid regions of West Africa and were most likely transported to the Caribbean in the 1600's with the slave trade (Bradford, 2005). These animals are now represented mainly by the Barbados Blackbelly and Virgin Island White (St. Croix) breeds. These hair sheep were maintained in the Caribbean under conditions that were conducive to high parasite infection, and only genetically-resistant animals survived. After 300 additional years of natural selection, the first documented importation of Barbados Blackbelly sheep into the U.S. occurred in 1904 (Wildeus, 1997). Importation of the St. Croix breed (SC) involving fewer than 10 animals in 1960 and 25 animals in 1975 have occurred since that time (Wildeus, 1997). Other hair sheep breeds, such as the Brazilian Santa Ines (Bricarello et al., 2005) and African Red Maasai (Mugambi et al., 1997) are also resistant to parasitic nematodes.

However, hair sheep breeds from arid regions tend to not be any more resistant than conventional wool breeds (Vanimisetti et al., 2004b). Compared to most wool breeds, hair sheep are generally small-framed, poorly muscled, and slow-growing, but they are desirable for their resistance to internal nematode parasites, higher reproductive performance, and lamb vitality (Notter, 1999). In addition, adaptation to hot, harsh climates and freedom from wool means hair sheep require little effort to maintain.

Parasite Resistance

Lambs. Caribbean hair lambs develop resistance sooner and have a higher level of resistance than wool lambs raised under the same conditions (Gamble and Zajac, 1992; Aumont et al., 2003; Vanimisetti et al., 2004b). No breed differences are observed between parasite-naïve 2-month-old St. Croix and Dorset lambs infected with *H. contortus* (Gamble and Zajac, 1992). These results suggest a similar innate immune response at this age. When these same animals receive a second challenge infection at 4 months of age, a substantial reduction in FEC from the initial infection is observed. The reduction in FEC is greater in St. Croix lambs compared to Dorset lambs. These results suggest that by 4 months of age, an acquired immune response develops in both breeds and is stronger in St. Croix lambs. Interestingly, 4-month-old naïve St. Croix lambs infected with *H. contortus* have a lower FEC than 2-month-old St. Croix and Dorset lambs, as well as naïve 4-month-old Dorset lambs infected with *H. contortus*. Therefore, breed difference may exist in the innate response of these animals by 4 months of age.

Enhanced resistance is found in 3- to 7-month-old Barbados Blackbelly lambs compared to lambs of a French wool composite breed, INRA401, during primary and secondary artificial challenge (Aumont et al., 2003; Gruner et al., 2003). Three- to 10-month-old St. Croix lambs are also more resistant to *H. contortus* infection than age-matched wool lambs of a composite breed (Courtney et al., 1985a). Gruner et al. (2003) observed a substantial effect ($P < 0.001$) of sex on parasite resistance in 3.5- and 7-month-old lambs. Females have lower FEC during the second challenge compared to the first, whereas males have no reduction in FEC. Under natural *H. contortus* infection, through grazing initiated at 8 weeks of age, St. Croix lambs shed fewer eggs and have lower worm burdens than Dorset lambs at 47 or 62 days PI, depending on year (Gamble and Zajac, 1992). At 9 to 10 months of age, St. Croix lambs again have lower FEC than Dorset lambs (Zajac et al., 1990; Vanimisetti et al., 2004b). Although hair lambs may not

differ from wool lambs in resistance to *H. contortus* within the first few weeks of life, innate and acquired resistance start to develop in hair but not wool breeds by 4 months of age. After 4 months of age, hair lambs are clearly more resistant to *H. contortus* than wool lambs under natural (grazing) and artificial infection.

Periparturient Ewes. Open, non-lactating adult hair or wool ewes previously infected with *H. contortus* have similar FEC during subsequent parasite infection (Courtney et al., 1985b). Compared to open ewes, pregnant or lactating ewes have decreased resistance to *H. contortus* around the time of lambing, although a higher level of resistance is maintained in hair ewes (Courtney et al., 1984). Two factors play a prominent role in the PPR: the development of arrested larvae and the acquisition and establishment of new larvae. Since St. Croix and Barbados Blackbelly ewes harbor fewer arrested larvae, development of these larvae does not cause a significant increase in FEC (Courtney et al., 1984). The same study showed that in previously parasite-free animals, St. Croix ewes also have lower FEC than wool ewes following ingestion of newly acquired infective larvae. These findings suggest that the PPR may not be as pronounced in hair sheep. Research involving resistant Florida Native wool ewes confirm the presence of lower numbers of arrested larvae in resistant breeds (Zajac et al., 1988). Pregnant Florida Native ewes maintained on pasture under relatively constant levels of parasite challenge have lower FEC than contemporary Rambouillet ewes for the entire year of the study (Amarante et al., 1999b). These results suggest that parasite-resistant breeds show a less pronounced PPR associated with lower numbers of arrested larvae, a reduced rate of larval establishment and, potentially, a maintenance of immune function during the periparturient period compared to most wool breeds.

Immune Response to *Haemonchus contortus*

Production of antibodies: IgA and IgE

One of the factors that may play a role in resistance of sheep to gastrointestinal parasites is production and specificity of antibodies, particularly IgA and IgE. Immunoglobulin A is the second most common antibody in serum (~1.8 mg/mL) and highest in total production, as most is produced and secreted at mucosal surfaces. Humans produce large amounts of IgA, averaging 66 mg/kg of bodyweight per day (Monteiro and Van De Winkel, 2003). Most IgA is found in

mucosal secretions to aid in protection from foreign pathogens. Binding of IgA to cell surface receptors can activate immune cells, such as eosinophils, and lead to parasite damage. Direct binding of IgA to the parasite in the mucus and on the mucosal surface can also cause damage to the parasite. Immunoglobulin E, on the other hand, has very low serum concentrations (~0.0003 mg/mL) and is primarily found attached to high affinity receptors on immune cells, mainly mast cells and basophils. These cells can produce a rapid immune response, typical of hypersensitivity and allergic reactions. These activated IgE-bound cells may provide an additional mechanism to damage invading parasites and facilitate their expulsion.

IgA. Comparisons of IgA levels in hair and wool breeds are limited, and IgA levels have only been compared in abomasal mucus (Zajac et al., 1990; Amarante et al., 2005). Results from studies of wool breeds involving animals less than 1.5 years old under varying nematode infection levels will provide information on the IgA response and differences in parasite resistance in susceptible breeds. Serum IgA peaks around 10 days after infection in previously infected sheep (Henderson and Stear, 2006), with a higher specificity for larval antigen compared to adult worm antigen (Schallig et al., 1995; Gomez-Munoz et al., 1999; Amarante et al., 2005). These specific antibodies would likely be most effective at the mucosal surface, the location of parasitic larvae. High IgA levels may be advantageous for the host and decrease parasite burdens, as FEC have negative correlations with both serum (-0.44) and mucus (-0.71) IgA levels. Relative amounts of IgA in serum and in the gut are associated with FEC, which would allow for an easily accessible assessment of resistant animals (Martinez-Valladares et al., 2005). These studies suggest that IgA in mucus may damage parasitic larvae and are consistent with reports that increased mucus IgA levels are associated with reduced FEC (Rocha et al., 2005) and reduced worm length (Strain and Stear, 1999, 2001).

Zajac et al. (1990) and Amarante et al. (2005) measured IgA in abomasal mucus of hair and wool sheep, and found no significant breed differences. These results could be a result of the time of sample collection, as IgA levels may fluctuate with the presence of parasite antigens. The breed differences assessed by Amarante et al. (2005) were between Santa Ines (hair sheep), Suffolk, and Ile de France lambs after 6 weeks of natural (mostly *H. contortus*) infection. The continual infection and difference in parasite intake and establishment may mask potential breed differences in IgA levels. Artificial primary (IgA measured 1 week PI) and secondary (IgA measured 2 weeks PI) challenges with *H. contortus* of a small number of Florida Native, St.

Croix, and Dorset/Rambouillet breeds did not elicit breed differences in IgA levels when evaluated by Zajac et al. (1990). Since IgA is produced in response to invading larvae, breed differences may only be apparent with high infection levels and at the earliest stages of infection.

IgE. Although anti-ovine IgE antibodies have now been produced from three sources (Shaw et al., 1996; Kooyman et al., 1997; Bendixsen et al., 2004), little research has addressed breed comparisons. The general response to gastrointestinal parasite infection in wool lambs is an increase in total serum IgE (Kooyman et al., 1997; Kooyman et al., 2000) and an increase in larval- and adult-antigen specific serum IgE (Kooyman et al., 1997; Bendixsen et al., 2004). Average total serum IgE concentration in uninfected Romney sheep is 1.8 µg/mL before infection and increases to between 17 and 40 µg/mL in serum during *T. axei* infection (Shaw et al., 1997). Increased serum IgE may also be an indication of resistance, as it is associated with reduced *H. contortus* worm burdens (Kooyman et al., 2000).

Immunoglobulin E produced in local lymph nodes tends to increase with infection. During secondary infection with *T. circumcincta* in lambs, total IgE in the lymph nodes peaks higher, 60 µg/mL, and earlier, 2 to 6 days PI, compared to the primary infection (Huntley et al., 1998a; Huntley et al., 1998b). A majority of total IgE present is specific to the L3 stage of *T. circumcincta*, indicating a rapid and specific response during the first few days of infection (Huntley et al., 1998a; Pernthaner et al., 2005b).

Since IgE can be produced by cells in the lymph nodes or the abomasum, Pernthaner et al. (2005b) measured IgE in lymphatic fluids draining these two areas. The lymph system is similar to the circulatory system, as lymph moves through tissues and lymph nodes. The lymph system facilitates movement of immune cells to and from tissues in order to aid in response to foreign antigens and inflammation. Pernthaner et al. (2005b) found that Romney lambs have higher levels of total IgE in lymph fluid draining from the small intestine compared to lymph coming from the intestinal lymph nodes, and concluded that the mucosa was the major site of IgE production. When *T. colubriformis* larval-specific IgE was measured in the same study, higher concentrations were produced in resistant compared to susceptible lines of Romney sheep. These findings support Bendixsen et al. (2004), who compared intestinal tissue of resistant and susceptible Scottish Blackface ewes infected with *T. colubriformis*. After 9 weeks of infection, increased larval-specific IgE was observed in resistant animals versus random-bred and susceptible sheep. Polymorphisms exist within the IgE gene, and specific alleles are associated

with *T. colubriformis* resistance in one flock of sheep (Clarke et al., 2001). This result could not be confirmed by Clarke et al. (2001) in a separate flock, which may suggest that the IgE polymorphism is not linked to loci influencing resistance in this sheep flock. These studies suggest that local production of IgE in the mucosa and lymph nodes occurs in response to nematode infection, and can be measured systemically through circulating IgE. Resistant wool lambs of some breeds tend to have higher levels of IgE, which is associated with fewer adult worms (Kooyman et al., 2000). Increased IgE may therefore be partially responsible for resistance in some breeds of sheep.

Eosinophils, mast cells, and globule leukocytes

Eosinophils, mast cells, and globule leukocytes (GL) are immune cells that protect the body from infection. Eosinophils are mainly found within tissues, and only make up a small portion of circulating leukocytes. On the other hand, mast cells, and activated, degranulated intraepithelial mast cells, known as GL (Huntley et al., 1984a), are found exclusively in tissue. The lifespan of these cells varies from a few hours to a few months. Eosinophils are short lived in circulation, with a half-life of about 18 hours, but stimulated eosinophils in tissue can persist for up to a few weeks. Mast cells and GL are much longer lived cells that can survive for a month or more. In order for any of these cells to protect the body from infection, they must be activated. Activation and subsequent degranulation occurs through antigens cross-linking IgA or IgE that has been bound to eosinophil and mast cell antibody receptors, respectively (Prussin and Metcalfe, 2003). Activation, and the resulting release of potent chemicals contained in cellular granules, occurs in local tissue sites and in the lumen of the gut. These powerful chemicals can cause damage to foreign organisms and surrounding self tissue (Meeusen and Balic, 2000). Eosinophils, mast cells, and GL increase in sheep during parasitic infection (Zajac et al., 1990; Kooyman et al., 2000; Amarante et al., 2005; Bricarello et al., 2005). These cells are negatively associated with FEC (Amarante et al., 2005) and worm burdens (Zajac et al., 1990) and may affect resistance to gastrointestinal nematodes.

Eosinophils in circulation. Sheep infected with gastrointestinal nematodes have increased concentration of circulating eosinophils (Zajac et al., 1990; Kooyman et al., 2000; Henderson and Stear, 2006) that result from production and differentiation of these cells in bone marrow in response to infection. Zajac et al. (1990) found increased circulating eosinophil levels

in hair compared to wool lambs after secondary infection, although the breed difference was not significant. More frequent measurements of circulating eosinophils suggests that eosinophils increase within 24 hours and peak by 8 to 10 days after *T. circumcincta* infection in 9-month-old wool lambs (Henderson and Stear, 2006). Circulating eosinophil concentration profiles in the previous study parallel concentrations of serum IgA, suggesting potential co-regulation or need for both immune effectors during infection. A correlation between circulating eosinophil levels and *T. circumcincta* larvae-specific serum IgA ($r = 0.514$) occurs in wool sheep, and these two factors together account for 53 % of the variation in worm burden (Henderson and Stear, 2006). The coexpression of each of these immune components suggest that eosinophils require IgA for optimal activation. Circulating eosinophils most likely have no direct effect on *H. contortus* larvae, so it is assumed that infiltration of these cells into mucosal tissue permits interaction with invading larvae.

Tissue levels of eosinophils, mast cells, and globule leukocyte. Enumeration of mast cells and GL in sheep during nematode infection have yielded varying results, whereas tissue eosinophils tend to increase during secondary infection. The inconsistencies are most likely the result of differences in breed and age of sheep, time of sample collection, type (natural versus artificial) of infection, severity of infection, and species of parasite. Most research shows increased numbers of eosinophils, mast cells, or GL in more resistant animals and/or negative correlations with measures of worm burden (Zajac et al., 1990; Gill et al., 2000; Amarante et al., 2005; Bricarello et al., 2005; Balic et al., 2006). One of the few studies to evaluate differences in eosinophils, mast cells, and GL in hair and wool lambs was performed by Zajac et al. (1990). Differences in mast cells and GL between hair and wool breeds were not significant at 14 days PI in older animals, although eosinophils were not measured and only a small number of animals were used per group. In a second study, differences in eosinophils or mast cells were not found, but significantly more GL occurred in hair lambs after 9 and 15 weeks of natural infection (Gamble and Zajac, 1992).

Other studies suggest that the timing of cell infiltration is critical, with resistant animals having a faster response (Lacroux et al., 2006). There is a short amount of time in which the host can respond to the parasitic larvae before the larvae develop into adults and move to the lumen of the abomasum. Tissue eosinophil counts tend to peak around 3 to 5 days after artificial infection with *H. contortus* (Balic et al., 2000; Balic et al., 2003), corresponding to the time larvae occupy

their tissue niche. Mast cells tend to respond more quickly (within hours) compared to eosinophils. If sheep can rapidly mobilize mast cells and effectively expel parasites using this mechanism, eosinophils would not need to be recruited (Meeusen and Balic, 2000; Balic et al., 2006). Larvae escaping damage from mast cells move to the gastric pits of the mucosa, where the host has more time to respond to and damage the parasite. Balic et al. (2006) found that within 24 hours of abomasal infection eosinophils not only surround *H. contortus* larvae in the gastric pits and overlying mucus layer, but the larvae were already damaged and undergoing structural collapse. Additionally, the same study showed that animals that remained parasite-free for 22 weeks before infection have the same number of tissue eosinophils as sheep infected after a 7-week parasite-free period. However, only animals infected after a 7-week parasite-free period have tissue eosinophils found in close association with and causing damage to invading larvae. Rainbird et al. (1998) showed that antigen-specific antibodies interact with activated eosinophils, leading to a significant increase in larval immobilization and larval damage *in vitro.* Sheep that undergo a prolonged parasite-free period may have decreased production of antigen-specific antibodies needed for eosinophil-induced parasite damage.

These studies suggest that eosinophils can damage and potentially aid in expulsion of invading larvae within the first few days of infection. Further exploration of the mechanisms of activation and degranulation in eosinophils and mast cells during early infection may provide a better understanding of resistance within different breeds of sheep.

Local gene expression during gastrointestinal nematode infection

Extracellular parasite infection typically leads to production of local and systemic antibodies by plasma cells, infiltration of mast cells and eosinophils, and differentiation of $CD4^+$ T-helper lymphocytes into T-helper type-2 (TH2) cells (Balic et al., 2000; Lacroux et al., 2006). These cells communicate, either through direct contact or chemical messengers, such as cytokines, to coordinate a response to the invading parasite. Cytokines produced by TH2 cells include IL-3, IL-4, IL-5, IL-10, and IL-13, and help develop and maintain the typical response to extracellular parasites. Some of these cytokines can also downregulate the opposing cellular immune response, which is characterized by naïve $CD4^+$ Th-cell differentiating into TH1 cells.

Cytokines produced by TH1 cells include interferon gamma (IFN-γ), IL-2, IL-12, IL-18, and IL-23. Although Gill et al. (2000) claimed a clear TH2 immune response was induced during gastrointestinal nematode parasite infection, more recent studies show a slightly modified TH2 response to be produced with greater IL-13, IL-5, and TNF-α, but not IL-4 or IL-10 (Schallig, 2000; Meeusen et al., 2005; Pernthaner et al., 2006).

T-cells

In addition to eosinophils and mast cell infiltration into the abomasal tissues during *H. contortus* infection, effector T- helper cells ($CD4^+$ T-cells) also accumulate. Effector TH cells are important for producing cytokines, activating immune cells, and helping to regulate the immune response. Lambs infected with *H. contortus* have more $CD4^+$ T-cells in the lymph nodes and tissue of the abomasum within 3 to 5 days after infection (Balic et al., 2000). These cells may be necessary for coordination of parasite damage and/or parasite expulsion in sheep, since depletion of $CD4^+$ cells leads to higher FEC (Pena et al., 2006).

Cytokines and the MHC

IL-13 and IL-5. Cytokine levels in infected hair sheep have not been studied, but comparisons of resistant and susceptible wool sheep found increased expression of IL-13 and IL-5 in resistant animals in response to *H. contortus* and *T. colubriformis* infections (Meeusen et al., 2005; Pernthaner et al., 2005a; Lacroux et al., 2006; Pernthaner et al., 2006). These two cytokines are important in the immune response to these parasites, since IL-13 promotes antibody class switching to IgE and IL-5 stimulates and activates eosinophils (Sanderson, 1992). A general upregulation of IL-5 and IL-13 in lymph nodes and abomasum occurs within 3 days after *T. colubriformis* infection (Meeusen et al., 2005; Lacroux et al., 2006). These results agree with the documented increase in IgE production (Kooyman et al., 2000) and the greater number of eosinophils (Henderson and Stear, 2006) in resistant animals during infection. Microsatellite markers in the area associated with the IL-3, IL-5, IL-4, and IL-13 genes on sheep chromosome 5 are linked to decreased FEC in two sheep flocks (Benavides et al., 2002). These findings further validate the importance of these TH2-type genes in parasite resistance.

Interferon gamma. The role of IFN-γ in parasite resistance has not been determined. The predominant function of IFN-γ, in general, is to drive a TH1-type immune response by downregulating IL-4, inhibiting TH2 cells, and promoting TH1 cell proliferation (Pulendran,

2004). Interleukin-12 is the initiator of TH1 development, causing $CD4^+$ TH0 cells to become TH1 cells and increase expression of IFN-γ (Collins et al., 1999). Production of IFN-γ remains stable or increases in abomasal and lymph fluid during infection even though there is an increase in opposing TH2 cytokines (Meeusen et al., 2005; Pernthaner et al., 2006). Other studies observe an expected decrease in IFN-γ as IL-5 increased, although these were the only two cytokines measured (Gill et al., 2000). The previous study measured cytokines produced by sheep lymph node cells collected 0, 5, or 28 days after parasite infection and cultured with parasite antigen (Gill et al., 2000). The inconsistencies observed between the studies may be due to differences in breeds and potentially due to polymorphisms in the region of the IFN-γ gene.

A quantitative trait loci (QTL) in the IFN-γ gene is associated with reduced FEC and increased IgA was located in a flock of Romney sheep (Dominik, 2005), free-living Soay sheep (Coltman et al., 2001), and Scottish blackface sheep (Davies et al., 2006). The transcription factors for IFN-γ have also been shown to affect parasite resistance in mice (Behnke et al., 2006). IFN-γ expression or cytokine production were not measured in sheep used in the previous studies. Production of IFN-γ by animals with different IFN-γ haplotypes needs further exploration to define the role of this cytokine in parasite resistance mechanisms.

Major Histocompatibility Complex. Although few studies have looked at the expression of individual major histocompatibility complex (MHC) haplotypes and effects on parasite burdens, QTL and microarray studies show that genes in this region affect levels of parasite resistance (Diez-Tascon et al., 2005; Dominik, 2005; Keane et al., 2006). Different genes of the MHC are upregulated and downregulated in resistant sheep under natural infection, as well as in resistant naïve animals. These genes were not evaluated further, and their impact on resistance is not clear. Alleles at the DRB1 locus of the MHC are associated with decreased FEC in Suffolk, but no association was found in the Texel breed. Since the MHC is highly variable, there may be multiple alleles within different breeds that alter resistance to gastrointestinal nematodes.

The recruitment of mast cells and eosinophils to the abomasal mucosa in response to *H. contortus* or other larvae may be the driving force in local cytokine production. Both of these cell types can produce a wide variety of cytokines, including those typical of TH1 and TH2 cells. Eosinophils and mast cells can produce TH2 cytokines (e.g. IL-4, IL-13, and IL-10) and TH1 cytokines (e.g., IL-12, IL-16, and IFN-γ), cytokines influencing cell growth and recruitment (e.g., IL-3, IL-5, and granulocyte-macrophage colony stimulating factor (GM-CSF)), and

cytokines involved in inflammation and tissue repair (e.g., IL-1, IL-6, IL-8, transforming growth factor β, and TNF-α) (Behm and Ovington, 2000; Henz et al., 2001). The presence of a large number of these cells in resistant animals during infection may be the reason for a mixed TH1-TH2 response. Some authors have suggested (from studies of mouse models) that increased levels of inflammatory cytokines produce an inhospitable environment for the parasite. Under inflammatory conditions, TH2 mechanisms present during infection aid in parasite damage (Maizels et al., 2004). If mast cells alone cannot expel the larvae before they reach the tissue, then eosinophils and mast cells can provide both inflammatory and TH2 signals needed for parasite expulsion. In conjunction with IL-4 or IL-13, TNFα, which is usually associated with inflammation, may also help to activate surrounding eosinophils (Luttmann et al., 1999). Potential problems with this type of response may occur when sheep do not respond to parasites fast enough and recruited immune cells reach the tissue after larvae have migrated to the lumen of the abomasum. Activation and degranulation of immune cells resulting from binding to the shed cuticle/sheath of the larvae would also be problematic, since surrounding self tissue would be damaged without damage to the parasite (Garside et al., 2000; Balic et al., 2006). The latter problem occurs in mice infected with certain parasite species, where local gut inflammation leads to little damage to the parasite (Garside et al., 2000).

SUMMARY

Infection by the abomasal parasite *H. contortus* imposes economical constraints on sheep production and reduced health of the infected animal. Prevalence of parasites resistant to dewormers is increasing and alternative parasite control methods need to be implemented. Selection of parasite-resistant animals is one such control method. Periods of suppressed immune function (young animals and periparturient ewes) occur, and resistant compared to susceptible animals have reduced parasite burdens during those times. Variation in resistance exists among breeds, and Caribbean hair sheep have greater parasite resistance compared to conventional wool breeds; therefore, hair sheep provide a genetic resource for increased parasite resistance.

Studies involving hair and wool breed comparisons, as well as comparisons of resistant and susceptible wool sheep have provided insight into proposed genes and mechanisms involved

in parasite resistance. If larvae reach their tissue niche, the best response appears to be a rapid infiltration of effector $CD4^+$ T cells, eosinophils, and mast cells along with parasite-specific IgA and IgE antibodies. These cells infiltrating the abomasum and cells in surrounding abomasal lymph nodes produce an altered TH2 response defined by increased IL-5, IL-13, TNFα, and potentially IFNγ. These cytokines are responsible for recruitment of eosinophils, antibody class switching to IgE, increased vascular permeability and blood clotting, and promotion of TH1 and inflammatory responses, respectively. The combination of these immune effectors, along with other unknown factors, leads to damage and expulsion of invading parasites.

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CHAPTER 2

Mucosal immunity in resistant hair and susceptible wool sheep parasitized by *Haemonchus contortus.*

ABSTRACT: Greater resistance to gastrointestinal parasites occurs in Caribbean hair sheep in comparison to conventional wool breeds. However, breed differences in response to infection in hair sheep, such as the St. Croix, and wool sheep are not well characterized. Our objective was to evaluate breed differences in immune effector cell populations and antibody concentrations in hair (n = 12) and wool (n =12) sheep infected with the abomasal parasite *Haemonchus contortus* and in uninfected animals. Six infected animals of each breed were sacrificed on either day 3 or 27 post-infection (PI) to represent the response to larvae or adult worms, respectively. An additional 14 hair and 14 wool animals remained uninfected and served as controls. Fecal egg counts were lower, although not significantly, and packed cell volumes were higher in infected hair compared to wool lambs, indicating increased parasite resistance in hair sheep. Abomasal lymph node weights were higher in infected compared to control animals ($P < 0.05$), and those of infected hair sheep were heavier compared to infected wool sheep ($P < 0.05$). Differences in numbers of eosinophils in abomasal tissue approached significance ($P = 0.07$) with more cells in hair compared to wool sheep by 3 days PI, but no difference were found for numbers of globule leukocytes. Differences in antibodies were apparent, with greater total IgE and IgA in serum of control hair sheep compared to control wool sheep ($P < 0.05$). During infection, hair sheep had increased total serum IgA compared to wool sheep by 3 days ($P < 0.01$) and at 5 and 21 days ($P < 0.05$) PI, but no difference in serum IgE were observed between breeds. However, after 27 days of infection, total IgE in lymph nodes of infected hair sheep had increased ($P < 0.01$) over concentrations found in infected wool sheep. Thus, resistant Caribbean hair sheep compared to conventional wool sheep have measurable differences in the immune response.

KEYWORDS: *Haemonchus contortus*, IgA, IgE, mast cell, eosinophil, sheep

INTRODUCTION

Caribbean hair sheep have greater resistance to gastrointestinal nematode (GIN) parasites compared to wool sheep, with breed differences apparent by 4 months of age (Zajac et al., 1990; Vanimisetti et al., 2004b). In hot, humid areas the most common and problematic GIN of sheep is *Haemonchus contortus,* a blood-feeding, abomasal parasite. The general immune response of sheep to *H. contortus* infection is a predominant T-helper cell type 2 (TH2) immune response characterized by eosinophilia, mastocytosis, increased IgA and IgE production, and increased TH2 cytokines (Kooyman et al., 1997; Schallig, 2000; Meeusen et al., 2005; Lacroux et al., 2006). However, reports of differences in the immune response between resistant hair and susceptible wool breeds to *H. contortus* infection are inconsistent (Zajac et al., 1990; Gamble and Zajac, 1992). Antibody production and accumulation of immune effector cells, such as eosinophils, mast cells, and globule leukocytes (degranulated intra-epithelial mast cells), varied across studies.

Immunoglobulin E and IgA production increased after GIN infection in wool sheep selected for increased parasite resistance, indicating these antibodies are associated with greater resistance (Bendixsen et al., 2004; Pernthaner et al., 2005b; Pernthaner et al., 2006). Resistant wool sheep appear to have greater infiltration of eosinophils, mast cells, and globule leukocytes in gastrointestinal tissue during infection, but results are not consistent among studies (Bisset et al., 1996; Amarante et al., 1999a). Greater numbers of these cells are associated with lower fecal egg counts (FEC) and worm burdens in resistant strains of both hair (Zajac et al., 1990) and wool sheep (Amarante et al., 1999a; Amarante et al., 2005). Increased resistance may result from direct parasite damage by eosinophils, and most likely mast cells, which can bind to the parasite and degranulate (Rainbird et al., 1998; Balic et al., 2006). Additionally, both eosinophils and mast cells may affect the animals resistance status by production of TH2-type cytokines such as IL-4, IL-5, IL-10, IL-13, and induction of IgE production (Henz et al., 2001; Rothenberg and Hogan, 2006).

This study was designed to compare Caribbean hair sheep and conventional wool sheep to determine differences in immune responsiveness during infection with *H. contortus*. Eosinophil and globule leukocyte infiltration into the abomasal mucosa and total IgE (lymph node and serum) and IgA (serum only) concentrations were assessed in control animals and

during infection. These immune components were then evaluated for their association with FEC in both breeds of sheep.

MATERIALS AND METHODS

Animals and tissue collection

St. Croix hair lambs (n = 26) and wool (n = 26) lambs of 50 % Dorset, 25 % Rambouillet, and 25 % Finnsheep breeding were maintained at the Virginia Polytechnic Institute and State University Sheep Center in Blacksburg, VA. All procedures were approved and carried out in accordance with the Animal Care Committee of Virginia Tech. January-born lambs were raised under field conditions with no effort to prevent parasitic infection. The overall design of the study is presented in Figure 2.1. At approximately 4 to 5 months of age, lambs were infected with 3,000 *H. contortus* infective third stage larvae (L_3) weekly for 4 consecutive weeks to ensure that all lambs had been exposed to the parasite. One week after the last infection, lambs were moved to drylot to limit infection with nematode parasites. Because macrolide resistance was present, a combination of levamisole (8 mg/kg body weight) and fenbendazole (10 mg/kg body weight) was used for deworming. All animals were dewormed when moved to drylot and again 3 days later. Lambs were subsequently moved to raised indoor pens 1 week prior to collection of samples, to provide a more controlled environment, and were kept in these pens for the remainder of the study. Five days after the last deworming and 3 days prior to experimental infection, lambs were dewormed for a third time to completely remove existing infections. Immediately prior to experimental infection, no eggs were detected in lamb fecal samples. Small numbers of coccidial oocysts were seen throughout the study, but symptoms of coccidiosis were not apparent.

Three days after the last deworming, 12 lambs of each breed were orally infected with 10,000 *H. contortus* L_3 larvae and 14 lambs of each breed were left as uninfected controls. Due to space limitations, control animals remained in drylot for an additional 2 weeks and were moved to indoor pens on day 7 (relative to infected animals). Control animals were dewormed on day 8 to approximate treatments in infected animals. However, control lambs were

accidentally infected on day 11 and were dewormed on day 12 and 14. At all time points assessed, control animals had no parasitic nematode eggs present in the feces. However, levamisole is known to affect immune function (Kurakata and Kitamura, 1983; Cabaj et al., 1995) and the effect of fenbendazole is not known.

Infected animals of each breed (n = 6) were euthanized at 3 or 27 days post-infection (PI). These days were selected to represent the response to larvae (day 3) and adult worms (day 27). Control animals of each breed were sacrificed at day 17 (n = 4), 27 (n = 6), and 38 (n = 4), relative to day 0 of infected animals. All animals were killed by captive-bolt pistol followed by exsanguination.

The gastrointestinal tract was removed immediately and processed for tissue collection. The abomasum was tied off at both ends and removed from the remaining digestive tract. Lymph nodes were removed from the lesser curvature of the abomasum and weighed. Additional adipose tissue was removed, and the abomasum was cut along the greater curvature and washed with room temperature PBS. A 2.5 cm^2 section of tissue, including the full thickness and one fold of the abomasum, was removed from the fundic region of the abomasum. The tissue was fixed in 10 % formalin and stored at 4°C.

Fecal samples were collected from the rectum of infected animals on day 0, 16, 21, and 27 PI and controls on day 14, 17, 21, 27, and 38 PI and processed as described below. Blood samples were obtained from available animals by jugular venipuncture on days 0, 3, 5, 16, 21, and 27 for infected animals and on days 14, 17, 19, 27, 30, 35, and 38 for control animals (corresponding to similar time intervals of infected animals). Packed cell volumes (PCV), or percentage of red blood cells, were determined by micro-hematocrit centrifugation. Additional blood samples were allowed to clot at room temperature, centrifuged, and serum was removed and stored at -20°C.

Parasitologic techniques

Adult *H. contortus* were collected from pasture infected sheep. Worms were pulverized in an ice-cold glass tissue homogenizer to release developing eggs. Homogenate was mixed with

egg-free feces to obtain a mono-specific larval culture. The Baermann technique was used to collect *H. contortus* L_3 larvae. These larvae were used to infect two worm-free donor lambs. At least 21 days after infection, feces was collected from donor lambs and cultured at 30 °C for 7 to 8 days. Larvae were collected as described above, stored at 4°C in deionized water, and used within one month to orally infect experimental animals. Fecal egg counts were determined by the modified McMaster's technique (Whitlock, 1948). Each egg observed represented 50 eggs per gram of feces.

Histology

Formalin-fixed sections, comprising the full thickness of the abomasum, were stained with hematoxylin and eosin for eosinophil and globule leukocyte enumeration. A graticule (10 x 10 mm) was used to count a total of 100 different fields, under a 100x oil immersion lens and a 4x eyepiece. Only acceptable longitudinal sections of the mucosa were used, and fields were selected to cover three separate areas of the tissue section, when possible. Data were analyzed as the total number of cells counted, which covered a 0.0625 mm^2 area.

Total IgA and IgE ELISA

IgA. Enzyme-linked immunosorbent assay (ELISA) was used to determine total IgA in serum. Optimal dilutions were determined previously by checkerboard titration for the coating antibody, sheep serum, and the conjugated antibody (data not shown). Nunc immuno 96-well flat bottom plates were coated at 4°C overnight with 100 µl of affinity purified rabbit anti-sheep IgA (2 µg/mL; Bethyl Laboratories, Inc.). Plates were washed 3 times with 300 µL of 0.05 % Tween-20 in phosphate buffered saline (PBS) solution between all steps and washed an additional 2 times before TMB substrate (Pierce Biotechnology, Inc., Rockford, IL) was added. To limit non-specific antibody binding, 200µL of a 1 % bovine serum albumin (in PBS-Tween) solution was used to block the plates and was incubated for 1 hour at room temperature. Sheep sera were diluted 1:4000 in PBS-Tween and run in duplicate with 100 µL of the serum dilution added to each well. Horseradish-peroxidase conjugated rabbit anti-sheep IgA (Bethyl Laboratories, Inc.) was used as the detection antibody; 100 µL (50 ng/mL) was added to each well and incubated at room temperature in the dark for 1 hour. After addition of 100 µl of TMB

substrate, the reaction was allowed to develop for 45 minutes. The reaction was stopped with 50 µL 2M H_2SO_4, and the plate was read at wavelengths of 450 nm and 630 nm. Background absorbance due to plate imperfections per well (630 nm) was subtracted from the absorbance at 450 nm to determine sample concentration of total IgA as described below.

Sheep IgA (Accurate Chemical Co., Westbury, NY) was used as a standard on each plate. Blank wells (only blocking solution) were also run on each plate to measure background absorbance per plate, allowing for plate to plate comparisons. The standard was serial diluted (2x) down the plate, in duplicate, from a starting concentration of 3 µg/mL. A standard curve was determined and used to calculate sample concentration. Samples whose absorbance values did not fall within the range of the standards were further diluted and reanalyzed. Mean values for duplicates were used for further analysis.

IgE. Lymph node tissue (1 g) was homogenized at 4°C with 4 mL of PBS using a glass tissue homogenizer. Samples were centrifuged at 4°C for 30 minutes at 21,000 g. The supernatant was removed and stored at -20°C until further processed. Total IgE in serum and lymph node supernatants were determined as described by Huntley et al. (1998a).

Statistical analysis

PCV, FEC, and lymph node weights. The FEC data were not normally distributed, so data were transformed as ln(FEC + 100) before further evaluation. Analysis of FEC in infected lambs was carried out with a repeat-measures analysis of variance using the mixed models procedure of SAS (SAS Inst. Inc., Cary, NC). The model included fixed effects of day, breed (hair or wool), and breed by day interaction with day as the repeated effect. The FEC means are presented as back-transformed means and standard errors. PCV's were initially analyzed for breed differences within infection status with a repeat-measures analysis of variance using the mixed models procedure of SAS. The model included fixed effects for day, breed, and day by breed interaction with day as the repeated effect. Significance of differences in least square means was determined using a student t-test. Lymph node weights were not significantly different between days within breed and infection status, therefore data from lambs sacrificed at different times were combined. Combined lymph node weights were then analyzed using the

generalized linear model procedure of SAS with fixed effects of breed, infection status, and breed by infection interaction.

Cell counts. Counts of eosinophils and globule leukocytes were not normally distributed, therefore data were transformed as ln(count + 1) before further evaluation. Cell counts were analyzed using the generalized linear model of SAS. The model included fixed effects of breed, group (infection status by day of sacrifice, where there were 2 infected and 3 control groups), and the breed by group interaction. The data are presented as back-transformed means and standard errors.

ELISA. Serum immunoglobulin concentrations were initially analyzed for breed differences within infection status with a repeat-measures analysis of variance using the mixed models procedure of SAS. The model included fixed effects for day, breed, and day by breed interaction with day as the repeated effect. Significance of group comparisons of least square means was determined using a student t-test. Lymph node total IgE concentrations at sacrifice were evaluated for group (infection status by day) differences using the generalized linear model of SAS. The model included fixed effects of breed, group, and breed by group interaction.

Correlations (r) and significance values between measurements for all hair and wool lambs were obtained using the PROC CORR function of SAS (SAS Institute Inc., Cary, NC). Correlations with FEC were determined using data from hair and wool sheep infected for 27 days. All values were determined to be significant at $P < 0.05$ unless stated otherwise.

RESULTS

PCV, FEC, and lymph node weights

Fecal egg counts for control animals remained at zero throughout the study. In contrast, all experimentally infected hair and wool sheep were successfully infected and had measurable FEC by 16 days PI. Infected hair sheep had lower FEC than wool sheep throughout the study, although not significantly (Table 2.1). Others have reported similar results, with significantly lower FEC in hair sheep compared to wool sheep of the same and different lineages (Zajac et al.,

1990; Gamble and Zajac, 1992; Vanimisetti et al., 2004b). The average PCV's of uninfected control hair (36.3 ± 0.7) and wool (35.5 ± 0.5) sheep were similar. On day 16 and 21 PI, infected animals had significantly lower PCV's compared to control animals at all time points. As expected, PCV's of infected animals were higher in hair compared to wool sheep (Fig. 2.2), and breed differences between infected animals approached significance ($P < 0.10$) on day 21 PI. Abomasal lymph nodes from all parasite-infected sheep were significantly heavier than those of all control animals ($P < 0.001$, Table 2.2). Breed differences in lymph node weight were not apparent within control animals, but lymph nodes from infected hair sheep were heavier than those of infected wool sheep ($P = 0.04$). Lymph node weight was associated with FEC on day 21 (r = -0.72), initial PCV (r = 0.58), PCV on day 16 PI (r = 0.61), number of eosinophils (r = 0.45), and total IgE concentration in the lymph node (r = 0.36).

Cell counts

Significant differences were found for eosinophil counts between infected animals and all control sheep on day 27 and 38 (Fig. 2.3). Three days after infection, a somewhat larger number of eosinophils were found in abomasal tissue of hair compared to wool sheep ($P = 0.069$). Both hair and wool sheep maintained higher eosinophil counts through 27 days of infection compared to control sheep on day 27 and 38 ($P < 0.01$). After infection with *H. contortus,* changes in the number of globule leukocytes in abomasal tissue were less striking than those found for eosinophils. Globule leukocyte counts for control animals of both breeds were similar and data were combined across days within each breed. There were no observable differences in cell counts by 3 or 27 days PI in either breed compared to control animals of the same breed (Fig. 2.4).

Immunoglobulin concentrations

Breed and infection status have a significant effect on total IgA concentration in serum. Total IgA concentrations in serum ranged from 5.6 to 9.6 mg/mL in control hair sheep and from 1.1 to 3.1 mg/mL in control wool sheep. Concentration of total IgA was significantly higher in control hair sheep compared to control wool sheep (Fig. 2.5). IgA levels for infected hair sheep fell between values found in control animals, and concentrations did not differ between infected and uninfected wool sheep. In contrast, infected hair sheep had elevated IgA compared to

infected wool sheep at 3 days PI ($P < 0.01$), and breed differences approached significance on day 5 and 21 PI ($P < 0.10$, Fig. 2.5).

Haemonchus contortus infection did not lead to significant differences in serum total IgE between hair and wool sheep (Fig. 2.6). Control hair sheep had greater ($P < 0.05$) circulating IgE for the first 13 days of sample measurement compared to wool sheep (Fig. 2.6). However, IgE concentration started to drop in all animals by day 27. In contrast, there was a significant effect of breed and infection status on total IgE concentration in abomasal lymph node tissue. Higher IgE was found for infected animals compared to control animals ($P = 0.06$) and hair sheep compared to wool sheep ($P = 0.01$, Fig. 2.7). There was no change in total IgE concentration within the lymph nodes by 3 days PI in either breed, compared to controls. By 27 days PI, hair sheep have greater ($P < 0.01$) total IgE concentration in the lymph nodes compared to wool sheep. Total IgE in lymph nodes of hair sheep increased from 39 ng/mL to 106 ng/mL from 3 to 27 days of infection (Fig. 2.7). Wool sheep infected for 27 days have slightly elevated total IgE, but this was not significant and was similar to control values. In comparison to wool sheep, hair sheep have higher production of IgE in serum of control animals and in abomasal lymph nodes of infected animals.

DISCUSSION

St. Croix hair sheep are more resistant to *H. contortus* compared to conventional wool breeds such as the Dorset, Suffolk, Dorper, and Dorset x Rambouillet crosses (Zajac et al., 1990; Gamble and Zajac, 1992; Burke and Miller, 2002; Vanimisetti et al., 2004b). Similar to previous studies, we found that 6-month-old St. Croix hair lambs have increased resistance to *H. contortus* compared to wool lambs with Dorset, Rambouillet, and Finnsheep ancestry. Even though breed differences were not significant in this study, hair sheep have lower FEC and higher PCV than wool sheep after infection at all time points assessed. Previous evaluation of these same lines of hair and wool sheep (although the line of wool ewes was crossed with male Dorpers, another hair breed) show hair sheep have significantly lower FEC than the wool composite (Vanimisetti et al., 2004b). It is not clear from our results if breed differences in FEC are due to decreased worm burdens and/or reduced fecundity of female worms; both mechanisms may occur in resistant

breeds such as the St. Croix (Zajac et al., 1990; Gamble and Zajac, 1992). Although resistance status of Caribbean hair sheep is well documented, studies of the immune response including changes in eosinophils, mast cells, globule leukocytes, and antibodies during *H. contortus* infection in these sheep are few and show inconsistent results (Zajac et al., 1988; Zajac et al., 1990).

Abomasal lymph nodes of infected animals almost doubled in size compared to control animals, and lymph nodes were heavier in infected hair sheep compared to wool sheep. Balic et al. (2000) observed a similar two-fold increase in weight of abomasal lymph nodes in wool sheep 5 days after *H. contortus* infection. Similar cell populations were observed in infected and control animals in that study, although the absolute number of immune cells was greater in infected animals. Therefore, the results we observed suggest greater infiltration and/or proliferation of immune cells in the lymph nodes of parasite-resistant hair compared to wool sheep. Increased numbers of immune cells could lead to greater parasite damage, which could explain the association between greater lymph node weight and lower FEC.

We found a substantial increase in the number of tissue eosinophils in the abomasum of infected hair sheep. Eosinophils are implicated in increased parasite resistance due to the negative correlations with FEC (r = -0.85) and worm burdens (r = -0.29) in infected wool sheep (Buddle et al., 1992; Bricarello et al., 2005). Direct damage of parasitic larvae by eosinophils occurs *in vitro* and *in vivo* (Rainbird et al., 1998; Balic et al., 2006). The mechanisms involved in eosinophil-parasite binding and degranulation have not been completely determined, but the presence of IL-5, complement, and antibodies increases the ability of eosinophils to kill parasitic larvae (Rainbird et al., 1998). The proportion of different antibody classes used in that study was not assessed, but it is likely IgA was present and effected eosinophil activation.

Henderson and Stear (2006) found that eosinophils and IgA have similar concentration profiles in circulation and their combined effects account for 53% of the variation in worm (*Teladorsagia circumcincta*) length. Other studies show an increase in the number of eosinophils and IgA during nematode infection (Zajac et al., 1990; Amarante et al., 2005) and an association with decreased worm length and FEC (Martinez-Valladares et al., 2005). Eosinophils have cell surface receptors for IgA (Prussin and Metcalfe, 2003), however, receptors for IgE have not been found on mouse or sheep eosinophils (Jones et al., 1994; Pettit et al.,

2005). These findings suggest the presence of both eosinophils and IgA may be needed for increased resistance to GIN parasites.

Total IgA in circulation was higher in control hair sheep compared to control wool sheep and higher than infected animals of both breeds. Concentrations of total serum IgA in uninfected hair sheep were somewhat higher than those reported for wool sheep in previous studies (1.1 mg/mL, Cripps et al., 1985), but within the range found for cattle (0 to 32 mg/mL, Williams et al., 1975). These results suggest hair sheep, in general, produce more total IgA.

The decrease in serum total IgA in hair sheep after infection could result from IgA binding to eosinophils or IgA being diverted to the lumen of the abomasum. Serum IgA is derived from plasma cells in the gastrointestinal tract (Sheldrake et al., 1984), and the need for IgA in the gastrointestinal tract during infection may supersede transport into serum. Additionally, transport of IgA from serum to intestinal tissue has been shown to occur in sheep, although samples were from lactating ewes of unknown infection status and IgA transport to the intestine was low (5% , Sheldrake et al., 1984). Further evaluation of IgA concentration in the abomasal mucus may clarify the relationship of IgA with parasite resistance in Caribbean hair sheep.

Hair sheep have greater total IgA 3 days PI compared to wool sheep. The breed difference suggests a stronger antibody response by hair sheep that could lead to increased damage to invading parasitic larvae. Other authors report that larval-antigen specific IgA production increases and peaks between one and two weeks PI (Gomez-Munoz et al., 1999; Henderson and Stear, 2006). Differences in IgA production during the first week of infection have not been measured between resistant and susceptible animals. However, contradictory to our observation of total serum IgA, similar concentrations of abomasal mucus antigen-specific IgA were found in both resistant and susceptible breed types after one week of infection and under a natural challenge (Zajac et al., 1990; Amarante et al., 2005).

Globule leukocytes tended to increase, although not significantly, in the abomasum of infected hair sheep after 27 days of infection compared to controls. Gamble and Zajac (1992) saw an increase in the number of globule leukocytes in the abomasum of infected hair compared to wool lambs at 4 months of age. However, a 15- to 40-fold increase in cells was found for these animals, where we only found an approximate 2-fold increase. Variation between studies could be due to measurement of older animals in our study, since breed differences diminish

between animals by one year of age (Zajac et al., 1990). Greater numbers of globule leukocytes occur in infected lambs of several breeds compared to control animals under 9 months of age (Bricarello et al., 2004; Lacroux et al., 2006). Studies involving infected sheep greater than 9 months of age did not find differences in globule leukocytes compared to controls (Amarante et al., 1999a; Amarante et al., 2005; Balic et al., 2006), with the exception of one study of 2- to 3-year-old sheep (Balic et al., 2003). Associations of increased globule leukocytes with lower FEC and decreased female worm length are present in younger animals and suggest a role for these cells in resistance (Lacroux et al., 2006).

Globule leukocytes are described as intraepithelial, partially degranulated mast cells (Huntley et al., 1984b). Many authors suggest mast cells and globule leukocytes are responsible for larvae damage and expulsion within the first few days of infection, unfortunately studies have not been performed to confirm this hypothesis. However, mast cells do bind IgE (Prussin and Metcalfe, 2003), leading to a similar co-dependency as that suggested for eosinophils and IgA. We found similar profiles for abomasal globule leukocyte counts and total IgE within the abomasal lymph nodes. These results suggest co-regulation of the two effectors, potentially with mast cell-IgE binding, allowing for activation and degranulation leading to formation of globule leukocytes.

Measurement of sheep IgE was first reported by Shaw et al. in 1996 and Kooyman et al. in 1997. Since then, multiple studies show that sheep infected with GIN parasites have increased total and worm-antigen-specific IgE (Kooyman et al., 1997; Huntley et al., 1998b; Shaw et al., 1998; Bendixsen et al., 2004; Pernthaner et al., 2005b). Bendixsen et al. (2004) found that total IgE ranges from 0.5 to 11 ng/mL in serum and from 45 to 620 ng/g in intestinal homogenate of 4.5-month-old wool lambs. Higher total IgE concentrations occur in serum (0.7 to 2.8 μg/mL) and lymph fluid (up to 60 μg/mL) of nematode-infected wool lambs (Huntley et al., 1998b). Comparison of wool sheep selected for increased parasite resistance versus those selected for susceptibility shows resistant animals have greater amounts of antigen-specific IgE in lymph fluid (Pernthaner et al., 2005b) and intestinal homogenate (Bendixsen et al., 2004). We also found that resistant hair sheep have greater IgE in lymph nodes during infection when compared to susceptible wool sheep. As seen with IgA, control hair sheep have greater IgE in serum compared to wool sheep. Increased antibodies in control hair sheep may indicate these animals

respond to infection faster and more effectively than wool sheep. However, after infection serum total IgE did not increase in either breed when compared to controls.

Caribbean hair sheep have increased resistance over a conventional wool breeds, and we show that breed differences in immune parameters exist between these animals. The abomasal lymph nodes of resistant hair sheep were larger, indicating a higher level of immune responsiveness. Overall, the immune response of parasite-infected hair sheep can be characterized by greater infiltration of eosinophils, increased total IgA in serum, and greater total IgE in abomasal lymph nodes compared to wool sheep. Interaction of these immune cells and antibodies may be an important aspect of resistance. Further evaluation of immune cell populations, differences in cell signaling, and cytokine production will help to determine resistance mechanisms in hair sheep infected with *H. contortus*.

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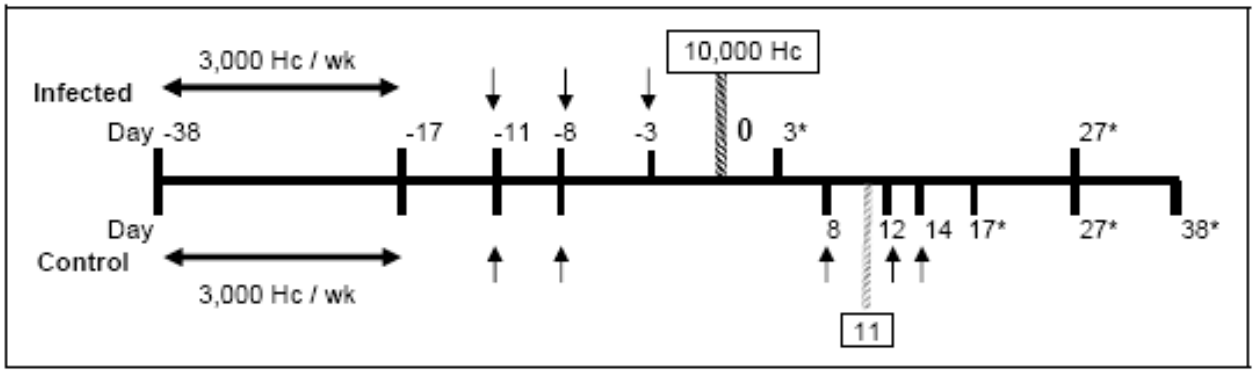
Figure 2.1. Depiction of infection, deworming, and sampling of infected and uninfected (control) hair and wool sheep. Days are relative to the final dosing of infected animals with *Haemonchus contortus* larvae (Hc). Control animals were accidentally infected on day 11.



↑ Infected and/or control animals treated with anthelmintic

* Animals euthanized and tissue samples collected

Figure 2.2. Packed cell volumes (PCV, %) of *Haemonchus contortus* infected and control hair and wool sheep.

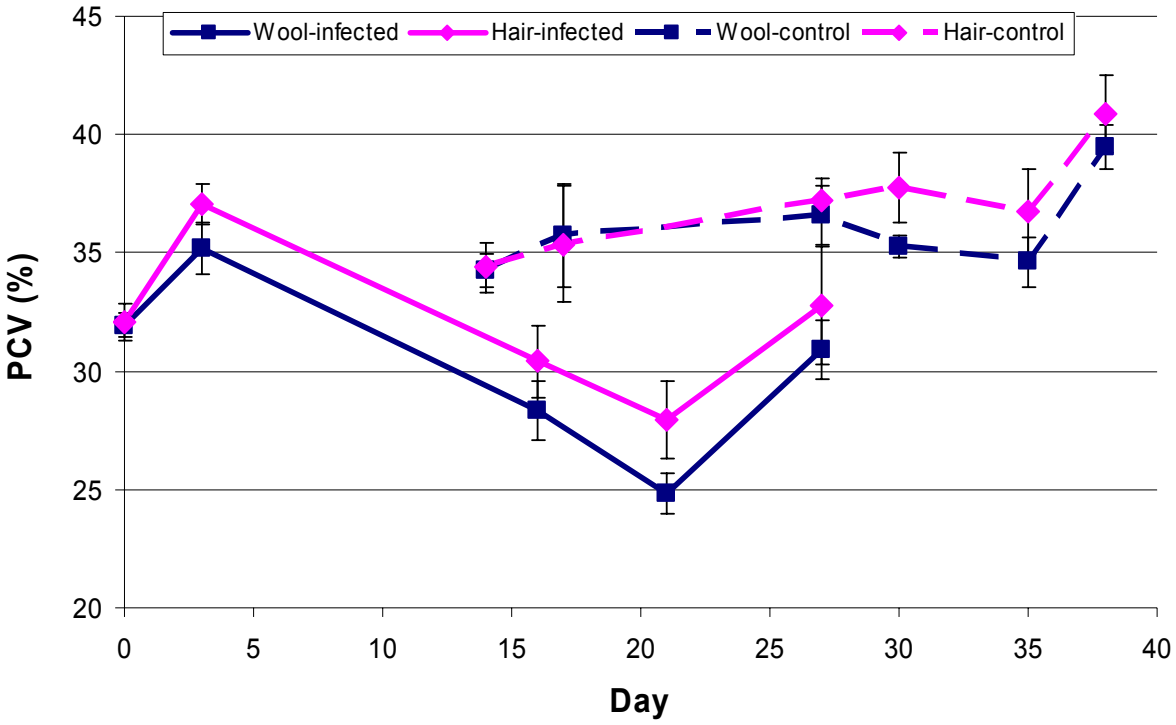
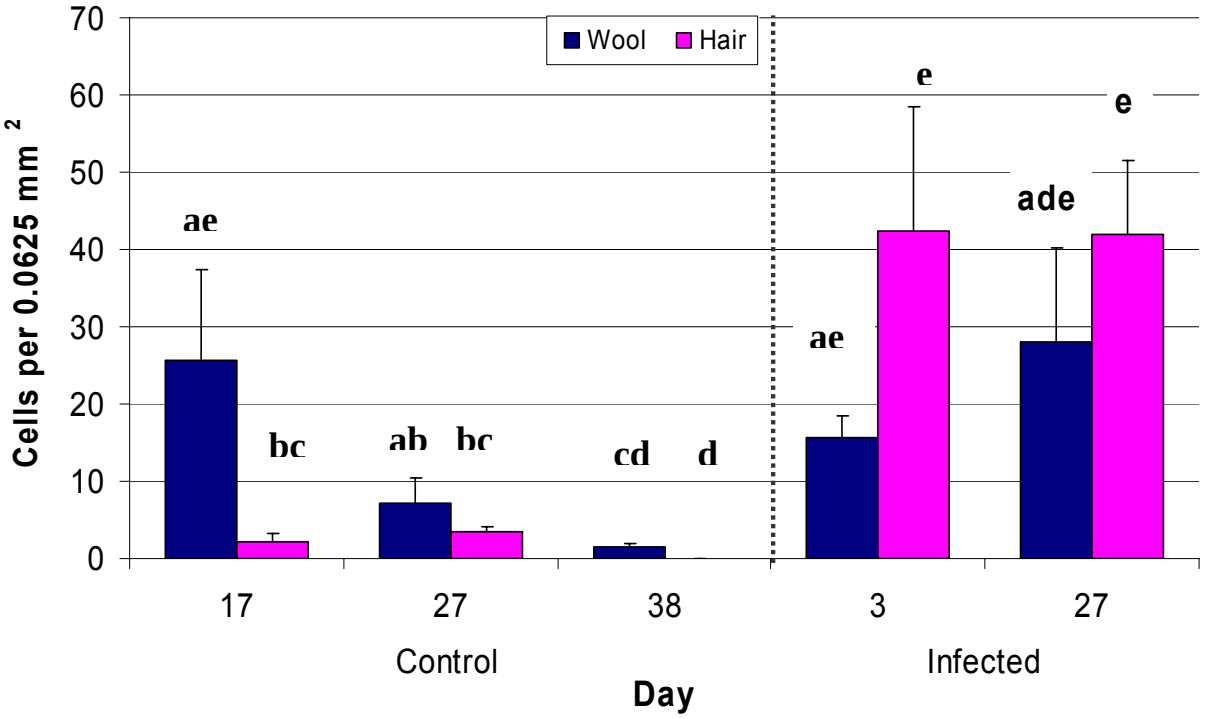
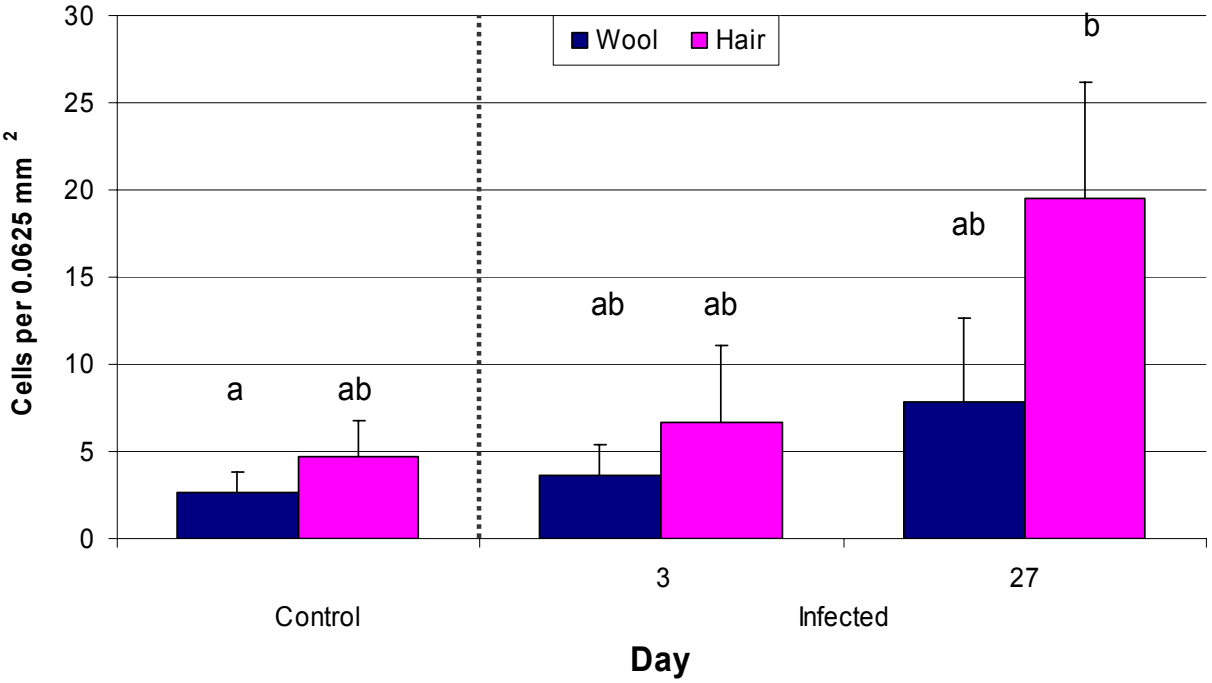


Figure 2.3. Back-transformed log eosinophil counts in abomasal tissue of control and *Haemonchus contortus*-infected hair and wool sheep.



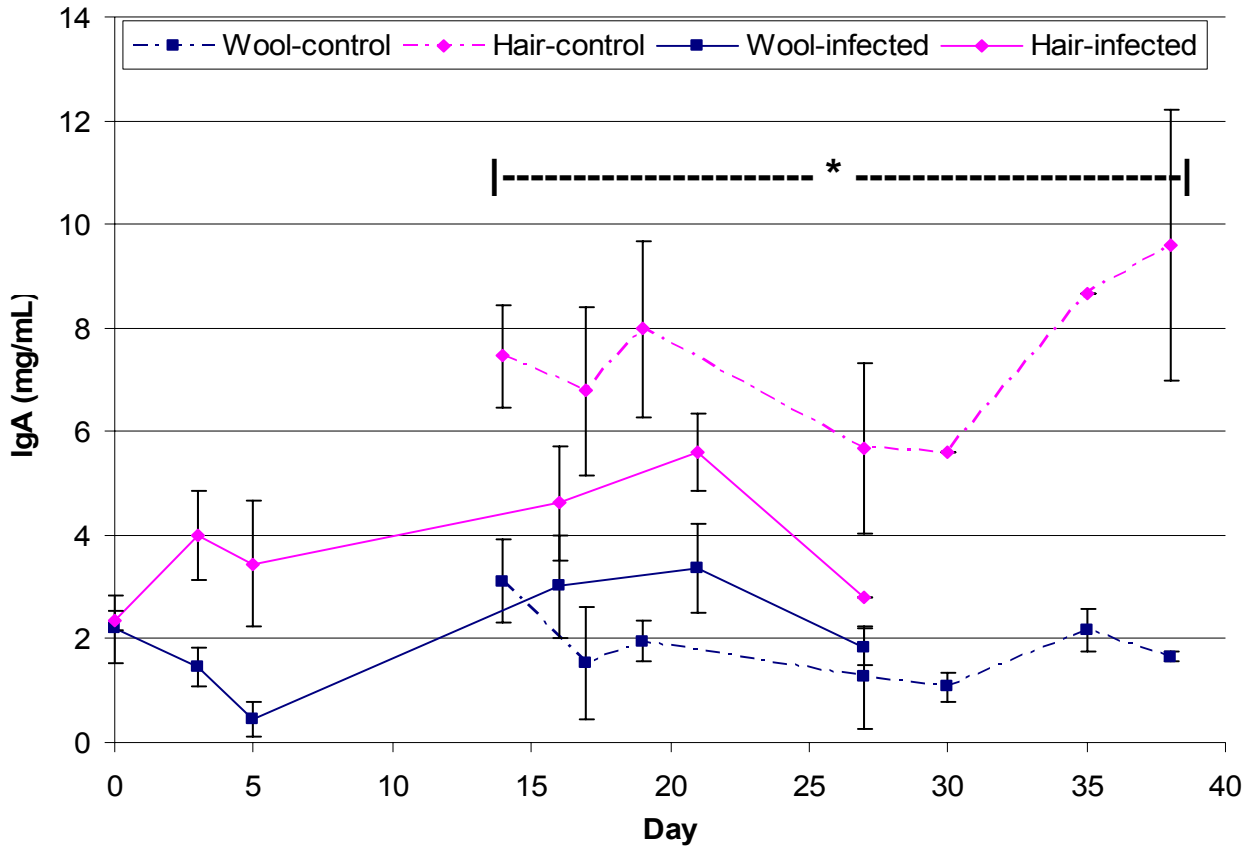
[abcde] Columns with different letters have significantly different least square means at $P < 0.05$

Figure 2.4. Back-transformed log globule leukocyte counts in abomasal tissue of *Haemonchus contortus*-infected and control hair and wool sheep.



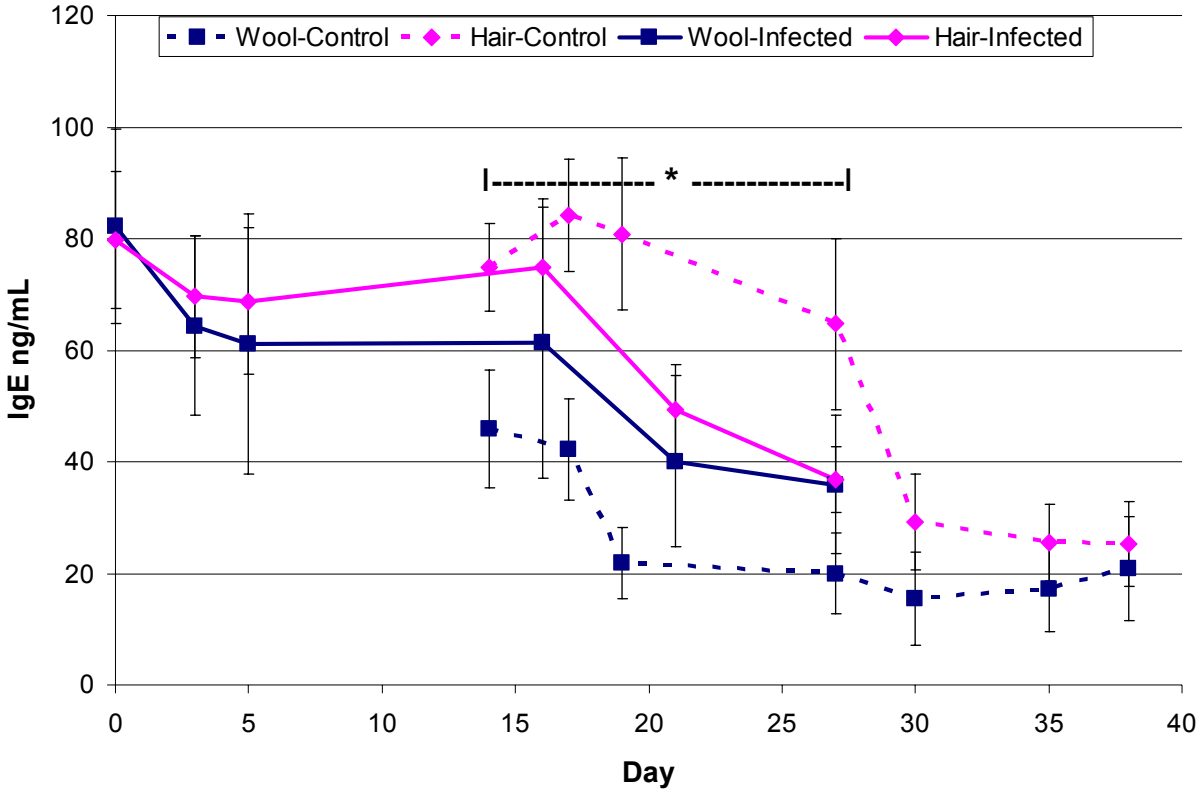
[ab] Columns with different letters have significantly different least square means at $P < 0.05$

Figure 2.5. Total IgA in serum of control and *Haemonchus contortus*-infected hair and wool sheep.



* Significant difference between breeds within control animals at $P < 0.05$.

Figure 2.6. Total IgE in serum of control and *Haemonchus contortus*-infected hair and wool sheep.



* Significant difference between breeds within control animals at $P < 0.05$.

Figure 2.7. Total IgE in the lymph node supernatant of control and *Haemonchus contortus*-infected hair and wool sheep.

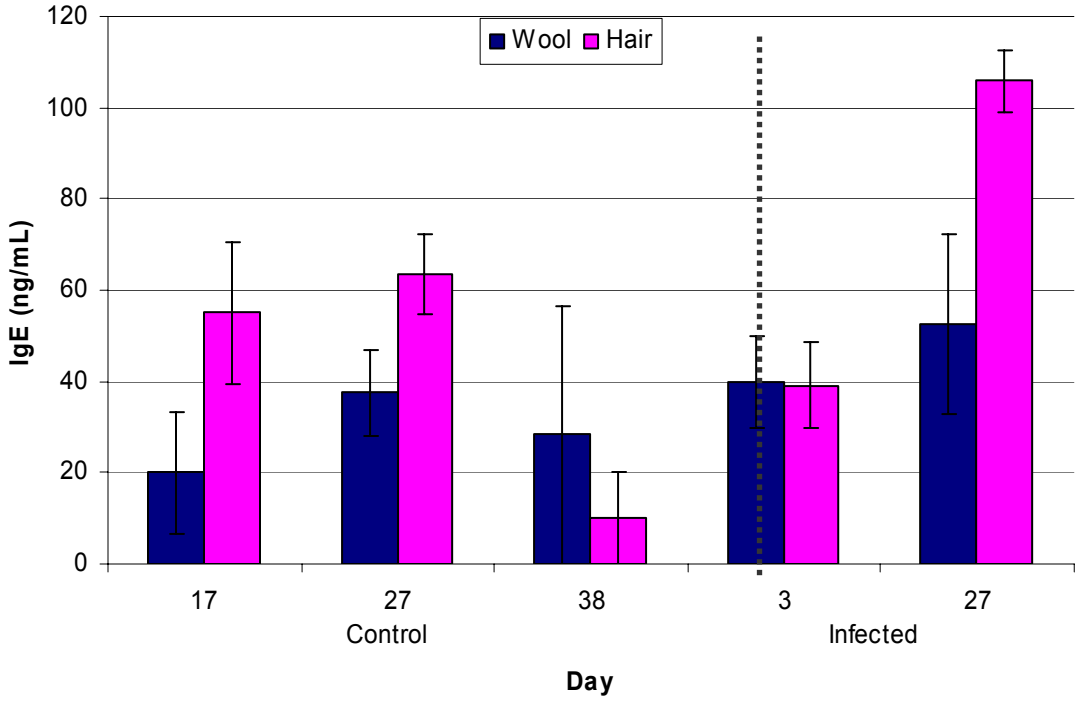


Table 2.1. Back transformed log FEC (mean ± standard error) of hair and wool lambs at 16, 21, and 27 days after infection with the abomasal parasite *Haemonchus contortus*.

	16	21	27
Wool	1213 ± 330[ab]	3647 ± 770[b]	3136 ± 1599[ab]
Hair	919 ± 100[a]	1280 ± 867[ab]	1267 ± 837[ab]

[ab] Different letters indicate significant differences between least square means at $P < 0.05$

Table 2.2. Weight (g) of abomasal lymph nodes (mean ± standard error) from *Haemonchus contortus* -infected or control hair and wool lambs.

	Control	Infected
Wool	2.24 ± 0.25[a]	3.92 ± 0.16[b]
Hair	2.89 ± 0.28[a]	4.70 ± 0.34[c]

[ab] Different letters indicate significant differences between least square means at $P < 0.05$

CHAPTER 3

Microarray analysis reveals difference in gene expression profiles of hair and wool sheep infected with *Haemonchus contortus*

ABSTRACT: Sheep moderately infected with the abomasal parasite *Haemonchus contortus* have reduced growth rate, decreased wool production, and anemia, and under heavy infections, may even die. Anthelmintic treatment can remove worm burdens, but the cost of treatment and the prevalence of drug-resistant worms have led to a greater focus on genetic resistance to parasitism. Variation in parasite resistance exists within and among sheep breeds, with Caribbean hair sheep having increased resistance compared to conventional wool breeds. Our objective was to investigate differences in gene expression between 24 parasite-resistant hair and 24 susceptible wool sheep to determine genetic mechanisms involved in resistance to *H. contortus*. Half of the animals of each breed were infected and sacrificed at 3 or 27 days post-infection; the remaining animals were uninfected controls. Bovine cDNA microarrays were used to examine breed differences in gene expression of abomasum and abomasal lymph node tissues. Over 60 transcripts were found to differ between breeds for each tissue and infection status. Ontology classification of differentially expressed genes with known function revealed transcription, immune response, transporter activity, and muscle function to be important processes in the abomasal tissue of infected animals. Expression profiles of lymph node tissue from infected animals revealed differential expression of genes involved in apoptosis, transcription, receptor activity, and immune response. These data likewise suggest breed differences for genes involved in the clotting cascade, cell survival, endosome function, and gut motility in both abomasum and lymph node tissues. Changes in these processes may be responsible for the increased parasite resistance seen in Caribbean hair sheep. Exploration of cell signaling pathways and expression changes within specific cell populations will help to determine immune mechanisms needed to damage and expel invading parasites.

KEYWORDS: *Haemonchus contortus*, gene expression, immune response, cDNA microarray, parasite, sheep

INTRODUCTION

Ruminants and internal parasites have co-existed for thousands of years, but an increase in stocking density has contributed to a greater parasite burden for the host and decreased revenue for livestock producers. Parasitism by gastrointestinal worms was identified as the primary animal health concern by 62 % of US sheep producers (NAHMS, 1996). The parasite of greatest concern in many areas of the world is the blood-feeding nematode *Haemonchus contortus*. Infection with *H. contortus* may cause severe anemia, anorexia, loss of condition, reduced growth rate, and even death. Health problems associated with gastrointestinal parasites leads to an estimated loss of $369 million (Australian dollars) per year to the Australian sheep industry (Australian Wool Innovations, 2007). The problem is expected to become worse as the prevalence of parasites that are resistant to anthelmintics increases (Jackson and Coop, 2000).

An alternative to the use of commercial anthelmintics would be to obtain or select sheep that are resistant to gastrointestinal nematodes. Selection within existing wool breeds has led to lines of sheep with a 6 to 36-fold difference in fecal egg counts (FEC), a common measure of parasite burden (Woolaston, 1992; Bisset et al., 1996; Pernthaner et al., 2005a). Unfortunately, selection to obtain meaningful reductions in FEC has taken many years and in some cases led to decreased wool production and carcass merit (Morris, 1997, 2001). Variation in parasite resistance also exists among breeds of sheep. For example, Caribbean hair sheep develop resistance sooner and at a higher level than wool sheep under the same conditions (Gamble and Zajac, 1992; Vanimisetti et al., 2004b).

Differences in the response of resistant and susceptible animals during nematode infection have not been well described. However, wool sheep infected with nematode parasites, in general, tend to express a T-helper cell type-2 (TH2) immune response, characterized by antibody production, eosinophilia, mastocytosis, and production of the cytokines IL-4, IL-5, and IL-13 (Lacroux et al., 2006). Immune mechanisms and the particular effector cells and cytokines involved in increased resistance to *H. contortus* have yet to be determined. Our objective was to compare hair and wool sheep to determine differences in gene expression in abomasum and abomasal lymph node tissues associated with parasite infection.

MATERIALS AND METHODS

Animals and tissue collection

The animals and tissues used are described in detail in chapter 2, and will be described briefly. The experimental design is depicted in Figure 3.1. From approximately 4 to 5 months of age, 24 St. Croix and 24 wool-cross lambs were orally infected with 3,000 *H. contortus* infective third stage larvae (L_3) once a week for 4 weeks and were then moved to a drylot. All animals were dewormed one week after the last infection and again 3 days later with levamisole (8 mg/kg body weight) and fenbendazole (10 mg/kg body weight) and moved to raised indoor pens to provide a more controlled environment. Five days after the last deworming and 3 days prior to experimental infection, lambs were dewormed for a third time to completely remove existing infections. Immediately prior to experimental infection, fecal egg counts (FEC) were determined as described below and were zero in all lambs.

Twelve hair and 12 wool lambs were then orally infected with 10,000 *H. contortus* L_3 and 12 hair and 12 wool lambs were left as uninfected controls. Control lambs were accidentally infected on day 11 (relative to infected animals) and were dewormed on day 12 and 14 as described previously. At all time points assessed, FEC and worm burdens were found to be zero in all control animals. However, the effect of the additional anthelmintic treatments at day 12 and 14 on gene expression is not known.

Six infected animals of each breed were euthanized at both 3 and 27 days post-infection (PI). Control animals were sacrificed on day 17, 27, and 38, relative to day 0 of infected animals (Fig. 3.1). All animals were killed by captive-bolt pistol, followed by exsanguination.

The gastrointestinal tract was removed immediately and processed for tissue collection. The abomasum was tied off at both ends and removed from the remaining digestive tract. Lymph nodes lining the lesser curvature of the abomasum were removed from surrounding adipose tissue, rinsed in PBS, and processed as described below. The abomasum was cut along the greater curvature and washed with room temperature PBS. A 2.5 cm^2 section of tissue,

including the full thickness and one fold of the abomasum, was obtained from the fundic region. Lymph node and abomasum tissues were homogenized separately on ice cold metal trays and 0.1 g samples were weighed, immediately frozen in liquid nitrogen, and stored at -80°C until processed.

Parasitologic techniques

Adult worms were collected from the abomasum of pasture-infected sheep and pulverized in an ice-cold glass tissue homogenizer to release developing eggs. Homogenate was mixed with egg-free feces to obtain a mono-specific larval culture. The Baermann technique was used to collect *H. contortus* L_3 larvae, which were then used to orally infect two worm-free donor lambs. After at least 21 days, feces was collected from donor lambs and cultured at 30 °C for 7 to 8 days. Larvae were collected as described above, stored in deionized water at 4°C, and used within 1 month to infect experimental animals. Fecal egg counts were determined by the modified McMasters technique (Whitlock, 1948).

RNA extraction, florescence labeling, and microarray hybridization

Total RNA was extracted from abomasal tissue using TRIzol reagent (Invitrogen Inc., Carlsbad, CA) and from lymph node tissues using RNeasy Miniprep kit (Qiagen) according to manufacturer's protocol. RNA was examined at 260 and 280 nm wavelengths using a UV spectrophotometer to determine concentration and purity (lack of protein contamination). The quality of RNA was assessed by visualization of distinct 28S and 18S rRNA bands following gel electrophoresis and ethidium bromide staining. All abomasal and lymph node RNA used for microarray analysis was of high quality, with minimal degradation (Fig. 3.2).

Abomasum and lymph node total RNA (12 µg or 10 µg, respectively) was used as a template for reverse transcription (Superscript III, Invitrogen). Resulting amino-allyl modified cDNA was then labeled with either Cy3 or Cy5 dye (Amersham Pharmacia Biotech, Piscataway, NJ). Unincorporated dye was removed and labeled cDNA was concentrated to a volume of less than 10 µL using Microcon 30 spin columns (Millipore Corp.). Concentrated cDNAs were mixed with 100 µL of SlideHyb #3 (Ambion, Inc., Austin, TX). Labeled cDNA was incubated at 70°C for 5 minutes before hybridization to glass microarrays. Abomasal samples were

hybridized to Michigan State University's National Bovine Functional Genome Consortium (NBFGC) bovine cDNA microarray as described by Suchyta et al. (2003). Michigan State University's Bovine Total Leukocyte, version 3, (BOTL-3) bovine cDNA microarray was used for transcript quantification of lymph node samples as described by Madsen et al. (2004). Ovine samples were expected to specifically hybridize with bovine cDNA microarrays due to the high (96 %) homology of ovine and bovine sequences (McEwan and Crawford, 2004), and studies involving cross-hybridization of ovine cDNA to bovine cDNA have been successful in determining differential gene expression (Diez-Tascon et al., 2005). Hybridization of 91 to 95 % of sheep cDNA's were observed by Tao et al. (2004) using other bovine cDNA microarrays.

The NBFGC array was spotted with over 18,000 transcripts from mostly bovine sequences and some published ovine sequences representing genes from the entire bovine genome. Transcripts found on the BOTL-3 array were spotted in triplicate and represented over 1200, mostly immune related, genes. Hybridized arrays were washed at room temperature in 0.2X saline-sodium citrate (SSC), rinsed in double distilled water, and dried by centrifugation. Images were scanned using the GeneTAC LS IV (Genomics Solutions) scanner and accompanying software (version 3.01). Spot alignment was performed using MolecularWare DigitalGENOME Pro 2.5 (MolecularWare, Cambridge, MA) and spots were visualized for abnormalities. Spots found to be abnormal (i.e., shape, intensity saturation, array imperfections, etc.) were removed from subsequent analysis. Total intensity values were obtained for each dye channel and normalized as described below to determine differences in gene expression.

Statistical analysis

The cDNA from one wool and one hair lamb were hybridized to a single slide. Pairings were made randomly within the same infection status and collection day, leading to 24 microarrays per tissue. To minimize effects of dye bias an equal number of hair and wool sheep samples were labeled with Cy3 or Cy5 dye. Thus for one half of the animals in the same collection time, breed, and infection status group, random assignment of Cy3 dye labeling for hair sheep or Cy5 dye labeling for wool sheep was made. The remaining hair sheep samples were labeled with Cy5 dye and remaining wool sheep samples were labeled with Cy3 dye. Therefore, an equal number of hair and wool sheep samples were labeled with both Cy3 and

Cy5.

Individual array data were $\log_2$ transformed to correct for right-tailed skewness. These data were visualized for Cy3 versus Cy5 dye bias per array using M vs. A scatter plots, where log intensity ratios $M = \log_2(Cy3/Cy5)$ were plotted against mean log intensities $A = (\log_2(Cy3) + \log_2(Cy5))/2$ for each spot on the array (Yang et al., 2002). Adjustments were made for differential dye binding using a locally weighted regression procedure (PROC LOESS) of SAS (SAS Institute, Cary, NC). Spot intensities generated from microarray hybridization and scanning had a small Cy5 dye bias that was corrected by LOESS normalization (Fig. 3.3). Boxplots of normalized data from each array were compared and median intensity and variance were found to differ (Fig. 3.4). To directly compare arrays, the median intensity value was subtracted from all intensities per array to obtain a median intensity of "0". Deviations from the geometric mean of each array were calculated. The mean of the absolute value of the deviation was used to adjust the variance of each array. The adjustment equalized variance across arrays within day and infection status (Fig. 3.4).

A two-step mixed model analysis using PROC MIXED (SAS Institute, Cary, NC) was then performed, where the first step adjusted for array-specific variation using the following model:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \varphi_{ijk}$$

where y_{ijk} is the normalized intensity value, μ is the overall mean, α_i is the random effect for array i, β_j is the fixed effect for dye j, and φ_{ijk} is the residual. Residuals were carried over to the second step, which tested for gene-specific effects of the sheep breeds:

$$\varphi_{ijkl} = \mu_l + \alpha_{il} + \beta_{jl} + \gamma_{kl} + \beta\gamma_{jkl} + \varepsilon_{ijkl}$$

where all effects have the same definition as before except effects are now gene-specific, indicated by subscript l, γ_{kl} is the fixed effect for breed k, and ε_{ijkl} is the residual. Breed differences per transcript were assessed using two tailed t-tests of least square means for each experimental group. Differential gene expression was considered to be significant for abomasal tissue on the NBFGC microarray if $P < 0.01$ and for lymph node tissue on the BOTL-3

microarray if $P < 0.05$. Due to multiple hypothesis testing, a large number of false positives (approximately 180 for the NBFGC array and less than 60 for the BOTL-3 array) were expected for the p-values selected. However, the use of lower significance value cut-offs would reduce the number of genes found with true differences in expression. Therefore, we selected the P-value cut-offs listed above to obtain a reasonable number of differentially expressed genes for further evaluation of similarities in gene function.

Ontology clustering

Genes found to have significant differential expression between resistant hair sheep and susceptible wool sheep were annotated using the The Institute for Genomic Research (TIGR) database (http://compbio.dfci.harvard.edu/tgi/) to reveal gene identities. Ontology classification was obtained using the links provided in the TIGR database for the given gene identities. Broad classification of the biological process for each gene was based on gene ontology of matching sequences. These classifications were used to determine gene function categories with the greatest representation.

RESULTS

Abomasal tissue

Characterization of microarray data. Gene expression differences in abomasal tissue exceeded the $P < 0.01$ level of significance for 65, 60, 333, 127, and 143 transcripts between hair and wool sheep infected for 3 or 27 days and for controls at 17, 27, and 38 days, respectively. At this level of stringency, no genes were differentially expressed in more than one group. However, if the level of significance was relaxed to $P < 0.05$, there were significant breed differences for 9 transcripts in sheep infected for both 3 and 27 days and for 156 genes in at least 2 of the control groups (data not shown). The two genes with known function present in both infected groups were CD2, which is found on the surface of T cells and natural killer cells and has adhesion and co-stimulatory properties (Yang et al., 2001) , and cationic amino acid transporter 4. Both genes have increased expression at 3 days PI and decreased expression by 27

days PI in hair versus wool sheep. The remaining 7 sequences in both infected groups have no known gene annotation. There was only one sequence with differential expression ($P < 0.01$) in both infected (3 day) and control (27 day) groups, but this sequence could not be matched to any known gene sequence in the TIGR database. To obtain a reasonable number of genes differentially expressed in control and infected groups, the P-value cut-off was relaxed to 0.025, leading to 13 genes with known function present in both groups (Table 3.1).

Ontology classifications. Sequences of transcripts differing ($P < 0.01$) between hair and wool sheep were subjected to a BLASTn search of the Genbank database. The search revealed between 18 and 33 % of sequences within infection status and day of sacrifice group have unknown functions. Major ontology clusters of annotated genes differing in abomasal tissue of animals after 3 days of infection included transcription factors (14 %), immune response (11 %), muscle function (11 %), and other (20 %) (Fig. 3.5). The category of "other" was used to group all categories with only one member. Differentially expressed genes from animals infected for 27 days have high representation within transporters (17 %), transcription factors (15 %), calcium ion binding (10 %), immune response (10 %), and other (20 %) (Fig. 3.6).

Differences in gene expression between uninfected hair and wool sheep revealed a large number of ontological categories (Day 17, 27, and 38 have 17, 22, and 20 clusters, respectively). Over all three control groups, clusters of genes involving transcription factors, transporters, and transferases were the most highly represented.

The top ontological functions on the entire array included transcription factors, transferases, RNA binding, and protein binding, contributing 5.3, 3.9, 3.7, and 3.6 %, respectively, to the 2799 genes of known function (Suchyta et al., 2003). Transporters and cytokines were less well represented, and made up 2.4 and 1.2 % of the genes with known function on the NBFGC array.

Due to their high representation within abomasal tissue of infected animals, we were particularly interested in genes belonging to the immune response category. Two of these genes, IL-4 receptor α and IL-12 receptor β1, both have increased expression in hair compared to wool sheep at 3 days PI. Increased expression of both genes was of interest since they are receptors for opposing TH1 and TH2-type cytokines and IL-12 receptor β1 has involvement with inflammatory pathways (Furuzawa-Carballeda et al., 2007).

Lymph node tissue

Characterization of microarray data. There were fewer sequences on the BOTL-3 array than on the NBFGC array. Therefore, gene expression differences between parasite-resistant hair sheep and susceptible wool sheep were accepted as significant at $P < 0.05$. Differences in gene transcription between hair and wool sheep were found for 81, 127, 267, 106, and 97 sequences in animals at 3 and 27 day PI and in controls at 17, 27, and 38 days, respectively. At this level of stringency, 10 sequences were represented in both infected groups and 53 sequences were represented in at least two control groups (data not shown). There were 73 genes with differential expression ($P < 0.05$) in at least one of the infected and one of the control groups. Of these genes, ten differed at $P < 0.01$ (Table 3.2). Six genes have higher expression in control animals of one breed with greater expression in the opposing breed while infected. Although the gene expression in control and infected animals were not compared directly on the microarray, genes with dramatic changes in expression across these groups may be important for determining parasite resistance mechanisms.

Ontology classifications. Fourteen to 19 % of transcript sequences with significant breed differences in abomasal lymph node tissue have an unknown function. We anticipated that of the genes with known function, a large proportion would be related to immune response given the high number of immune genes on the microarray. However, the top two categories of differentially expressed genes dealt with receptors (13%) and apoptosis (10%) in animals 3 days PI (Fig. 3.7) and apoptosis (16%) and transcription (11%) after 27 days of infection (Fig. 3.8). Genes involving the immune response still had a moderate representation, accounting for 7 % of genes with known function in animals 3 days PI and 10 % at 27 days PI. Immune genes with differential breed expression after 3 days of infection included those related to the MHC, tumor necrosis factor, and chemokine receptor type 5. Interferon-γ receptor β, IL-4, IL-2, IL-6 receptor-α, an IgG receptor, and 3 different receptors for transforming growth factor β have differential expression in animals after 27 days of infection (Table 3.3). Half of the immune genes differing in the animals infected for 27 days were responsible for regulation of inflammation.

Functional classification of genes with differential expression in uninfected wool and hair sheep revealed a large number of ontological categories (days 17, 27, and 38 have 23, 18, and 13

clusters, respectively). Over all three control groups, clusters of genes involving transcription factors, immune response, and apoptosis were the most highly represented.

DISCUSSION

We compared gene expression profiles in tissues of parasite-resistant hair sheep to those of susceptible wool sheep in control animals and animals infected with *H. contortus*. Gene expression profiles were obtained using microarray analyses of both abomasum and abomasal lymph node tissues. When compared to conventional wool sheep, parasite-resistant hair sheep not only have differential expression of hundreds of genes following infection with *H. contortus,* but also have even greater expression differences when uninfected. Resistant and susceptible selection lines within breeds of wool sheep have been compared using microarray analysis during nematode parasite infection (Diez-Tascon et al., 2005; Keane et al., 2006). However, hair sheep developed parasite resistance over centuries of natural selection and may possess novel resistance mechanisms compared to wool sheep intensively selected for resistance over periods of only 10 to 20 years.

Clotting factors, muscle function, apoptosis, cell membrane motility, and intracellular transport appear to be processes that are affected in abomasal tissue of sheep infected with *H. contortus* (Table 3.1). These genes not only exhibited differential expression in infected animals, but overcame increased expression in control animals of the opposing breed. Compared to wool sheep, infected hair sheep have lower expression of von Willebrand Factor, a chaperone for coagulation factor VIII, indicating reduced platelet adhesion (Sadler, 2005). Increased annexin A3 in infected hair sheep may also lead to reduced coagulation. As discussed by Park et al. (2005), annexin A3 leads to increased endothelial cell migration, blood vessel formation, and anti-coagulation. Both of these genes suggest hair sheep initiate anti-coagulation pathways in the first few days of infection, which may appear to be counter-productive during infection with a blood-feeding parasite. However, this may allow for faster externalization of immune cells and antibodies that can damage invading parasites (Amarante et al., 2005; Balic et al., 2006).

Torsin-3A, a chaperone with neuroprotective activity (Cao et al., 2005), has greater expression in infected hair compared to wool sheep 27 days PI and may aid in adult parasite

expulsion through regulation of enteric motility. Diez-Tascon et al. (2005) obtained similar results related to gut motility in resistant wool sheep with greater expression of smooth muscle genes, transgelin and actin-γ2. Immune cell survival may be lengthened in wool sheep by increased casein kinase 1 α1 expression, which is an anti-apoptotic gene (Chen et al., 2005). However, expression may be induced too late in wool sheep since differences were only apparent 27 days PI. Bridging integrator 3 (BIN3) and Hermansky-Pudlak syndrome 4 (HPS4) are needed for lysosome/endosome formation and migration (Routhier et al., 2001; Nazarian et al., 2003). Additionally, HPS4 may act to increase coagulation in wool sheep through production and mobilization of platelet dense granules (Nazarian et al., 2003). Both BIN3 and HPS4 may be involved in intracellular trafficking of granules to be exteriorized upon cell activation. Granulocytes, including eosinophils and mast cells, infiltrate abomasal tissue in infected animals with greater cell accumulation in hair compared to wool breeds (MacKinnon et al., *In preparation*, Balic et al., 2000). Increased numbers of these particular cells and not increased gene expression within cells of infected hair sheep may cause differences in gene transcription and should be further evaluated.

Differences in expression profiles were not consistent between animals infected for 3 versus 27 days. Changes in the host response could be caused by the presence of different parasite stages. Adult and larval stages of *H. contortus* reside in different locations (lumen versus abomasal crypts, respectively) and produce different proteins (Meeusen et al., 2005). In addition, only adult and late larval stages of *H. contortus* directly damage the abomasal lining causing inflammation and blood loss.

Although the genes that were differentially expressed differed between days, the functional gene categories stayed consistent across tissues. The particular ontological categories represent important processes that differed between breeds during infection including transcription factors, immune response, and transporters in abomasal tissue and transcription factors, apoptosis, and receptors in lymph node tissue. Thus, individual genes with differential expression in the abomasum or lymph node tissues appeared to be involved in similar processes.

Expression of von Willebrand factor in the abomasum differed between breeds. This protein serves as a ligand for integrin-α9 (Singh et al., 2004), which is involved in clotting processes and have differential breed expression in lymph node tissue (Table 3.2). Genes involved in blood vessel formation, annexin (Table 3.1) and WNT2B (Wang et al., 2007),

differed between breeds in abomasum and lymph node tissues, respectively. Expression of genes involved in blood diffusion may have a fundamental role in increasing resistance of hair sheep not only in the abomasal tissue, but also in the outlying lymph nodes. Both tissues appear to have altered apoptotic pathways between breeds, through expression differences of casein kinase 1 α1 in abomasal tissue and B-cell translocation gene (Yoshida et al., 1998) and MAP4K2 (Yuasa et al., 1998) in lymph node tissue.

We have found unique breed differences associated with parasite-resistant hair sheep that, to the best of our knowledge, have not previously been observed. Abomasal and lymph node tissues from infected hair and wool sheep have differential expression of genes involved in the clotting cascade, cell survival, endosome function, and gut motility compared to wool sheep. Exploration of these expression changes within abomasum and lymph node tissues may partially explain breed differences between resistant hair and susceptible wool sheep.

Genes belonging to the immune function category, differentially expressed in lymph node tissue 27 days PI, were mostly involved in wound healing and inflammatory processes. Receptors for the anti-inflammatory cytokine transforming growth factor β were observed in both breeds along with activin receptors, also involved in inflammatory processes and wound repair (Bamberger et al., 2005). Pernthaner et al. (2005a) observed greater expression of the pro-inflammatory gene TNF-α in lymph of resistant versus susceptible lines of wool sheep infected with a nematode parasite, suggesting that inflammation may affect resistance to infection. Similar results are found in studies of resistant cattle infected with an abomasal nematode parasite (Li et al., 2007). A stronger TH2 immune response may be produced in lymph node tissue of wool sheep 27 days PI through greater IL-4 and reduced IFNγ-receptor β production compared to hair sheep. This result, however, could be due to an increased worm burden and/or a delayed response in susceptible wool sheep. Multiple genes involved in the inflammatory process showed expression differences in lymph node tissue of hair and wool sheep. These breed differences may simply result from tissue damage caused by the parasite, or they may suggest mechanisms involved in resistance to *H. contortus*. A closer examination of cell signaling pathways and the response of specific cell populations to parasite antigen has yet to be completed and may provide answers to these questions.

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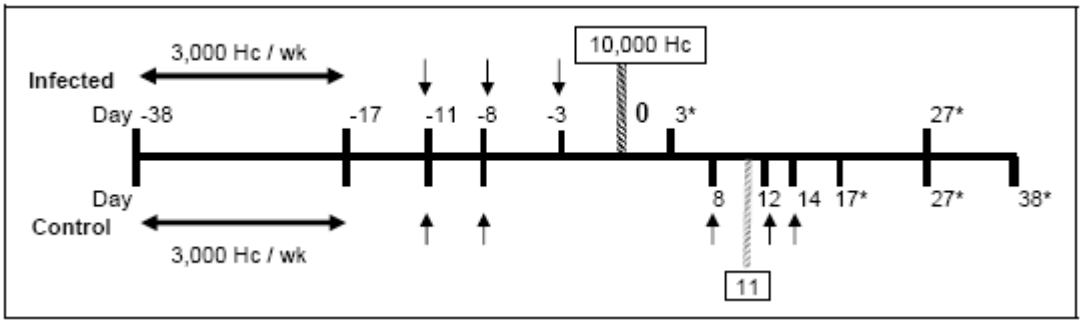
Figure 3.1. Depiction of infection, deworming, and sample collection through the course of the study for infected and uninfected (control) hair and wool sheep. Days are relative to the final dosing of infected animals with 10,000 *Haemonchus contortus* larvae (Hc).



↑ Indicates that animals were dewormed

* Indicates that animals were sacrificed and tissue samples collected

▒ Control animals were accidentally infected on day 11 and subsequently dewormed on day 12 and 14.

Figure 3.2. Ethidium bromide staining of abomasal and lymph node total RNA. Clear 28S and 18S rRNA bands suggest minimal degradation of sample.

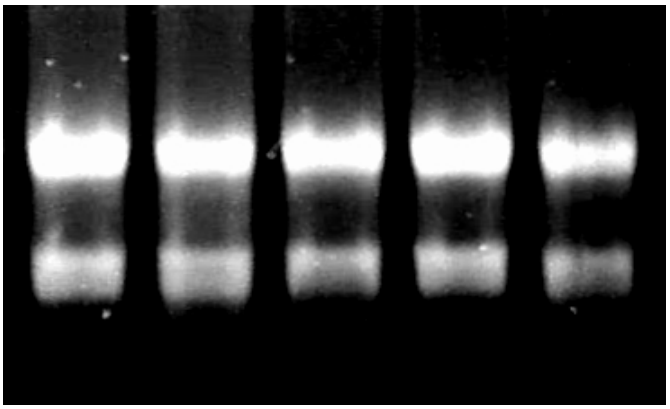


Figure 3.3. M vs A scatterplots from a typical NBFGC bovine cDNA microarray before (a) and after (b) LOESS normalization of Cy3 and Cy5 dye intensities obtained. Points represent the log of the ratio of Cy3 to Cy5 intensity (M) by the average intensity of the Cy3 and Cy5 dye channels (A).

(a) (b)

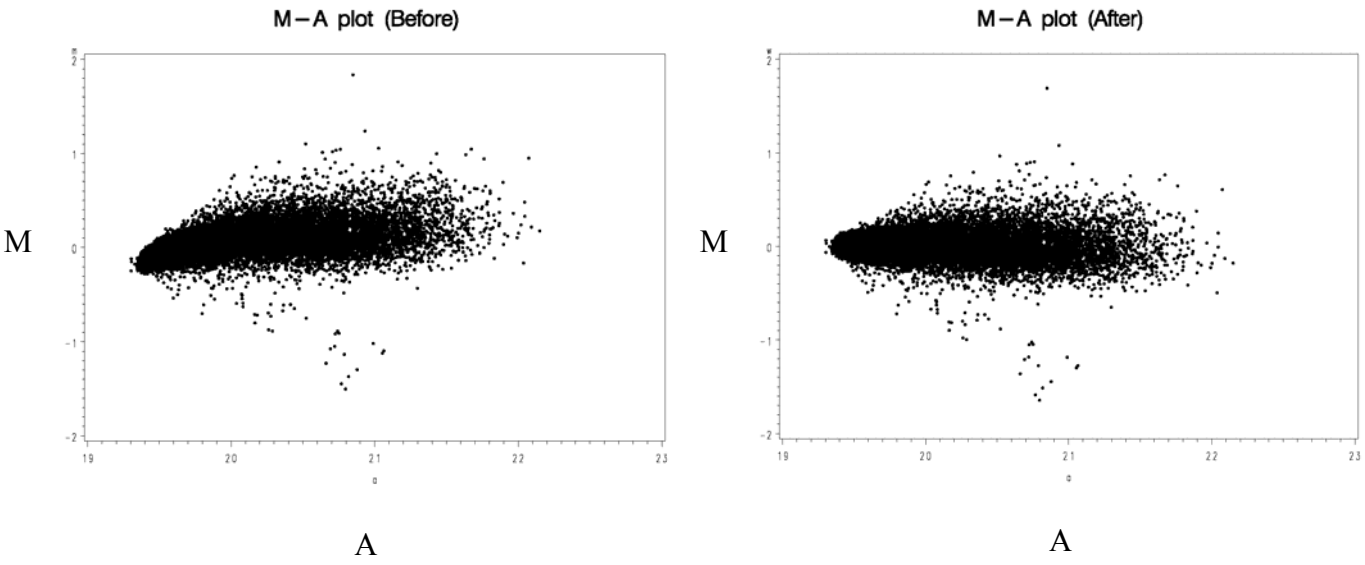


Figure 3.4. Boxplots of Cy3 and Cy5 spot intensities for the six microarrays (1 to 6) from hair and wool sheep 27 days post-infection before (a) and after (b) normalization by median and variance adjustments. Results are representative of microarray differences found for other day by infection status groups.

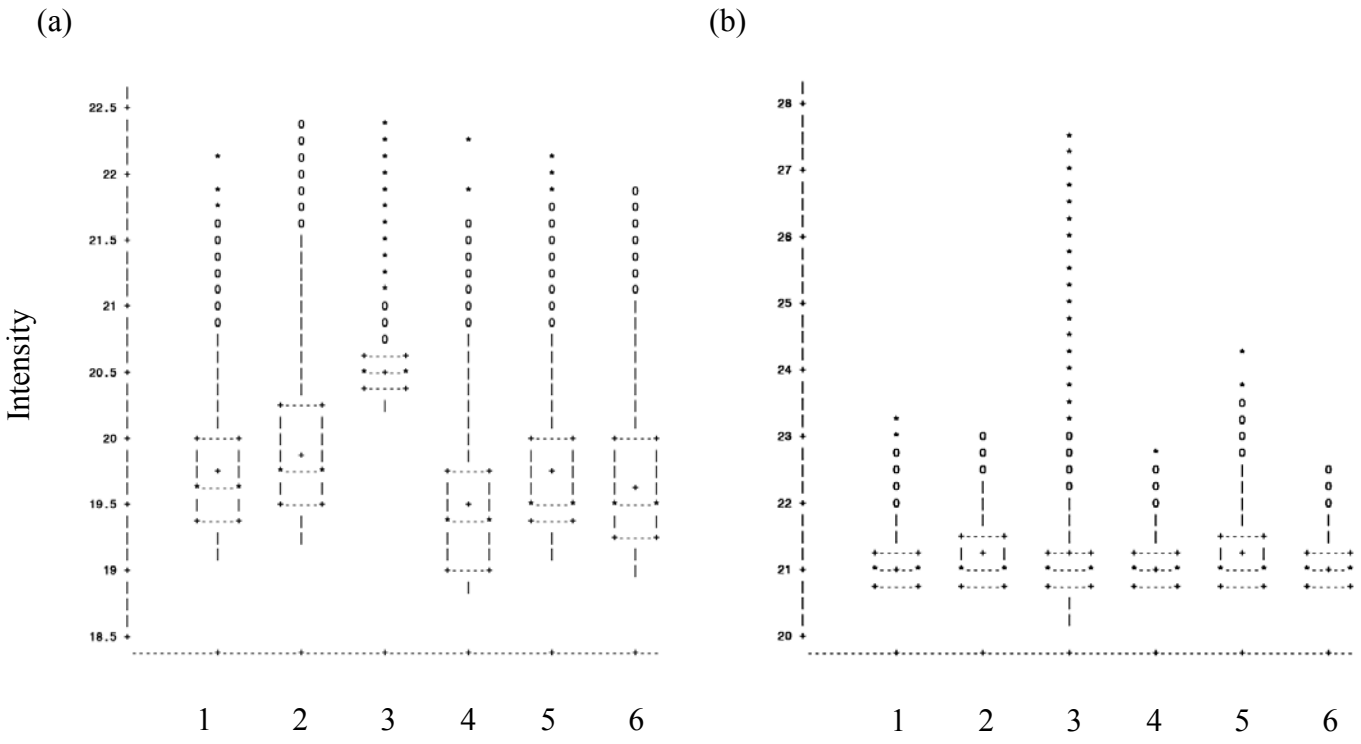


Figure 3.5. Ontological grouping of genes with known function differentially expressed ($P < 0.01$) in abomasal tissue of parasite-resistant hair sheep and susceptible wool sheep 3 days after infection with *Haemonchus contortus*.

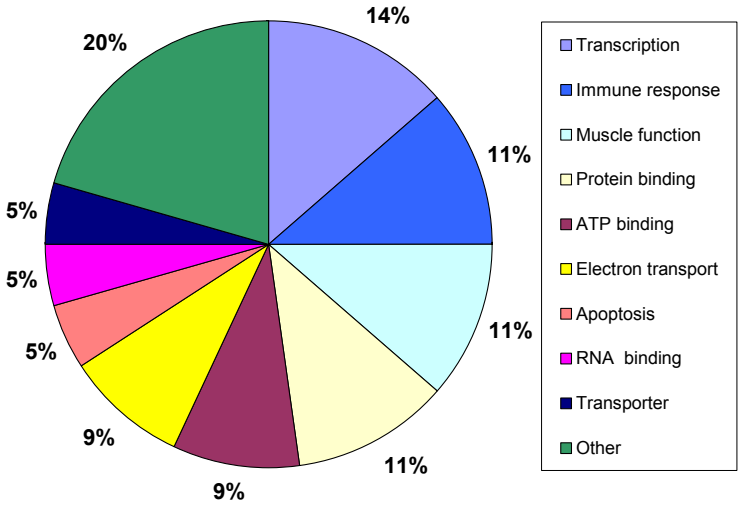


Figure 3.6. Ontological grouping of genes with known function differentially expressed ($P < 0.01$) in abomasal tissue of parasite-resistant hair sheep and susceptible wool sheep 27 days after infection with *Haemonchus contortus*.

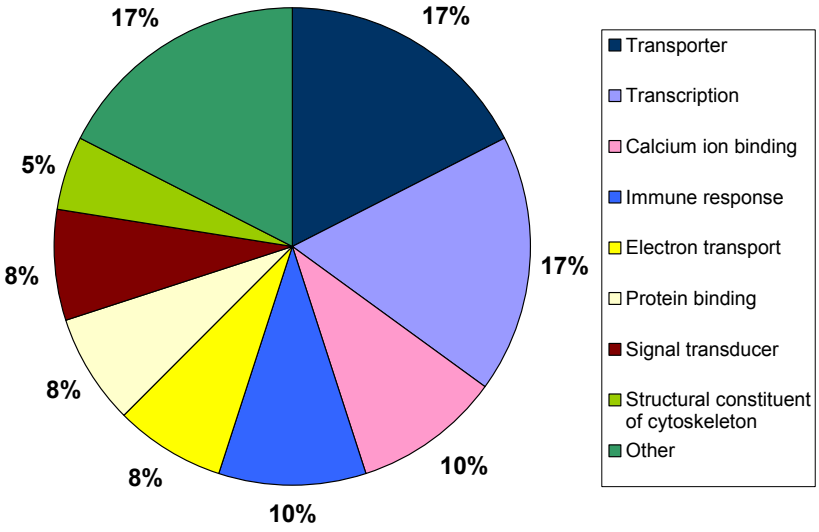


Figure 3.7. Ontological grouping of genes with known function differentially expressed ($P < 0.05$) in abomasal lymph node tissue of parasite-resistant hair sheep and susceptible wool sheep 3 days after infection with *Haemonchus contortus*.

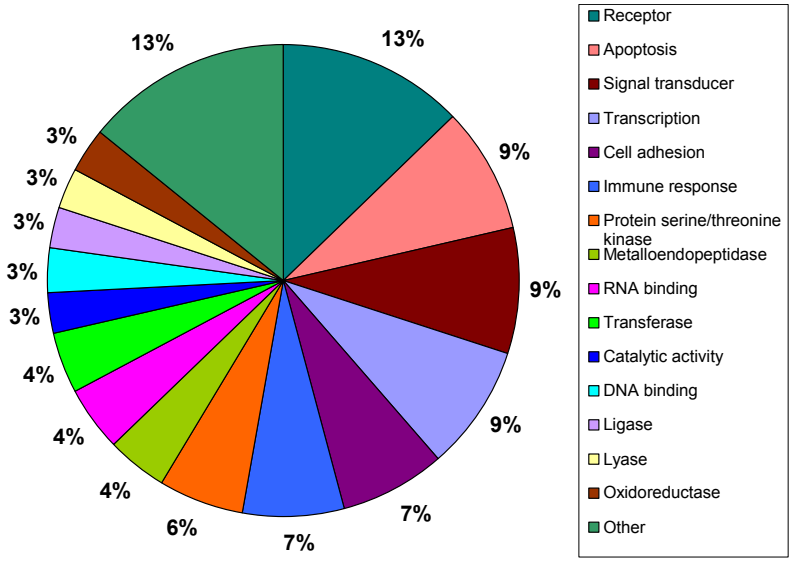


Figure 3.8. Ontological grouping of genes with known function differentially expressed ($P < 0.05$) in abomasal lymph node tissue of parasite-resistant hair sheep and susceptible wool sheep 27 days after infection with *Haemonchus contortus*.

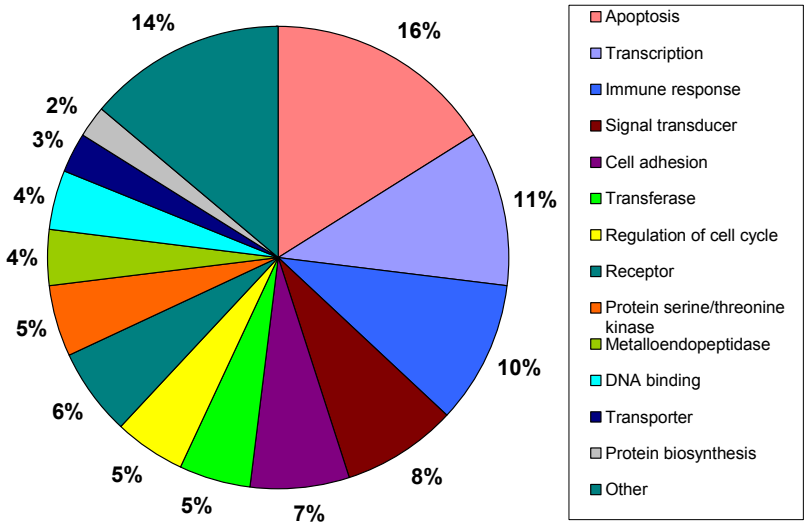


Table 3.1. Genes of known function with differential expression ($P < 0.025$) in abomasal tissue of parasite-resistant hair and susceptible wool sheep. Genes listed appeared in at least one uninfected and one infected group. Hair or wool indicates the breed with higher expression.

Genbank accession no.	TC in TIGR	Uninfected			Infected		Gene/Protein name
		17	27	38	3	27	
Consistent breed expression							
BF076603	TC306559		Wool		Wool		Ubiquinone biosynthesis methyltransferase COQ5
BE681976	TC337076	Wool			Wool		Prolactin-related protein
AW655623	TC301821	Wool			Wool		Sestrin-2
AW652951	TC331930		Wool			Wool	Alpha-adaptin A
AW658039	TC329204	Hair			Hair		Tat
BE236367	TC328011	Hair			Hair		Rapamycin associated protein FRAP2
BE751146	TC315712	Hair				Hair	GTPase-like protein
Opposing breed expression							
BE722217	TC335700		Hair		Wool		von Willebrand Factor
BE476329	TC307997		Hair		Wool		Bridging integrator 3
BF604182	TC354102		Hair			Wool	Hermansky-Pudlak syndrome 4 protein
BG688624	TC312392	Hair				Wool	Casein kinase 1 α1
BF231475	TC302448			Wool	Hair		Annexin A3
AW660517	TC313799	Wool				Hair	Torsin-3A

Table 3.2. Genes of known function having differential expression ($P < 0.01$) within lymph node tissue of parasite-resistant hair and susceptible wool sheep. Genes present appear in at least one uninfected and one infected group. Hair or wool indicates the breed with higher expression.

BOTL-3 accession no.	Uninfected			Infected		Gene/Protein name
	17	27	38	3	27	
Consistent breed expression						
BOTL0100001XB10R	Hair				Hair	H3 histone, family 3B (H3.3B)
BOTL0400421_PCR		Hair			Hair	Toll-like receptor 2
BOTL0400083_PCR			Wool		Wool	Serine-protein kinase ATM
BOTL0400540_PCR	Wool			Wool		Protein-kinase, interferon-inducible double stranded RNA dependent inhibitor,
Opposing breed expression						
BOTL0100010_E07	Hair				Wool	B-cell translocation gene 3
BOTL0400102_PCR	Hair				Wool	Immunoglobulin superfamily, member 1, isoform 1
BOTL0400492_PCR	Hair				Wool	Wingless-type MMTV integration site family, member 2B (WNT2B)
BOTL0400431_PCR	Hair			Wool		Pyruvate dehydrogenase kinase, isozyme 3
BOTL0100006XE08R	Wool				Hair	Mitogen-activated protein kinase kinase kinase kinase 2 (MAP4K2)
BOTL0400300_PCR	Wool			Hair		Integrin $\alpha 9$

Table 3.3. Genes involved in immune response having differential expression ($P < 0.05$) within lymph node tissue of infected parasite-resistant hair and susceptible wool sheep 3 and 27 days post-infection. Hair or wool indicates the breed with greater expression.

BOTL3 accession no.	TC in TIGR	Breed with Inc. expression	Gene/Protein name
Infected - 3 days post-infection			
BOTL0100002XH12R	TC371934	Wool	MHC class II DM β-chain precursor
BOTL0400566_PCR	TC365207	Wool	macrophage stimulating 1 receptor
BOTL0400010_PCR	TC303147	Hair	GRO- β mRNA/CXCL1
BOTL0400067_PCR	TC313464	Hair	Tumor necrosis factor ligand superfamily member 12
BOTL0400105_PCR	TC302829	Hair	C-X-C chemokine receptor type 5
Infected - 27 days post-infection			
BOTL0400064_PCR	TC302065	Wool	TGF β receptor I/Activin A receptor I
BOTL0400279_PCR	TC335681	Wool	IL-4
BOTL0400456_PCR	TC336188	Wool	TGF β receptor III
BOTL0400614_PCR	TC382194	Wool	IL-6 receptor α
BOTL0100012_B09	TC306576	Hair	IFN-γ receptor β
BOTL0400065_PCR	TC302108	Hair	Activin A receptor II
BOTL0400222_PCR	TC317760	Hair	Fc γ receptor
BOTL0400310_PCR	TC311583	Hair	IL-2
BOTL0400421_PCR	TC355823	Hair	Monocyte differentiation antigen CD14
BOTL0400642_PCR	TC316824	Hair	TGF β receptor II

CHAPTER 4

Gene expression profiles of hair and wool sheep reveal importance of IL-13 and other TH2 immune mechanisms for increased resistance to *Haemonchus contortus*

ABSTRACT: One of the main issues faced by U.S. and foreign sheep producers has been management of gastrointestinal parasite burdens in sheep. With an increased prevalence of drug-resistant worms, treatment of infected animals has led to greater economic loss compared to previous years. Therefore, other methods of parasite control need to be assessed, including the incorporation of genetically resistant animals into current breeding stocks. Hair breeds, such as the St. Croix, express greater parasite resistance compared to conventional wool breeds. However, the immune mechanisms involved in determining parasite resistance in hair or wool breeds are unknown. Additionally, information on cytokine expression profiles of wool sheep selected for increased resistance and for hair sheep, in general, is lacking. Our objective was to investigate gene expression differences between 26 parasite-resistant hair and 26 susceptible wool sheep to determine genetic mechanisms associated with resistance to *H. contortus*. Half of the animals of each breed were infected and sacrificed at 3 or 27 days post-infection; the remaining animals were uninfected controls. Breed differences in expression of genes associated with a TH2-type immune response (IL-4, IL-4 Rα, IL-13, IL-5, IgE, IgE Rα) and a TH1-type immune response (IFN-γ, IL-12 p35, IL-12 Rβ1, IL-12 Rβ2) in abomasum and abomasal lymph node tissues were determined. For both hair and wool sheep, infection with *H. contortus* led to greater expression of IL-5, IL-13, and IgE, and reduced IFN-γ and IL-12 p35 in lymph node tissue. In abomasal tissue, parasite infection led to greater IL-13, IgE, and IgE Rα expression compared to control animals. Between breeds, hair sheep have a stronger TH2 response after infection, with increased IgE and decreased IFN-γ in lymph node tissue and increased IL-13 and decreased IL-12 p35 in abomasal tissue compared to infected wool sheep. We have found novel differences in expression of immune genes in hair and wool sheep infected with *H. contortus*.

KEYWORDS: Gene expression, *Haemonchus contortus*, immune response, real-time RT-PCR, cytokine, sheep

INTRODUCTION

Management of gastrointestinal parasites is one of the top concerns of U.S. sheep producers, especially in hot, humid areas where sheep can have substantial worm burdens year round (NAHMS, 1996). The blood-feeding nematode, *Haemonchus contortus,* is one of the most detrimental parasites of sheep in tropical and subtropical regions and infection can lead to animals with decreased production and under a heavy worm burden, even death. Additionally, the prevalence of drug-resistant worms has increased with some parasite strains resistant to all classes of anthelmintics (Jackson and Coop, 2000). A dramatic increase in the cost to treat infected animals will most likely occur, and unless new anthelmintics are developed, chemical treatment may become completely ineffective. Therefore, other methods of parasite control need to be assessed, including the incorporation of genetically resistant animals into current breeding stocks.

An alternative to the use of chemical anthelmintics is to produce sheep that have enhanced genetic resistance to internal parasites. Selection within wool breeds has led to significant reductions in parasite load (Woolaston, 1992; Bisset et al., 1996). Unfortunately, selection to meaningfully reduce fecal egg counts (FEC), a measure of parasite burden, has taken many years and in some cases may have led to decreased wool production and reduced carcass merit (Morris, 1997, 2001). The resistance level of sheep to internal parasitism differs among breeds, and Caribbean hair sheep develop resistance sooner and at a higher level than wool sheep maintained under the same conditions (Gamble and Zajac, 1992; Vanimisetti et al., 2004b). However, the immune mechanisms involved in development and expression of parasite resistance in hair or wool breeds remain to be determined.

Infection with nematode parasites tends to elicit a T-helper cell type-2 (TH2) immune response, characterized by antibody production, eosinophilia, mastocytosis, and production of the cytokines IL-4, IL-5, and IL-13 (Kooyman et al., 2000; Pernthaner et al., 2005a; Balic et al., 2006; Lacroux et al., 2006). However, Meeusen et al. (2005) also found an increase in IFN-γ, a TH1 cytokine, in addition to increased IL-4, IL-5, and IL-13 in abomasal tissue of sheep 3 days after infection with *H. contortus*. Eventhough differences in immune response between resistant and susceptible lines of wool sheep are known (Pernthaner et al., 1996; Diez-Tascon et al., 2005; Pernthaner et al., 2005a; Keane et al., 2006), to our knowledge, gene expression studies

involving Caribbean hair sheep have not been performed. Our objective was to identify differences in expression of TH1 and TH2 immune genes and their receptors in hair and wool sheep during infection with *H. contortus*. Differences in immune gene expression were evaluated in abomasum and lymph node tissues. By comparing resistant hair and susceptible wool sheep, we will provide insight into genetic mechanisms that regulate resistance to the extracellular gastrointestinal parasite *H. contortus*.

MATERIALS AND METHODS

Animals, tissue collection, and RNA extraction

Details of the experimental procedure are as described in Chapters 2 and 3. Briefly, 24 St. Croix and 24 wool lambs of 50 % Dorset, 25 % Rambouillet, and 25 % Finnsheep breeding were maintained at the Virginia Polytechnic Institute and State University Sheep Center in Blacksburg, VA. All procedures were approved and carried out in accordance with the Animal Care Committee of Virginia Tech. From approximately 4 to 5 months of age, lambs were infected with 3,000 *H. contortus* infective third stage larvae (L_3) once a week for 4 consecutive weeks. Animals were then moved to a drylot to preclude additional *H. contortus* infection. All animals were dewormed one week after the last infection and again 3 days later with both levamisole (8 mg/kg body weight) and fenbendazole (10 mg/kg body weight). Lambs were then moved to raised indoor pens to provide a more controlled environment. Prior to the experimental infection, nematode eggs were not present in any lambs feces.

Three days after the last deworming, 12 hair and 12 wool lambs were orally infected with 10,000 *H. contortus* L_3 larvae and an additional 14 hair and 14 wool lambs were left as uninfected controls. Control animals were dewormed on day 8 (relative to infected animals) to approximate treatments in infected animals. However, control lambs were accidentally infected on day 11 and were dewormed as described above on days 12 and 14. At all time points assessed, no parasitic nematode eggs were detected. Levamisole is known to have effects on immune function, with reduced complement production and changes in T-cell populations (Kurakata and Kitamura, 1983). Fenbendazole has not been implicated in altering immune

responsiveness. However, the effects of levamisole and/or fenbendazole on gene expression are not known.

Six infected animals of each breed were euthanized at both 3 and 27 days post-infection (PI). Specific days were selected to represent the response to larvae (day 3) and adult worms (day 27). Control animals were sacrificed on days 17, 27, and 38, relative to day 0 of infected animals. All animals were killed by captive-bolt pistol, followed by exsanguination.

The gastrointestinal tract was removed immediately and processed for tissue collection. The abomasum was tied off at both ends and removed from the remaining digestive tract. Lymph nodes lining the lesser curvature of the abomasum were removed from surrounding adipose tissue, rinsed in PBS, and processed as described below. The abomasum was cut along the greater curvature and washed with room temperature PBS. A 2.5 cm^2 section of tissue, including the full thickness and one fold of the abomasum, was removed from the fundic region of the abomasum. Lymph node and abomasum tissues were homogenized separately on ice cold metal trays and 0.1 gram samples were weighed and immediately frozen in liquid nitrogen and stored at -80°C until processed.

Total RNA was extracted from abomasal tissue using TRIzol reagent (Invitrogen Inc., Carlsbad, CA) and from lymph node tissues using RNeasy Miniprep kit (Qiagen) according to manufacturer's protocol. RNA was examined at 260 and 280 nm wavelengths using a UV spectrophotometer to determine concentration and purity (lack of protein contamination). All abomasal and lymph node RNA used for real-time reverse transcriptase (RT)-PCR was of high quality

Parasitologic techniques

Eggs collected from adult *H. contortus* were used to produce a mono-specific larval culture. Larvae were collected using the Baerrman technique, stored in deionized water at 4°C, and used within 1 month to infect experimental animals. Fecal egg counts were determined at 16, 21, and 27 days PI by the modified McMaster's technique (Whitlock, 1948).

Primer design and validation

Primer sequences for house keeping (HK) genes (GAPDH, β-actin, and ribosomal protein L19) were obtained from the Michigan State, Center for Animal Functional Genomics primer database (http://cafg.msu.edu). Target genes (Table 4.1) were selected based on results from cDNA microarray analysis (MacKinnon et al., *In preparation)* and from previous literature. Oligonucleotide sequences for IL-4, IFNγ, and IL-5 were obtained from Coussens et al. (2004). Primer Express software (Applied Biosystems) was used to design remaining primers based on known bovine and ovine sequences (constant heavy chain of IgE, α chain of the Fcε-R1 receptor (IgE Rα), α chain of the IL-4 receptor (IL-4 Rα), IL-13, IL-12 p35 (IL-12), β1 chain of the IL-12 receptor (IL-12 Rβ1), β2 chain of the IL-12 receptor (IL-12 Rβ2), and TNF-α). Primer pairs were selected to have one of the two primers spanning an exon-intron junction, where applicable, to limit DNA amplification. Primer sequences, presence or absence of DNA amplification, and melting temperatures of the corresponding amplicons are listed in Table 4.1.

All primer pairs were evaluated for ovine and bovine cDNA and DNA amplification, as well as contamination by inclusion of a blank (negative) control. Amplification efficiency for each primer pair was assessed to ensure similiar HK and target gene amplification. To determine amplification efficiency, 1, 3.16, 10, 31.6, and 100 ng of cDNA were used as described below and the slope of the cycle threshold (C_t) values for the $\log_{10}$ cDNA concentrations was obtained. A slope of -3.33 suggested 100 % amplification efficiency. Multiple primer pairs for TNFα were evaluated, but none met the above criteria. Therefore, TNFα was excluded from our analysis.

To determine the appropriate HK gene to use, multiple HK genes were tested for approximately equal amplification in abomasal and lymph node tissue of infected and control animals. GAPDH was found to have consistent expression ($C_t \pm SE = 19.34 \pm 0.36$) in samples tested and was used for further analysis.

cDNA synthesis and real-time RT-PCR

First strand cDNA was reverse transcribed from 2.2 μg total RNA using a High Capacity cDNA Archive Kit (Applied Biosystems) according to manufacturer's protocol. Reverse transcribed samples were diluted to a concentration of 10 ng/μl using RNase-free DEPC water

and stored at -20°C. Each real-time RT-PCR reaction was carried out in a total volume of 25 µl using 2 µl (10 ng/µl) of reverse transcribed RNA, 12.5 µl SYBR Green PCR master mix (Applied Biosystems), 1.5 µl (5 µM concentration) forward and reverse primers, and 7.5 µl DEPC water. All samples were loaded into optical 96-well plates (Applied Biosystems) in duplicate and real-time RT-PCR was carried out on an ABI PRISM 7300 sequence detection system (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. A melting curve was obtained at the end of each run to ensure amplification of only one product. If variation in C_t values between duplicates exceeded 0.125 or multiple products were found, the sample was rerun until acceptable values were obtained. Mean C_t values for sample duplicates were used for all analyses.

Experimental design and statistical analysis

The HK gene, GAPDH, was used as an internal control to adjust for differences in cDNA sample (animal by tissue) concentration. For each sample, the difference in C_t values of the HK and target genes (ΔC_t) was calculated. Analysis of ΔC_t values was performed for each gene using a generalized linear model (SAS Institute Inc., Cary, NC) including effects for breed (hair or wool), group (infection status by day of sacrifice), and breed by group interaction. A two-tailed student t-test was used to determine significant differences between group least square means. Correlations and significance values between measurements were obtained by breed using the PROC CORR function of SAS (SAS Institute Inc., Cary, NC). Values were considered significant at $P < 0.05$ unless stated otherwise. For presentation purposes only, $2^{-\Delta\Delta Ct}$ values were calculated to express fold changes in gene expression for infected and control hair and wool sheep on each day of sacrifice in comparison to control wool sheep. The $\Delta\Delta C_t$ value was calculated as the difference between the least square means of control wool sheep and the least square means of the other day by breed by infection status groups. Significance values for group differences were determined from analysis of ΔC_t values.

RESULTS

Parasitology

Parasitic nematode infection, as assessed by FEC, was not observed in any control animals. All sheep infected with *H. contortus* larvae had positive FEC by 16 days PI. Fecal egg counts measured 27 days PI were not significantly different, but were 1.4-fold higher in wool compared to hair lambs (MacKinnon et al., *In preparation*). A significant breed difference is reported by Vanimisetti et al. (2004b) in these same lines of hair and wool sheep (although the line of wool ewes were crossed with male Dorpers, another hair breed), where hair sheep have lower FEC than the wool composite.

Gene expression in abomasal and lymph node tissue

Gene expression of 10 cytokines, IgE, and associated receptors was measured in hair and wool sheep using real-time RT-PCR (Tables 4.2 and 4.3). All genes had quantifiable expression in lymph node tissue. However, expression of IL-4, IL-5, IL-12 Rβ1, and IL-12 Rβ2 was too low to measure in abomasal tissue. Relatively few breed differences were observed in control animals on individual collection days or when all were grouped together by breed. However, control hair sheep had lower expression of IFN-γ ($P = 0.04$) and IgE Rα ($P = 0.005$) in lymph node tissues compared to control wool sheep, with no significant differences in abomasal tissue of control animals (Table 4.1 and 4.2). When control animals of a given breed were grouped together, hair sheep also had significantly lower IgE Rα ($P < 0.05$) expression in abomasal tissue compared to control wool sheep (Fig. 4.2). Even though expression of IgE Rα was lower in control hair sheep, greater IgE was observed in lymph node tissue ($P = 0.0002$) of hair compared to wool sheep (Fig. 4.1).

Infection with the abomasal parasite, *H. contortus*, caused dramatic changes in gene expression of the abomasum and lymph node tissues in both breeds. At 3 days PI, significant increased expression of IL-5 (1.9-fold), IL-13 (6-fold), and IgE (3.4-fold) and decreased IFN-γ (1.4-fold) and IL-12 p35 (1.4-fold) was found in lymph node tissue of all infected sheep compared to all controls. Gene expression changes in abomasal tissue of all infected animals 3 days after infection included significant increases in IL-13 (7.7-fold), IgE (2.4-fold), and IgE Rα (2.1-fold) compared to all control animals.

Although changes in gene expression were observed in both breeds by 3 days after infection, differences between breeds were also observed. The expression pattern of IL-12 p35, a cytokine that drives a TH1 immune response, differed between lymph node and abomasum tissue 3 days PI. As expected, a 1.4-fold reduction in expression was found for IL-12 p35 in lymph node tissue in both breeds (Fig. 4.1F). However, a 3.5-fold increase in IL-12 p35 production was found in abomasal tissue of infected wool sheep, whereas expression remained constant in hair sheep (Fig. 4.2D). A general increase in IgE expression was observed after infection, however, hair sheep have much greater expression of IgE in lymph node tissue compared to wool sheep (Fig. 4.1A). Lower production ($P < 0.10$) of IgE Rα by 3 days PI was also found in lymph node tissue of hair compared to wool sheep (Fig. 4.1B).

By 27 days after *H. contortus* infection, resistant hair and susceptible wool sheep were showing clear breed differences in gene expression for both lymph node and abomasum tissues. Although some differences only approached significance, greater expression of TH2-type genes, IL-13, IgE, and IgE Rα, and reduced TH1-type genes, IFNγ and IL-12, was observed in abomasal tissue of infected hair sheep (Table 4.3A). General gene expression patterns were similar in lymph node tissue of hair sheep with increased IL-13 ($P = 0.065$) and IgE and reduced IFN-γ compared to wool sheep (Table 4.2A).

Abomasal and lymph node tissues have similar relative gene expression between breeds and across days. For most genes, higher gene expression was found in the same breed on the same day in both tissues or there was no breed difference. However, this was not the case for the IgE Rα. Compared to control animals, expression of IgE Rα decreased in lymph node tissue and increased in abomasum tissue of infected animals (Fig. 4.1B and 4.2B). Abomasal tissue of infected hair sheep have a somewhat greater expression of IgE Rα compared to tissue from wool sheep (P =0.19, Table 4.3A). However, in lymph node tissue of infected animals, hair sheep maintained lower levels of IgE Rα expression compared to wool sheep (P =0.09, Table 4.2A).

Associations between gene expression levels and FEC

Associations of individual cytokine expression 27 days after infection with FEC varied between hair and wool sheep. The only gene significantly correlated with FEC of hair sheep was IL-12 in abomasal tissue ($r = 0.89$; $P = 0.017$). The positive correlation suggests that greater

expression (lower ΔC_t) of the TH1 inducing cytokine IL-12 was associated with lower FEC. In wool sheep, FEC was positively correlated with the ΔC_t value of IL-4 Rα (r = 0.90; $P = 0.013$). These results suggest that increased expression of IL-4 Rα, a receptor for TH2 cytokines IL-4 and IL-13, is associated with reduced FEC in susceptible wool sheep.

DISCUSSION

Wool sheep infected with gastrointestinal nematodes predominantly produce a TH2-type immune response (Meeusen et al., 2005; Pernthaner et al., 2005a; Lacroux et al., 2006). Mouse models of nematode parasite infection show that the particular effector cells and TH2 cytokines required for parasite expulsion vary among nematode species (Finkelman et al., 1997). In sheep, the essential immune mechanisms needed for increased resistance to *H. contortus* remains unknown. We show dramatic difference in immune gene expression of both breeds between uninfected control animals and sheep infected for 3 and 27 days. These differences suggest the involvement of certain cytokines, immunoglobulins, and receptors in the general immune response of sheep to *H. contortus* infection.

Infection with *H. contortus* induced a TH2-type response by 3 days PI in tissues of hair and wool sheep. Increased expression of TH2-type genes, IL-13, IL-5, IgE, and IgE Rα, but not IL-4, were observed in abomasum and/or lymph node tissues of infected animals. These animals also had reduced expression of the TH1 cytokines IL-12 and IFN-γ in lymph node tissue. Increased expression of IL-13, IL-5, and IL-4 occurs in lymph node cells of wool sheep infected with *Trichostrongylus colubriformis*, an intestinal nematode parasite (Pernthaner et al., 2006). However, these sheep also have increased production of IFN-γ after infection. In younger wool sheep of a different breed, Lacroux et al. (2006) reported increased IL-13, IL-5, and IL-4 with no difference in IFN-γ, IL-12, IL-10, or TNFα in abomasal and lymph tissues. Differences between studies in expression of IFN-γ may be a result of parasite-produced IFN-γ homologues that can alter the host immune response (Grencis, 2001). Production of this cytokine would facilitate a more favorable TH1 environment for the parasite. Maizels et al. (2004) pointed out a multitude of proteins produced by different gastrointestinal parasites that appear to be similar to host

cytokines. Therefore, careful analysis of the host immune response to parasitism and what does and does not confer resistance should be considered.

Overall there appears to be a predominant increase in IL-13 in response to *H. contortus* infection. These results are not surprising, as IL-13 is produced by TH2 cells and induces IgE-class switching in bovine cells (Trigona et al., 1999). The role of IL-13 is consistent with our data for increased IgE expression. Increased IL-13 also causes B-cell, but not T-cell, activation and proliferation (Trigona et al., 1999), mast cell activation (Kaur et al., 2006), IFN-γ regulation, and drives a TH2 response (Webb et al., 2007). Hair sheep also had an increase in IL-5 after infection, which can be produced by mast cells and TH2 cells, and is most closely associated with eosinophil recruitment, activation, and enhanced larval damage (Rainbird et al., 1998). Eosinophil viability can be increased through the combined effect of IL-13 and IL-5 compared to the presence of either cytokine alone (Luttmann et al., 1999). Production of IL-13 and IL-5 in local lymph nodes and IL-13 in abomasal tissue suggests initiation of a TH2 response, recruitment and anti-apoptosis of eosinophils, and an increase in IgE within the first few days of infection.

Uninfected hair sheep were found to have higher IgE expression compared to wool sheep. Greater antibody production may increase numbers of IgE bound mast cells in the gastrointestinal tract, enabling hair sheep to rapidly respond to invading parasites. Increased numbers of mast cells, which bind IgE, are found in association with parasitism (Lacroux et al., 2006). Activation and degranulation of these cells occurs through worm antigen-IgE binding, and has the potential to direct a cascade of immune events. Mast cells can cause increased IgE production by autocrine production of IL-4 and IL-13 (Henz et al., 2001). Even though the predominant immune response to parasitism appears to be consistent among studies, lacking is the role of particular cytokines and immune effectors in increasing resistance.

Caribbean hair sheep have increased resistance to gastrointestinal parasites compared to conventional wool breeds (Zajac et al., 1990; Gruner et al., 2003; Vanimisetti et al., 2004b). However, little research has been done to characterize the immune mechanisms involved in parasite resistance of hair sheep. Comparisons of eosinophils, mast cells, and abomasal mucus IgA of hair and wool sheep show St. Croix hair sheep have increased numbers of degranulated mast cells in the abomasal mucosa (Gamble and Zajac, 1992) and Santa Ines hair sheep lack differences in antigen-specific IgA compared to wool sheep (Amarante et al., 2005) after

infection. Although these studies involved hair sheep infected with *H. contortus,* there were differences in the dose of infective larvae, sheep age, breed of comparison, climate, and previous infection status. However, Gamble and Zajac (1992) provided evidence for a potential role of mast cells in increased resistance of hair sheep. These cells produce cytokines and could therefore be responsible for the changes in cytokine expression we observed in parasite infected sheep.

When infected with *H. contortus*, resistant hair sheep appear to have a stronger TH2 response compared to wool sheep in both the abomasum and lymph node tissues. There was no differential expression of any of the genes analyzed in the abomasal tissue by 3 days PI. On the other hand, lymph node tissue of hair sheep have a distinct TH2 polarization 3 days after infection, with greater expression of IgE and decreased TH1 cytokine, IL-12, and part of its receptor, IL-12 Rβ2, compared to wool sheep. Between parasite-resistant and susceptible selection lines of wool sheep, Pernthaner et al. (2005a; 2006) reported a stronger TH2 response after infection in resistant animals. However, resistant animals from these studies were infected with *T. colubriformis* and have slightly different gene expression profiles than those observed in our study. Pernthaner et al. (2005a; 2006) found that resistant wool sheep have increased IL-5, IL-13, and TNFα with consistent IL-4 and IFN-γ expression in lymph cells when compared to susceptible animals. Measurement of IgE antibodies in resistant and susceptible lines of Merino and Romney wool sheep show greater IgE production in the resistant line (Bendixsen et al., 2004; Pernthaner et al., 2005b) , which is consistent with our expression data. Cytokine receptors have not been measured in parasite-infected sheep. However, the expression of cytokine receptor may be of even greater importance to the overall immune response than the cytokines themselves, since they are responsible for cell signaling.

The TH2 response in lymph node and abomasal tissue was even more differentiated between breeds by 27 days PI, with hair sheep having greater IL-13 and IgE in both tissues, reduced IL-12 in abomasal tissue, and reduced IFN-γ in both tissues. Increased IL-13 and IgE in resistant animals is consistent with studies of wool sheep selection lines as described above (Pernthaner et al., 2005a; Pernthaner et al., 2005b; Pernthaner et al., 2006). Although IL-4 Rα was not differentially expressed in sheep after 27 days of infection, the positive association it had with FEC in wool sheep may be due to IL-13 signaling. Both IL-4 and IL-13 use IL-4 Rα as part of their cell surface receptors to induce a TH2 response. The observed decreased expression of

TH1 cytokines IL-12 and IFN-γ may be the result of downregulation by increased TH2 cytokines in infected hair sheep (Webb et al., 2007). Recombinant bovine IL-12 directly causes increased IFN-γ production by T-cells (Collins et al., 1999). Therefore, reduced IL-12 expression 3 and 27 days PI may have led to the observed decrease in IFN-γ by 27 days PI. Other research shows that increased IL-12 in the presence of IL-10 actually reduced IFN-γ production (White et al., 2002). The combined effect of IL-12 and IL-10 could explain the correlation between increased IL-12 expression and lower FEC in hair sheep. However, IL-10 expression was not measured in these sheep. Cytokine profiles at day 27 of infection may also be a function of total worm burden, as hair sheep potentially have fewer worms than wool sheep. Adult and late-larval stages of *H. contortus* have a dramatic influence on the abomasal pathology, damaging tissue, causing bleeding and inflammation as they feed. Even under these inflammatory conditions, hair sheep appear to maintain a stronger TH2 response versus more susceptible wool sheep. Further evaluation of larvae and worm burdens from these sheep may help to clarify the association of cytokines with increased resistance.

Our data, summarized in Table 4.4, suggest measurable differences in expression of immune genes between hair and wool sheep. Some differences were apparent in uninfected animals, such as higher IgE in lymph nodes of hair sheep. Infection with *H. contortus* led to a dominant TH2 profile with gene expression changes in abomasal and lymph node tissue by 3 days after infection. However, expression of all TH1 cytokines and receptors measured were generally higher, although not significantly, in susceptible wool sheep compared to resistant hair sheep after infection. These results support a stronger TH2 response in infected hair sheep, where a clear preference for increased IL-13 over IL-4. Breed differences in expression of TH1 and TH2-type genes exist in both infected and control animals. To the best of our knowledge, this study provides the first evidence of cytokine, IgE and IgE receptor gene expression differences between resistant hair and susceptible wool sheep. Further evaluation of the specific immune cell populations and associated cell signaling pathways may help to determine the role of these cytokines in controlling parasite infections.

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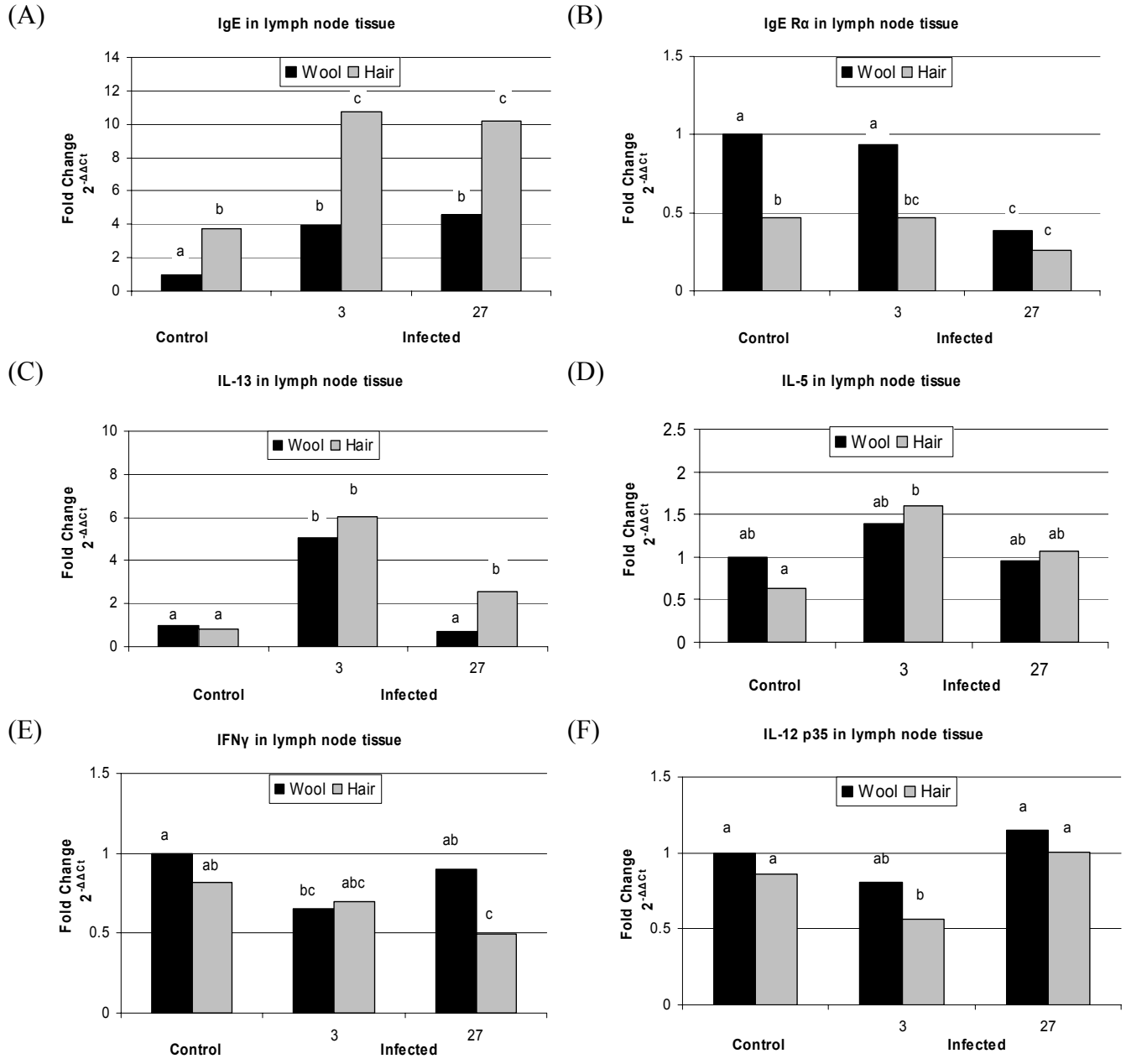
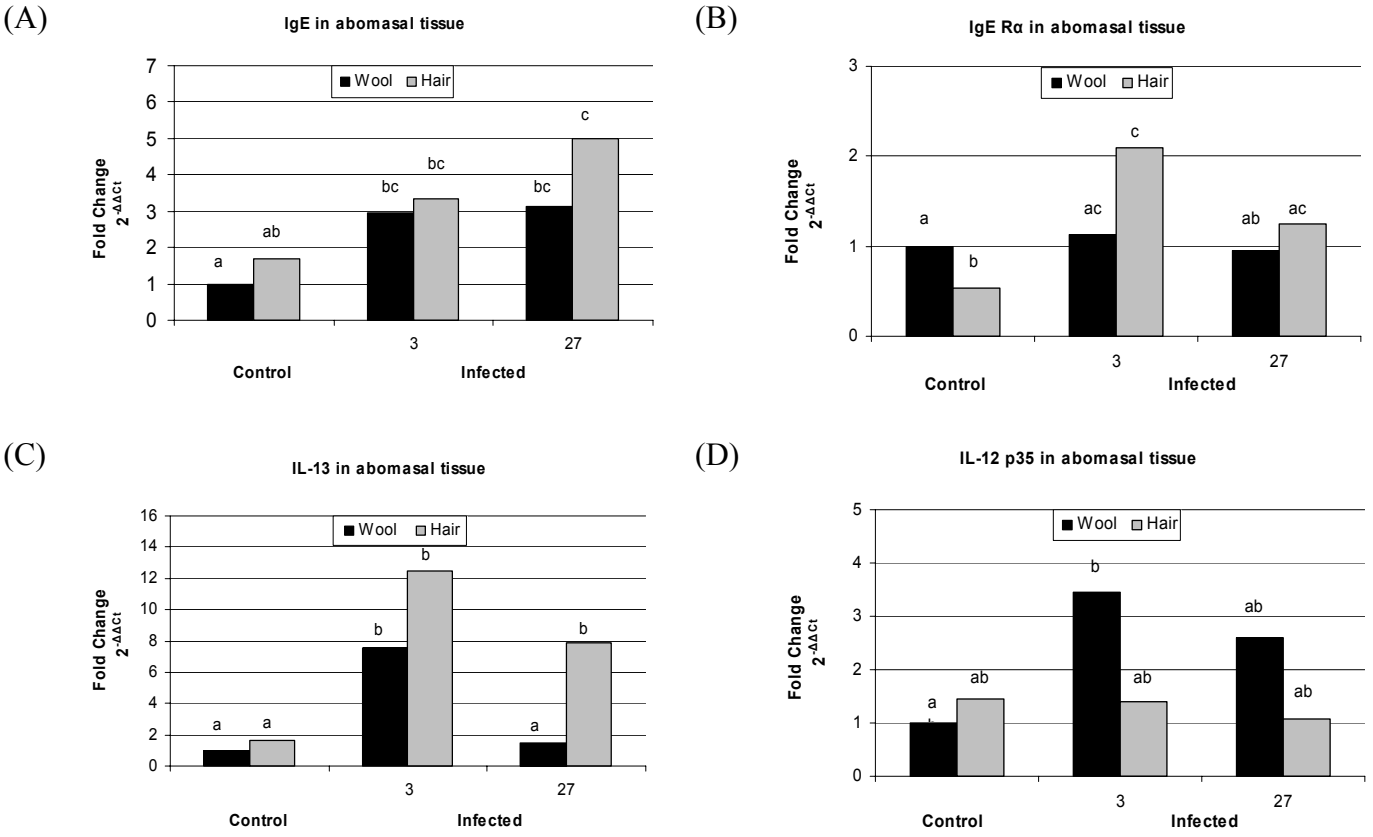
Figure 4.1. Relative gene expression of IgE (A), IgE Rα (B), IL-13 (C), IL-5 (D), IFN-γ (E), and IL-12 p35 (F) in lymph node tissue of hair and wool sheep. Control animals were grouped together by breed and designated as controls. Control wool lambs were arbitrarily assigned a value of 1.0 (using the $2^{-\Delta\Delta Ct}$ method) and other fold changes are relative to this group. Gene expression of infected animals is divided into data from animals at days 3 and 27 post-infection.



[abc] Columns with different superscripts indicate difference in least square means at $P < 0.05$

Figure 4.2. Relative gene expression of (A) IgE , (B) IgE Rα, (C) IL-13, and (D) IL-12 p35 in abomasal tissue of hair and wool sheep. All control animals of one breed were grouped together and designated as controls. Control wool lambs were arbitrarily assigned a value of 1.0 (using the $2^{-\Delta\Delta Ct}$ method) and other fold changes are relative to this group. Gene expression of infected animals is divided into data from animals at days 3 and 27 post-infection.



[abc] Columns with different superscripts indicate difference in least square means at $P < 0.05$

Table 4.1. Forward and reverse primer sequences for real-time RT-PCR. Melting temperature (T_m) of amplicon and presence or absence of ovine DNA amplification are indicated.

Gene	Forward sequence	Reverse sequence	T_m (°C)	DNA
IL-4	GCCACACGTGCTTGAACAAA	TGCTTGCCAAGCTGTTGAGA	78.9	no
IL-4 Rα	CCAAGCTCCTGCCCTGTTTA	CCATTTCTAGCAGCCTTAGAGAAGTC	78.0	yes
IL-5	TGGTGGCAGAGACCTTGACA	GAATCATCAAGTTCCCATCACCTA	78.8	no
IL-13	AAGCCCTCAGCTAAGCAGGTT	TGGGCCACTTCAATTTTGGT	79.2	no
IgE	GCGAGACCTACTACTGCAAAGTGA	CACGCTTGCCAACATCCTT	81.2	no
IgE Rα	TGCCGAATCAAAGGATTTGC	GATCAACCAGTCACTGATGACGTT	76.5	no
IFN-γ	TGGAGGACTTCAAAAAGCTGATT	TTTATGGCTTTGCGCTGGAT	77.1	no
IL-12 p35	GCTGCAGAAGGCCAGACAA	ATATCTTCATGATCAATCTCCTCAGAAG	74.3	yes
IL-12 Rβ1	CTTTGGGTACCTCGGCTTGA	CCTCAGTTTCCCCATCTTGAAA	75.9	no
IL-12 Rβ2	CCTGGGCACAACCCTGTTT	AACAACCCCGACGGAGATC	80.1	yes
β-actin	CGCCATGGATGATGATATTGC	AAGCCGGCCTTGCACAT	82.5	yes
RPL19	GCTCCTCAGCCAAGCACATAC	GCCATGGTAATCCTGCTCAGTAC	79.2	yes
GAPDH	GCATCGTGGAGGGACTTATGA	GGGCCATCCACAGTCTTCTG	81.7	yes

Table 4.2. Gene expression (ΔCt ; least square means ± standard error) in lymph node tissue of hair (H) and wool (W) sheep. Sheep were infected (A) for 3 or 27 days with *Haemonchus contortus* or were uninfected controls (B) collected at 17, 27, or 38 days compared to day 0 of infected animals. Higher ΔC_t value designates lower gene expression. P-values (shaded) indicate significance of breed differences within day using t-test of group means.

(A) **Infected**

Day	3				27			
	H		W		H		W	
IL-4	13.67 ± 0.21		14.37 ± 0.78		14.08 ± 0.61		12.92 ± 0.90	
	0.401				0.306			
IL-4 Rα	4.77 ± 0.26		4.33 ± 0.19		4.80 ± 0.29		4.63 ± 0.22	
	0.203				0.659			
IL-13	10.94 ± 0.51		11.19 ± 0.46		12.17 ± 0.69		13.97 ± 0.53	
	0.723				0.065			
IL-5	13.12 ± 0.31		13.33 ± 0.19		13.72 ± 0.67		13.87 ± 0.61	
	0.569				0.865			
IFN-γ	9.21 ± 0.15		9.31 ± 0.23		9.70 ± 0.15		8.84 ± 0.30	
	0.725				**0.030**			
IL-12 p35	9.38 ± 0.18		8.87 ± 0.18		8.55 ± 0.22		8.36 ± 0.31	
	0.068				0.625			
IL-12 Rβ1	15.92 ± 0.24		15.60 ± 0.57		15.30 ± 0.67		14.52 ± 0.70	
	0.605				0.391			
IL-12 Rβ2	8.82 ± 0.15		8.29 ± 0.27		8.56 ± 0.46		7.84 ± 0.58	
	0.112				0.351			
IgE	0.47 ± 0.27		1.91 ± 0.37		0.54 ± 0.27		1.71 ± 0.40	
	0.010				**0.037**			
IgE Rα	9.65 ± 0.35		8.65 ± 0.41		10.05 ± 0.43		9.92 ± 0.53	
	0.092				0.415			

(B) **Control**

Day	17				27				38			
	H		W		H		W		H		W	
IL-4	14.09	± 0.25	14.95	± 1.33	13.59	± 0.56	12.79	± 0.65	14.68	± 1.09	12.55	± 0.77
	0.558				0.374				0.188			
IL-4 Rα	5.21	± 0.15	4.60	± 0.37	4.65	± 0.18	4.69	± 0.25	4.72	± 0.24	4.57	± 0.30
	0.198				0.894				0.712			
IL-13	13.89	± 1.26	13.34	± 0.23	13.69	± 0.39	13.68	± 0.48	14.33	± 0.14	13.41	± 0.40
	0.970				0.985				0.094			
IL-5	14.36	± 0.15	14.57	± 0.80	14.33	± 0.47	13.17	± 0.42	14.86	± 0.77	14.30	± 0.71
	0.802				0.095				0.620			
IFN-γ	9.12	± 0.50	8.80	± 0.31	9.00	± 0.15	8.44	± 0.18	8.77	± 0.53	9.07	± 0.29
	0.618				**0.039**				0.647			
IL-12 p35	9.06	± 0.25	9.00	± 0.26	8.62	± 0.20	8.29	± 0.18	8.80	± 0.16	8.66	± 0.05
	0.875				0.256				0.458			
IL-12 Rβ1	16.22	± 0.62	15.94	± 0.88	15.49	± 0.47	14.52	± 0.44	16.08	± 0.48	15.71	± 0.60
	0.807				0.160				0.654			
IL-12 Rβ2	9.07	± 0.51	8.70	± 0.52	8.38	± 0.25	7.52	± 0.29	8.58	± 0.47	8.25	± 0.31
	0.632				0.054				0.587			
IgE	1.10	± 0.63	3.07	± 0.56	1.82	± 0.34	3.94	± 0.31	3.23	± 0.37	4.63	± 0.49
	0.080				**0.001**				0.086			
IgE Rα	9.27	± 0.39	8.16	± 0.37	9.94	± 0.32	8.40	± 0.29	9.44	± 0.15	9.26	± 0.26
	0.110				**0.005**				0.554			

Table 4.3. Gene expression (ΔCt ; least square means ± standard error) in abomasal tissue of hair (H) and wool (W) sheep. Sheep were infected (A) for 3 or 27 days with *Haemonchus contortus* or were uninfected controls (B) collected at 17, 27, or 38 days compared to day 0 of infected animals. Higher ΔC_t value designates lower gene expression. P-values (shaded) indicate significance of breed differences within day using t-test of group means.

(A) **Infected**

Day	3		27	
	H	W	H	W
IL-4 Rα	5.92 ± 0.10	5.22 ± 0.77	5.64 ± 0.24	5.46 ± 0.12
	0.39		0.52	
IL-13	10.34 ± 1.01	11.07 ± 0.46	11.01 ± 0.71	13.46 ± 0.54
	0.52		**0.02**	
IFN-γ	9.00 ± 0.36	8.97 ± 0.26	8.92 ± 0.26	8.12 ± 0.34
	0.93		0.10	
IL-12 p35	13.55 ± 0.38	12.26 ± 1.17	13.94 ± 0.25	12.67 ± 0.26
	0.32		**0.01**	
IgE	5.86 ± 0.87	6.04 ± 0.73	5.28 ± 0.27	5.94 ± 0.25
	0.88		0.10	
IgE Rα	6.99 ± 0.55	7.87 ± 0.30	7.73 ± 0.42	8.13 ± 0.55
	0.19		0.58	

(B) **Control**

Day	17		27		38	
	H	W	H	W	H	W
IL-4 Rα	5.29 ± 0.19	5.34 ± 0.17	5.46 ± 0.24	5.21 ± 0.17	5.52 ± 0.19	5.15 ± 0.25
	0.86		0.42		0.30	
IL-13	13.27 ± 0.76	14.23 ± 1.11	13.28 ± 0.49	13.74 ± 0.44	13.35 ± 0.61	14.12 ± 0.50
	0.54		0.50		0.38	
IFN-γ	7.79 ± 0.26	8.28 ± 0.99	8.50 ± 0.60	8.33 ± 0.44	7.97 ± 0.53	9.29 ± 0.81
	0.70		0.83		0.25	
IL-12 p35	13.30 ± 0.25	13.58 ± 0.69	13.76 ± 0.91	13.84 ± 0.37	13.14 ± 0.87	15.01 ± 0.93
	0.75		0.94		0.26	
IgE	5.40 ± 1.04	7.09 ± 0.44	7.02 ± 0.23	7.77 ± 0.46	7.96 ± 0.56	7.92 ± 0.48
	0.16		0.18		0.96	
IgE Rα	8.81 ± 0.21	7.62 ± 0.79	9.00 ± 0.34	8.14 ± 0.26	8.98 ± 0.29	8.46 ± 0.47
	0.27		0.07		0.41	

Table 4.4. Relative trends in gene expression of infected hair compared to wool sheep for TH1 and TH2 cytokines, IgE, and associated receptors. The breed having higher expression within abomasums or lymph node tissue is indicated with the breed name. Similar trends on days 3 and 27 post-infection were observed in multiple genes and are designated with one breed name per tissue. Some genes (N/A) could not be measure in abomasal tissue.

	Lymph node		**Abomasum**	
Day	3	27	3	27
IL-4	**Hair**	**Wool**	N/A	
IL-4 Rα	**Wool**		**Wool**	
IL-13	**Hair**[#]		**Hair***	
IL-5	-		N/A	
IL-12 p35	**Wool**[#]		**Wool***	
IL-12 Rβ1	**Wool**		N/A	
IL-12 Rβ2	**Wool**		N/A	
IFN-γ	-	**Wool***	-	**Wool**[#]
IgE	**Hair***		**Hair**[#]	
IgE Rα	**Wool**[#]		**Hair**	

[*] $P < 0.05$ between breeds for either day 3 or 27

[#] $P < 0.10$ between breeds for either day 3 or 27

- No difference in breed expression

CHAPTER 5

GENERAL DISCUSSION AND IMPLICATIONS

Hair sheep infected with *Haemonchus contortus* tended to have greater numbers of eosinophils and globule leukocytes in the abomasal mucosa, circulating and tissue antibody concentrations, and expression of TH2 immune genes compared to wool sheep. Although some of these differences were present in uninfected sheep, an overall increase in immune responsiveness was found in hair sheep after infection with these gastrointestinal parasites. It appears that the immune response within the first few days of infection may be critical for parasite expulsion, as many parameters differed between hair and wool sheep after only 3 days of infection. Larval *H. contortus* are in close contact with the mucosa which may allow for host recognition. However, by 27 days after infection the host has limited direct contact with adult *H. contortus*, which are only present in the lumen of the abomasum. Therefore, sheep may have to respond with in the first few days of infection to cause damage to the parasite. We only observed a snapshot of the host response to invading larvae and adult worms. Monitoring immune development over the first week of infection may help to clarify specific timing of cell infiltration and cytokine production and how these parameters relate to resistance.

The host response to gastrointestinal parasitism is dynamic and the interactions of cells and cytokines are important to the overall response. Increased eosinophil infiltration and increased TH2 cytokine, IL-13, and decreased TH1 cytokines, IL-12 and IFNγ were observed in infected hair compared to wool sheep. Further evaluation of particular immune cells and individual cytokines and how they affect resistance status is needed. Each one of these cytokines can cause a cascade of changes in the immune response, with effects on antibody production, antibody class switching, and cell proliferation, infiltration, and activation. Differences in eosinophil and globule leukocyte populations most likely affect local cytokine concentrations, potentially changing the overall immune response. Additionally, if these cells of hair and wool sheep differ in signaling pathways, their functionality may be altered causing differences in the immune response. Increasing cytokine concentrations artificially or blocking cytokines and receptors in infected sheep will help to determine effects on other cells and cytokines, as well as their overall effect on resistance. Blocking of specific cytokines or receptors may be a useful way to direct the immune response and treat parasite infected

animals. However, altering the immune response may affect the response to other pathogens or vaccines, and should be carefully assessed.

We have only begun to uncover potential immune mechanisms involved in resistance to gastrointestinal nematode infection, and in particular those mechanisms present in resistant hair sheep. However, the results we have obtained are promising. Hair sheep have measurable differences in cell populations, antibodies, and expression of cytokines and receptors. If these differences are genetic and result from gene polymorphisms among breeds, animals with the potential for increased resistance can easily be selected at birth. Prior to implementation of selection procedures, gene variants conferring resistance will have to be assessed for detrimental effects on wool and carcass traits. It is possible that increased production and maintenance of immune effectors could lead to decreased energy for growth. As methods currently used for controlling parasites are limited, the possibility of administering cytokines and antibodies or selection based on genetic polymorphisms would provide another useful tool for limiting parasite infection.

The comparison of resistant hair and susceptible wool lambs is a useful model for determining differences associated with resistance to gastrointestinal parasites. Differences do exist between these animals when they are not infected, and these potentially have no effect on resistance status. However, differences in the immune response only observed during infection have provided a useful starting point for determination of mechanisms required for greater parasite expulsion.