Mechanisms of immune-mediated scouring in parasite-resistant Merino sheep

by

Andrew Williams

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Summary

The general hypothesis tested in this thesis was that challenging parasite-resistant Merino sheep with infective nematode larvae will cause a hypersensitive immune response, characterised by production of inflammatory granulocytes, mediators and antibodies that would be negatively correlated with both worm burdens and faecal dry matter.

Challenging parasite-resistant sheep in an animal house with a mixture of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* larvae caused faecal dry matter to decrease ($P<0.05$) compared to unchallenged sheep. This reduction in faecal dry matter occurred despite the absence of a significant worm burden. At post-mortem examination, challenged sheep had higher numbers of granulocytes (eosinophils and mast cells) in abomasal and intestinal tissue than unchallenged sheep, and also higher concentrations of inflammatory mediators in mucus from the abomasum and small intestine.

Twenty-month old sheep from the same parasite-resistant line were selected on the basis of estimated breeding values (EBV) for worm egg count (WEC) and also dag score. There were ten sheep in each of the following four groups – 1) High WEC, high dag, 2) High WEC, low dag, 3) Low WEC, high dag and 4) Low WEC, low dag. These sheep were challenged daily with *T. colubriformis* and *T. circumcincta* larvae for six weeks and then necropsied. An additional group of ten sheep acted as an unchallenged control. Challenging sheep with larvae reduced faecal dry matter at two, three and four weeks after challenge. Sheep with high-dag scores had reduced faecal dry matter compared to sheep with low-dag scores. The sheep showed good resistance to the parasite challenge as evidenced by low worm egg counts and low total worm counts at post-mortem, with the numbers of *T. colubriformis* particularly low. Sheep with low EBV for WEC had lower ($P<0.05$) numbers of total *T. circumcincta* larvae than sheep with high EBV for WEC, and sheep with high dag scores had lower ($P<0.05$) numbers of early *T. circumcincta* larvae than sheep with high dag scores. Within the low WEC EBV category, sheep with high and low-dag scores had similar total worm counts, indicating that some sheep are able to reject larvae without an increase in scouring. Sheep with low faecal dry matter had significantly higher numbers of eosinophils in small intestine tissue. Sheep with low total worm counts had significantly higher levels of bradykinin in abomasum mucus. Sheep with more granulocytes in tissue and inflammatory

mediators in mucus tended to have fewer numbers of *T. circumcincta* but there was little relationship with numbers of *T. colubriformis.* Larval challenge also increased the levels of parasite-specific antibodies and interleukin-5 in serum. Concentrations of antibody in serum tended to be negatively correlated with numbers of *T. circumcincta* but there was no relationship with faecal dry matter. These results showed that dag scores are correlated to a reduction in faecal dry matter, which can be attributed to the challenge with infective parasite larvae. Inflammation during worm infection is associated with rejection of the worm challenge and may result in more fluid faeces, and consequently scouring. However, sheep can be bred that have both low worm burdens and low-dag scores.

Parasite-resistant sheep were challenged with either *T. colubriformis*, *T. circumcincta* or both species. As in the first two experiments, larval challenge lowered ($P<0.05$) faecal dry matter and very few worms established. However, there were no differences in faecal dry matter between sheep challenged with a mixture of the two species, or either species by itself. Levels of parasite-specific IgA and IgE in serum were increased by the larval challenge ($P<0.05$) but there were no differences in sheep challenged with both species or either species alone. IL-5 in serum was increased by the larval challenge ($P<0.05$) and the highest levels were in sheep challenged with both species. Challenge with *T. colubriformis* alone did not increase levels of inflammatory cells in the abomasum compared to unchallenged sheep. By comparison, challenge with *T. circumcincta* alone resulted in slightly higher numbers of inflammatory cells in the small intestine compared to unchallenged sheep, and the highest numbers of inflammatory cells in the small intestine were in sheep challenged with both species. These results showed that challenge with both *T. circumcincta* and *T. colubriformis* can cause a reduction in faecal dry matter that may lead to scouring in the field. In addition, a mixed challenge will not necessarily result in worse scouring.

Overall, the results support the concept that scouring in parasite-resistant sheep is due to an inflammatory immune response to ingested larvae. This response is efficient at expelling incoming larvae but may also lead to a high build up of dags. However, there are some sheep that are able to effectively reject larvae without an increase in scouring. Therefore, sheep producers should focus on breeding sheep that have both low WEC and also low-dag scores.

Declaration

The work presented in this thesis is the original work of the author. It has not been submitted for examination at any other university. I carried out the experimental work and wrote the manuscript myself after discussions with my supervisors, Dr Ian Williams and Associate Professor Phil Vercoe, with additional advice from Drs Dieter Palmer and John Karlsson from the Department of Agriculture and Food Western Australia and Associate Professor David Emery from the University of Sydney.

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Publications

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Chapter 1 - General Introduction

Nematode parasitism is a major constraint on the profitability of sheep industries worldwide (Stear *et al.*, 2003). In the southern half of Australia, which is characterised by a Mediterranean, winter-rainfall climate, the main nematode species are the abomasal parasite *Teladorsagia circumcincta and* the small intestinal parasite *Trichostrongylus* spp. (Besier and Love, 2003). The main clinical signs in sheep infected with these nematodes are reduced weight gain and scouring (diarrhoea). There is a need to develop parasite-control methods that do not rely on chemical treatment, due to widespread anthelmintic resistance and consumer demand for organic animal products (Besier, 2006). Breeding for natural resistance to worms on the basis of low faecal worm egg count is an attractive solution as it has the potential to considerably reduce the need for anthelmintic treatment. The feasibility of such a research program has been demonstrated (Woolaston and Windon, 2001, Karlsson and Greeff, 2006). However, in winter-rainfall climates there is an increased tendency for resistant sheep to scour during the rainfall season (Bisset *et al.*, 1996, Karlsson *et al.*, 2004).

Scouring is a major management issue for sheep producers, as it leads to the build up of faecal material around the breech ('dags'). This accumulation of faeces leads to direct financial penalties for producers as the wool must be removed prior to shearing and its value is dramatically reduced (Larsen *et al.*, 1995). In addition, and perhaps most important, sheep with a heavy build up of dags are highly susceptible to flystrike (Scobie *et al.*, 2008).

In mature sheep (12 months age or older), scouring is mainly caused by the ingestion of nematode larvae from pasture (Larsen *et al.*, 1994). Thus, it was expected that by breeding sheep that had lower worm burdens scouring would be reduced. However, researchers in New Zealand and Western Australia have discovered that resistant sheep, while having very low faecal egg counts, consistently have more dags than unselected sheep (Bisset *et al.*, 1997, Greeff *et al.*, 1999).

The immune response of sheep to nematode parasites has hallmarks of a Th2, or hypersensitive immune response (Miller and Horohov, 2006). Researchers have shown that sheep bred for low worm egg counts have indicators of an enhanced Th2 immune response to worms, such as increased numbers of eosinophils and mast cells in mucosal tissue (Rothwell *et al.*, 1993, Bisset *et al.*, 1996, Gruner *et al.*, 2004). Larsen *et al.*

(1994) noted that Merino sheep with severe dag had more eosinophils in the gastrointestinal tract than sheep with little or no dags. Therefore, it is apparent that, in adult sheep, scouring can mainly be attributed to the immune response of the host rather than the direct effects of the parasite ('immune-mediated scouring').

The main objective of the work in this thesis was to investigate the mechanisms of immune-mediated scouring in parasite-resistant sheep. To do this, I used the Rylington Merino flock in Western Australia where sheep have been bred for low worm egg counts for over twenty years. The immune response and immunopathology of this flock has not yet been studied. I aimed to determine whether the immune response of these parasite-resistant sheep was also responsible for the increased scouring observed in this flock.

The general hypothesis tested in this project was that challenging parasite-resistant sheep with worm larvae will cause an inflammatory immune response that results in both softening of the faeces and rejection of the larval challenge.

Chapter 2 – Literature Review

2.1 Introduction and scope

In this review, I will develop the case that diarrhoea in sheep that are resistant to nematode parasite infection may be caused primarily by the inflammatory immune response to ingested parasite larvae. I will describe the current knowledge in this area and formulate hypotheses in order to investigate the mechanisms of scouring in parasite-resistant Merino sheep. Understanding these mechanisms will be a major step towards developing sustainable parasite-control in sheep production.

Three specific fields of literature need to be addressed. First, I will describe the life cycle and pathophysiology of the main nematode species in southern Australia and focus on the feasibility of breeding worm-resistant sheep as a sustainable solution. Second, I will review the current knowledge on scouring in adult, immune sheep. Finally, the immunobiology of nematode infection is discussed. This information is used to formulate research hypotheses on the mechanisms of immune-mediated scouring in parasite-resistant Merino sheep.

2. 2 Nematode parasitism in Australian sheep production

Gastrointestinal nematodes (GIN) are a major threat to the profitability of the Australian sheep industry, costing sheep producers around $A370 million annually (Sackett *et al.*, 2006). These costs are due to the reduced productivity of infected sheep and the increased labour and material costs associated with parasite control.

2.2.1 Biology of parasitic nematodes of sheep

In the agricultural regions of South Western Australia, which are characterised by a Mediterranean, winter rainfall climate, the most significant nematode parasites of sheep are *Trichostrongylus* spp. and *T. circumcincta,* due to their increased ability to withstand cold and desiccation (Besier and Love, 2003). Clinical signs of infection include decreased appetite, reduced weight gain and skeletal growth as well as decreased wool and milk production (Coop and Holmes, 1996). In clinical cases, death may result from diarrhoea or scouring (Parkins and Holmes, 1989). Ingesting larvae from contaminated pasture infects sheep. Larvae hatch out of eggs that are passed in the faeces of infected animals.

Life Cycle of Trichostrongylus spp. and T. circumcincta

T. colubriformis is the main species from the genus *Trichostrongylus* in South Western Australia, with *T. axei* and *T. vitrinus* present to a lesser extent (Noble and Noble, 1982). They are slender, small-mouthed nematodes that reside in the small intestine of domestic ruminants, except for *T. axei* which resides in the abomasum. Adult worms produce oval shaped eggs that are passed in the faeces. If conditions are optimal, eggs can hatch within 24 hours and develop from free-living organisms into infective third stage larvae within 14 days (Noble and Noble, 1982). Larvae migrate to pasture on films of water and can be widely dispersed by the sporangia of certain fungi (Stevenson and Hughes, 1988). In cool, damp conditions, larvae may survive for several months on pasture. They are extremely tolerant to cold, with larvae able to infect sheep after being stored for 308 days at 4ºC (Andersen *et al.*, 1966).

Eating pastures or drinking water contaminated with larvae infects grazing animals. Once ingested, larvae moult twice in the mucosa of the intestine and reach sexual maturity in two to four weeks (Stevenson and Hughes, 1988).

T. circumcincta has a similar life cycle to *T. colubriformis,* with infective third stage larvae (L_3) appearing on the ground 5-6 days after being passed in the faeces of infected animals (Stevenson and Hughes, 1988). In contrast to *Trichostrongylus*, *T. circumcincta* lives only in the abomasum and not in the intestine (Noble and Noble, 1982).

2.2.2 Patho-physiology of nematode infection in sheep

Lambs, weaners and pregnant ewes are most susceptible to nematode infection, but sub-clinical infections are observed in almost all sheep grazing pastures. The main signs of parasite infection in grazing sheep are reduced food intake and decreased efficiency of food utilisation (Coop and Holmes, 1996).

Reduced food intake

In sub-clinical infections, reductions of 10-30% in voluntary food intake are common (Coop and Sykes, 2002). This reduction in food intake is normally corrected once the animal has developed immunity to nematodes and has a low resident worm population. The mechanisms behind this inappetence are not well understood. It has been suggested that gut peptides such as gastrin and cholecystokinin may play a role through altering digesta motility in the reticulorumen, or affecting satiety signals from the hypothalamus (Fox, 1997, Dynes *et al.*, 1998). It has also been proposed recently that in immature animals, reduction in food intake occurs during the acquisition of immunity and can be attributed to the production of pro-inflammatory cytokines (Greer *et al.*, 2005).

Reductions in food intake are not normally seen in sheep with a well-developed immune response (Sykes and Greer, 2003). Therefore, it is unlikely that these reductions play any role in the pathology of immune-mediated scouring.

Reduced efficiency of food utilisation

Infection with GIN changes the intestinal microenvironment. These changes include reduced efficiency of the use of absorbed nutrients, altered gut motility, and increased loss of protein into the intestinal lumen.

Elevated plasma pepsinogen concentrations are a consistent feature of infection with abomasal parasites such as *T. circumcincta* (Anderson, 1972, Jeffcoate *et al.*, 1992). High plasma pepsinogen concentrations are indicative of increased mucosal permeability and lesions in the abomasum (Murray *et al.*, 1970). Pepsinogen is normally stored and released from granules in cells at the base of the gastric glands in the abomasum (Stear *et al.*, 1999a). Infection with abomasal parasites leads to a stretching of the abomasal mucosa and disruption of the junctions between epithelial cells, allowing pepsinogen to diffuse firstly into lymph and then into the bloodstream (Baker *et al.*, 1993). Anderson (1972) observed that increased plasma pepsinogen levels occurred in sheep that had low worm egg counts (<100 epg), and that the pepsinogen concentrations were strongly correlated with numbers of infective larvae on pasture, but not with worm burdens. This suggests that increased leakage of pepsinogen into the plasma is a consequence of ingesting larvae, and ultimately unrelated to the adult worm burden. This was one of the first observations that pathological signs of worm infection could be caused by the larval challenge *per se*, and not a burden of adult worms. It was proposed that hypersensitive immune responses to ingested larvae were responsible for

the increased leakage in the abomasum. Yakoob *et al.* (1983) and Jeffcoate *et al.* (1992) have also found elevated plasma pepsinogen levels in immune sheep with low worm burdens, and have ascribed this leakage to hypersensitive-type immune responses. Yakoob *et al.* (1983) also noted that in immune sheep challenged indoors with larvae, elevated plasma pepsinogen levels were accompanied by a softening of faeces that did not occur in unchallenged sheep. In this experiment, the establishment of incoming worms was only about 1-2%, so sheep were immune due to previous larval exposure on pasture. Greer *et al.* (2009) also noted that faecal dry matter decreased during larval challenge as sheep gained immunity, but this reduction did not occur in animals that were immunosuppressed through treatment with corticosteroids. This provides support for the hypothesis that in immune sheep, scouring can mainly be attributed to the immune response to larval challenge, and not to a large adult worm burden.

Gut motility is altered by parasite infection, although there are conflicting reports on what effects are apparent. Gregory *et al.* (1985) have reported that gut motility and peristalsis are decreased in sheep infected with *T. colubriformis.* Fox *et al.* (1989) also observed much slower rates of passage in calves infected with *O. ostertagi.* It would be unlikely that decreased peristalsis would be involved in the pathogenesis of diarrhoea, and, in fact, the opposite would be expected. However, these studies were conducted with young animals and the reductions in digesta in transit can probably be attributed to reduced food intake and the direct effects of large numbers of parasites in the gut. Gregory *et al.* (1985) noted that as sheep began to develop immunity (i.e. began to reject worms from the gut) the percentage of moisture in the faeces increased, but in this experiment faecal moisture was not correlated with digesta flow rate.

In immune animals, increased gut motility and peristalsis are clinical signs of parasite infection and are most likely part of the immune response (Emery *et al.*, 1993a). These changes are due to alterations in the migrating motor complex (MMC). The MMC normally operates during periods between food intake, and is responsible for triggering peristaltic waves that transport fibre and indigestible materials from the stomach and small intestine into the colon. Increased frequency of rapid spiking activity, the component of the MMC responsible for the flow of small-intestinal contents, has been observed within 24 hours of challenge with *T. colubriformis* in immune sheep (Tremain *et al.*, 1994, cited in Larsen, 1997). Bueno *et al.* (1982) have also reported increased activity of the MMC and increased abomasal emptying, and also duodenal flow rate, in sheep challenged with *H. contortus.* This increased peristalsis may be an effective

immune mechanism against newly ingested parasite larvae, and is probably mediated by immediate hypersensitivity reactions in the gut mucosa (Khan and Collins, 2006). MacKinnon *et al.* (2009) have reported that genes controlling gut motility are upregulated in parasite-resistant sheep compared to susceptible sheep. Therefore, it is probable that there is a positive correlation between the degree of peristalsis and gut motility and the effectiveness of the immune response. This may explain why sheep that are highly resistant to parasite infection still suffer diarrhoea.

Protein metabolism

It is well established that a loss of protein is a pathophysiological feature of infection with GIN. Protein is lost through leakage into the intestinal tract and increased sloughing of epithelial cells into the lumen due to increased turnover of the mucosa of the gastrointestinal (GI) tract (Coop and Sykes, 2002).

The extent to which plasma proteins leak into the GI tract is dependent on the magnitude and species of the infection. Poppi *et al.* (1981) showed that plasma protein accounted for only 20% of endogenous protein lost into the GI tract in sheep infected with *T. colubriformis* and, of this amount, only 60% was reabsorbed further down the tract. Rowe *et al.* (1982) reported that in sheep infected with *H. contortus*, leakage of plasma protein accounted for virtually all endogenous nitrogen loss and all of this was reabsorbed by the terminal ileum. Generally, in sheep affected with abomasal parasites, most endogenous protein losses will be absorbed in the small intestine (Rowe *et al.*, 1988). However most grazing sheep in Mediterranean environments are infected with a mixture of abomasal and small intestinal parasites and, as such, there may be a reduced capacity for the small intestine to reabsorb protein lost from the abomasum. Instead, protein lost into the lumen is degraded in distal regions of the intestinal tract and absorbed as ammonia which is later excreted as urea (Coop and Sykes, 2002). The amount of non-recoverable endogenous protein leaving the terminal ileum of a GI parasitised sheep may be as high as 4-5 g per day (Coop and Kyriazakis, 2001). In addition, even when endogenous protein is reabsorbed, protein deposition in the body is poor due to re-partitioning of energy for protein synthesis to repair GI tissue, and to replace endogenous secretions (Coop and Sykes, 2002, Liu *et al.*, 2003).

Protein deposition in the body is reduced as significant amounts of protein are required for maintenance and turnover of the GI tract. Yu *et al.* (2000) quantified the protein turnover of the ovine GI tract during nematode infection, using mass isotope tracer

kinetics with leucine as the marker amino acid. They found that sequestration of protein by the GI tract from arterial pools increased by 24% during infection with *T. colubriformis*, and that oxidation of leucine increased by 22-41%. Bermingham *et al.* (2000) reported that sheep infected with *T. colubriformis* had reduced weight gain, but not reduced whole-body loss rate of cysteine and valine. Liu *et al.* (2007) also observed increased rates of fractional protein synthesis in the abomasal lymph nodes, mesenteric lymph nodes and bone marrow in Merino sheep infected with a mixture of *T. circumcincta* and *T. colubriformis*, but decreased rates of fractional protein synthesis in the skin, muscle and liver. This indicates that nutrients are actively partitioned between growth and the immune response during nematode infection. The mechanisms behind this partitioning are not clear, but are probably driven by local hormones from the GI tract (Adams and Liu, 2003).

2.2.3 Anthelmintic resistance

Control of parasites is currently based on drenching programs with a number of chemical compounds. These include older broad-spectrum compounds (benzimidazole and levamisole) and the more recent macrocyclic lactone class of drenches (avermectin and milbemycin). Narrow spectrum compounds such as closantel are effective only against *H. contortus* (Besier and Love, 2003). Resistance has been reported for many years in the older benzimidazole and levamisole drenches (Sangster, 1999), and resistance to macrocyclic lactone compounds is now becoming widespread (Dobson *et al.*, 2001, Sutherland *et al.*, 2003). In Western Australia, the most recent survey of anthelmintic resistance indicates that more than 90% of farms that have sheep have some form of drench resistance (Palmer *et al.*, 2000). It is likely that the level of resistance has increased in the time since this study. In addition, there is now increasing consumer demand for animal products that have been produced with a minimum of chemical inputs (Besier, 2006). Harnessing the natural immunity of sheep by breeding for resistance to parasitic nematodes appears to be a long-term, sustainable solution.

2.3. Breeding worm resistant sheep

It has been recognised for the past fifty years that there is considerable variation among livestock in their ability to resist a range of diseases and pathogens. This variation is under genetic control, and selective breeding programs have been developed to breed for resistance to mastitis and ketosis in dairy cows (Solbu and Lie, 1990), trypanosomiasis and ticks in beef cattle (Murray *et al.*, 1991) and flystrike and fleece rot

in sheep (Colditz *et al.*, 2006). The rapid rise of anthelmintic resistance in intestinal parasites of sheep led to the initiation of experimental flocks of sheep that were bred to be genetically resistance to parasites. Flocks were established in the 1970s in Eastern Australia with Merino sheep, with the criteria for selection being low worm egg counts and/or antibody titre in response to an artificial challenge with either *H. contortus* or *T. colubriformis* (Woolaston *et al.*, 1990, Windon, 1991). In 1987 further flocks were established in New Zealand with Romney sheep (Baker *et al.*, 1990) and in Western Australia with Merino sheep (Karlsson and Greeff, 2006). Selection in these last two flocks was based on low worm egg counts following a natural parasite challenge due to grazing pastures contaminated with *Trichostrongylus* spp. and *T. circumcincta*.

Resistance and Resilience

Resistance refers to the ability of a sheep to suppress the establishment and/or development of a disease. Resilience can be defined as the extent that a parasitised animal can maintain production in relation to an uninfected control animal. Both of these have been proposed as possible breeding objectives, as the aim of any livestock system is to maximise production and economic return. However, selecting purely on production traits (i.e. selecting for resilience) in a population carrying a disease burden has the potential to allow the disease to reach unmanageable levels and cause significant stock losses. Albers *et al.* (1987) investigated the genetic progress possible by selecting for either low WEC or for production traits in Merino sheep infected with *H. contortus.* They found that selecting for low WEC made close to identical genetic progress in production traits as when these traits were selected for directly. Conversely, selecting on production traits made little or no progress in genetic gain of low WEC. Therefore, it appears that selecting for low WEC will not lead to unfavourable genetic correlations with other important traits. However, it must be considered that including extra traits in selection indices will automatically decrease the rate of genetic gain for the other traits in the index.

Breeding for resistance also offers the chance to control the epidemiology of the parasite. Selecting animals carrying lesser burdens of worms systematically reduces the fitness of parasites, as they require hosts to reproduce. Bishop and Stear (2003) suggested that the benefits of selecting for disease resistance relative to production traits may have been underestimated, due to the epidemiological effects of breeding for worm resistance. These authors used modelling which predicted that selection responses to breeding for worm resistance are higher than that predicted by quantitative genetic

theory. This is due to lower amounts of re-infection in resistant sheep due to reduced pasture contamination. This reduced contamination has been shown experimentally in genetically resistant Romney (Bisset *et al.*, 1997) and Merino sheep (Williams *et al.*, 2009, Kahn *et al.*, 2003).

Indicator Traits

The trait currently used to assess worm resistance is a faecal worm egg count (WEC). This indicates the number of worm eggs present, but not the diversity of species present. It is also apparent, especially in the case of *T. circumcincta,* that WEC is not always well correlated with the actual nematode burden present in the host (Barger, 1987). Despite this, WEC is an important measurement as it indicates the extent to which the host contributes to pasture contamination (Albers and Gray, 1987). Sheep selected on the basis of low WEC also have lower total worm burdens at post-mortem examination (Bisset *et al.*, 1996, Gruner *et al.*, 2002). Other advantages of using WEC as an indicator trait are the ease and low cost of the procedure. The heritability of WEC varies between environments, sheep breeds and nematode species, but is generally accepted to be around 0.3 (Table 2.1).

Table 2.1 Heritability estimates for faecal worm egg counts in Merino, Romney and blackface sheep.

Breed	Location	Infection Regime	Heritability	Reference
Merino	New South Wales	Artificial *H. contortus* challenge	0.29	(Woolaston and Piper, 1996)
Merino	New South Wales	Artificial *T. colubriformis* challenge	0.41	(Woolaston *et al.*, 1991)
Romney	New Zealand	Natural challenge, mainly *T. colubriformis* and *T. circumcincta*	0.27	(Bisset *et al.*, 1992)
Merino	Western Australia	Natural challenge, mainly *T. colubriformis* and *T. circumcincta*	0.28	(Pollott *et al.*, 2004)
Scottish blackface	UK	Natural challenge, mainly *T. circumcincta*	0.33	(Stear *et al.*, 2003).

Measurements of eosinophils and parasite-specific immunoglobulin in blood samples may also be a viable indicator trait of resistance. The heritability of eosinophil count is about 0.2 and has a genetic correlation of -0.62 with WEC (Woolaston *et al.*, 1996). Levels of immunoglobulins (total antibody and IgG_1) specific to *T. colubriformis, H. contortus* and *Cooperia curticei* are heritable and negatively correlated with WEC (Douch *et al.*, 1995a, Douch *et al.*, 1995b). At six months of age, h^2 values ranged from 0.18 to 0.43 and it was estimated that selection on serum antibody levels would result in 51-67% of the genetic gain possible from selection on WEC. While it is clear that serum immunoglobulin levels are heritable and related to immunological responsiveness, the extra cost and labour associated with collecting blood samples and performing an

ELISA test needs to be taken into consideration. It appears that WEC is still the most effective indicator trait that can be used to breed for worm-resistance.

2.3.1 Worm resistance – genetic correlations with other traits

Production Traits

There is little data on the genetic correlations between WEC and production traits, but it is most unlikely that breeding for worm resistance will lead to unfavourable correlated responses. Eady *et al.* (1998) carried out the first study of the correlations between WEC and production traits in Australian sheep. They noted a slightly favourable correlation with bodyweight, a neutral correlation with fibre diameter and a slightly unfavourable correlation with clean fleece weight. Morris *et al.* (2000) noted slightly unfavourable correlations between WEC and bodyweight and fleece weight in Romney sheep in New Zealand. It was postulated that the increased demand for nutrients, especially cysteine and methionine, needed for the increased immune response in resistant sheep resulted in less partitioning of nutrients towards wool growth and fat deposition. Greer and colleagues have demonstrated that in young sheep, suppressing the immune response through administration of corticosteroids can increase the efficiency of use of metabolisable energy for net energy deposition in sheep infected with either *T. colubriformis* (Greer *et al.*, 2005) or *T. circumcincta* (Greer *et al.*, 2008). The effect of immunosuppression diminished in older animals. These researchers postulated that, during the acquisition of immunity, sheep with a strong immune response would gain less weight and grow less wool. The mechanisms behind this relationship could be a competition for nutrients between production and the immune response. Alternatively, it may be due to a direct effect of the immune response on the central nervous system, whereby production of pro-inflammatory cytokines during the acquisition of immunity leads to inappetence (Colditz, 2003).

However, results from Western Australia give a different picture. Pollott and Greeff (2004) reported low but favourable correlations between WEC and clean fleece weight (-0.03) and body weight (-0.09). In addition, Greeff and Karlsson (2006) have noted that resistant sheep have significantly higher body weight at weaning (22.1 kg v. 20 kg) and at mature age (55.7 kg vs. 49.2 kg) when grazed separately from unselected animals, thereby preventing cross-contamination of pastures. Also, the maximum divergence in WEC between resistant and susceptible sheep has been shown to be when nutrient supply is limiting (Walkden-Brown and Eady, 2003). Clearly, the

epidemiological benefits of worm-resistant sheep, where immature sheep face a significantly reduced larval challenge, outweigh the metabolic cost of the immune response.

Scouring traits

Scouring (diarrhoea) traits in sheep are usually determined by dag scores and faecal consistency scores. Faecal consistency can be scored on a subjective scale where 1 is hard pellets and 6 is watery diarrhoea (Larsen, 1997). When the faeces is loose or watery, faecal material may adhere to the wool around the breech. This build up of faecal material is known as dags. Dags are also scored on a subjective scale where 0 or 1 refers to no faecal material around the breech, whereas 5 refers to a heavily soiled breech. Scouring is a symptom of infection with nematode species such as *T. colubriformis* and *T. circumcincta*. Therefore, it was assumed that sheep that were selected to be resistant to these parasites would be less prone to scour. However, it is apparent that this is not the case. Karlsson *et al.* (1995) reported that in the parasite-resistant Rylington Merino flock from Western Australia, faecal consistency score was higher ($P<0.1$) in the selected sheep compared to the control line. Subsequent genetic analyses in this flock by Greeff and Karlsson (1998) revealed a negative genetic correlation between WEC and faecal consistency score at weaning (-0.21) and at hogget age (-0.22). This suggests that breeding for worm resistance may in fact lead to an increase in scouring. Similarly, researchers in New Zealand found that flocks of Romney sheep bred for resistance had higher dag scores and softer faeces than unselected sheep (Douch *et al.*, 1995b, Bisset *et al.*, 1997, Morris *et al.*, 2000). However, within the parasite-resistant flocks there are sheep that have low WEC and also low-dag scores. Therefore, scouring is not an inevitable consequence of worm-resistance.

The studies on the correlations between worm-resistance and scouring in the Rylington flock and in New Zealand were undertaken in winter-rainfall climate zones. Karlsson *et al.* (2004) investigated the possibility of genotype by environment interactions by taking rams from two parasite-resistant flocks – the Rylington Merino flock located in the winter-rainfall region of south-western Australia and the CSIRO *H. contortus* selection flock located in the summer-rainfall area of Armidale, New South Wales, and running their progeny alongside each other, as well as unselected, control sheep, in the two different climatic regions. It was found that there was no difference in dag scores between the different lines in the summer-rainfall areas. However, in Western

Australia, the resistant Rylington sheep had significantly higher dag scores than the other lines. This suggests that the increased scouring observed in resistant sheep in winter-rainfall areas is partly attributable to environmental conditions. Karlsson *et al.* (2004) observed that dag scores increased rapidly following the start of winter rain. This suggests that the sudden increase in infective nematode larvae on pastures after the break of season, following a period of low infectivity during the warm summer months, is an important factor in scouring in resistant sheep.

In flocks of outbred sheep where scouring has been studied, no relationship has been found between dag score and WEC in ewes (Larsen *et al.*, 1994) or in lambs (French and Morgan, 1996, French *et al.*, 1998, Wolf *et al.*, 2008). Broughan and Wall (2007) found that dag scores were positively associated with WEC in lambs aged between three and six months. This suggests that the relationship between dag scores and WEC may interact with the age and/or immunological responsiveness of the sheep. In fact, it has been proposed that two sub-types of parasite-related scouring occur, one associated with high WEC in lambs and another associated with low WEC in adult sheep (Karlsson and Greeff, 2005). This is supported by the findings of Jacobson *et al.* (2009) who found that scouring in lambs in an abattoir in Western Australia was associated with a high WEC. Conversely, adult sheep that were scouring had lower WEC ($P<0.05$) than adult sheep that were not scouring.

2.4 Scouring

Scouring is a common feature in adult Merino sheep grazing improved pastures in winter rainfall areas of Australia. It occurs in sheep that otherwise appear healthy with no signs of clinical gastro-intestinal parasitism or bacterial infection (Larsen, 1997). Scouring is a major problem for sheep producers as it causes breech soiling and the formation of dags on the wool surrounding the breech leading to lost production, increased labour costs, and the increased likelihood of flystrike in affected sheep.

2.4.1 Prevalence and financial costs

Scouring in adult sheep is observed on virtually all sheep properties with high winter rainfall. This is mainly areas with a Mediterranean climate such as the southern half of Australia. In areas with year-round rainfall, scouring is less of a problem (R.B. Besier, pers comm.). Jacobson (2006) surveyed 138 farmers in the south-west region of Western Australia and found that all of them considered that dags, caused by scouring, were a major problem on their property. More than 90% of the sheep on their properties

were affected by dags, with dags classified as moderate or severe in nearly half of these cases. The most susceptible age group was hoggets (12 months of age), with 52% of sheep in this class having moderate or severe dag. The least affected age group was wethers, with 31% of sheep from this group having moderate or severe dag.

Larsen *et al.* (1995) quantified the financial penalties of severe dag (dag score 4 or 5) in Merino ewes in south-west Victoria. They concluded that in sheep with severe dag, the cost was between $A0.98 to $A1.45 per head, due to the increased costs of crutching and the reduced value of soiled wool which is heavily discounted. By multiplying the respective costs per head for sheep with different dag scores by the proportion of sheep in the flock with that dag score, it was concluded that the average loss due to dag was $A81.90 per 100 ewes. This represented a total loss of at least $A10 million per annum for sheep producers in Victoria alone. In addition to the direct financial costs, crutching is an unpleasant task for many farmers, and animal welfare is also significantly compromised due to the increased likelihood of flystrike (Larsen *et al.*, 1995). These are less tangible but very real costs involved with severe dag. Flystrike is an increasingly pertinent issue in the Australian sheep industry due to the commitment of the industry to phase out mulesing by 2010 (Paull *et al.*, 2008).

2.4.2 Scouring and dag formation

Faeces in sheep ranges from firm pellets in healthy sheep to faeces formed into stools and pasty or watery diarrhoea, which indicates that the intestinal tract is not functioning properly, and leading to breech soiling (dags). Once some faecal material is adhered to the wool, further build up is increasingly likely as faeces easily stick to other faeces already on the wool (Waghorn *et al.*, 1999). This creates a warm, moist environment that provides ideal conditions for newly hatched blowfly larvae to feed and grow. This can result in dermatitis, with the resulting inflammation causing protein-rich exudates to appear at the site of infestation. These in turn attract further larvae (Colditz *et al.*, 2006). In addition to the increased risk of flystrike, the soiled wool must be removed before shearing and is sold at a heavily discounted price, resulting in direct financial penalties for producers.

Waghorn *et al.* (1999) reported that consistency of the faeces was the most important factor in determining whether or not dags formed, rather than the composition of faeces. Strong genetic correlations of 0.93 and 0.63 have been found between faecal consistency score and dag score in Merino sheep by Karlsson and Greeff (1996), and

Pollott *et al.* (2004). The dry matter of faeces is variable, with pellets able to be formed with a dry matter content as low as 23%, while faeces with a more fluid consistency may have a dry matter content as high as 32% (Waghorn *et al.*, 1999). While there is the potential for some overlap between faecal consistency scores and faecal dry matter, there is generally a strong negative correlation between faecal dry matter and faecal consistency score (Larsen, 1997, Le Jambre *et al.*, 2007) i.e. the fluid faeces that causes dag formation is mostly a result of faeces with a lower dry matter percentage. The conformation of the breech is also an important factor, with sheep that naturally have less wool around the breech significantly less prone to dag formation (Scobie *et al.*, 2008).

The proportion of undigested feed, endogenous secretions and water in faeces are determined by a combination of food type and time of transit through the GI tract, in particular the caecum and centrifugal coils of the colon and rectum (Van Soest, 1994). In sheep, hard faecal pellets are normally formed through the regular contractions and associated water absorption in the spiral colon (Ruckebusch and Fioramonti, 1980).

Water absorption in the colon depends on an osmotic gradient driven by the increased concentration of ions in the surrounding epithelial tissue compared to the digesta. The establishment of this gradient is compromised when the rate of digesta flow through the colon is increased, which may be a result of local inflammation. Alternatively, increased water in the faeces may be a result of increased ion and volatile fatty acid (VFA) concentrations in digesta flowing through the colon, resulting in less osmotic pressure and less capacity for water absorption (Wesselink *et al.*, 1995). This occurs when rumen retention times are low and/or digesta flow rates are increased in the abomasum and small intestine, resulting in decreased VFA absorption in the rumen and nutrient absorption in the small intestine. This can be a result of increased food intake or different food types. It can also be a result of immune responses towards parasites resulting in altered smooth muscle contraction and increased peristalsis (Khan and Collins, 2004).

2.4.3 Causes of Scouring

Lush Green Pasture

As scouring is normally observed after the break of season in a Mediterranean environment, when lush green pasture becomes available after a hot and dry summer, it has been proposed that increased water content or some other component of green

pasture is responsible for the onset of diarrhoea. Watts *et al.* (1978) reported that although anthelmintic treatment reduced the prevalence of scouring lambs by up to 32%, certain components of lush pasture were still likely to be a contributing factor to the condition.

Glastonbury (1990) investigated the aetiology of scouring in weaner sheep grazing lush pastures and noted that there were no obvious signs of ill-health in affected sheep, and at post-mortem the only obvious anomaly was an increased volume of liquid digesta in the rumen, caecum and colon. Therefore, it was concluded that the scouring was due to the increased water and soluble carbohydrate content of lush pasture, resulting in a more rapid flow of digesta through the gastro-intestinal tract. Consequently, the high carbohydrate concentration of digesta in the colon and caecum resulted in decreased water absorption due to osmotic potential, with diarrhoea as a result.

Despite the findings of Glastonbury, there is strong evidence against the increased soluble carbohydrate content of green pasture being a causal agent of scouring. Larsen (1997), in a detailed review, concluded that increased levels of soluble carbohydrates in pasture were unlikely to be involved in the aetiology of scouring. This is because the prevalence of scouring is at its highest in winter when the soluble carbohydrate content of pasture is at its lowest, as found by Walsh and Birrell (1987) (4-6% w/w DM in winter, compared to 10-13% in late spring).

Increased water consumption from pasture is also unlikely to be a major factor in the onset of scouring. Even in mid-winter when the dry matter content of pasture is at its lowest, sheep are unlikely to ingest more than their normal *ad libitum* intake (Larsen, 1997). Suttle and Field (1967) have investigated the effects of intra-ruminal infusion of water on the concentrations of water and minerals in serum and faeces. While these authors did not monitor faecal consistency, they found only a small increase in faecal output of water (0.11 litres/day). The majority of excess water intake was lost in urine and through respiration and perspiration. Therefore, any excess water ingested from lush green pastures during winter and spring is most likely excreted as urine and is not responsible for diarrhoea.

One aspect of green pasture that may contribute to scouring is a low level of neutral detergent fibre, which can impair rumen function (Campion and Leek, 1997). Davidson *et al.* (2006) found that sheep grazing green pastures that were supplemented with fibre had significantly higher faecal dry matter and less dags than sheep grazing only pasture.

Interestingly, the group supplemented with fibre had a significantly higher WEC than the control group, although this may possibly be due to a dilution effect of the increased faecal moisture in the control group.

Capeweed

Capeweed (*Arctotheca calendula*) is a common pasture species in Western Australia during winter and spring. The presence of capeweed following the break of season is often thought to cause scouring.

Capeweed has high levels of calcium, magnesium, sodium and potassium compared to ryegrass and clover, however the concentrations of these minerals are at their highest during summer/early autumn, when scouring is not usually a problem (Walsh and Birrell, 1987).

Pethick and Chapman (1991) investigated the effect of feeding capeweed to sheep in a controlled animal-house environment. Two-year-old Merino wethers were offered 6 kg/day of fresh capeweed for seven days. There were no indications of diarrhoea, or any abnormal levels of liquid digesta in the caecum and colon at post-mortem. Therefore, it is unlikely that capeweed is a major cause of scouring in adult sheep.

Other possible causes of scouring

Increased levels of sodium, potassium, calcium and magnesium in pasture may play a role in scouring as they are essential in maintaining the electrolyte and acid-base balance in the body. It could be hypothesised that alterations in the mineral composition in pasture at different times of the year alter the osmotic potential of the tissue and fluids surrounding the GI tract lining, resulting in decreased water absorption. However, work by Walsh and Birrell (1987) suggests that there is little seasonal variation in the concentrations of these minerals in ryegrass and subterranean clover, the predominant pasture species in southern Australia. Given the low prevalence of scouring during summer it appears then that there is little relationship between mineral intake from pasture and scouring. Further evidence of this is the work of Waghorn *et al.* (1994) who reported that increasing dietary sodium to 0.51% DM (compared with a nutritional requirement of 0.1% DM) increased urine volume but had no effect on faecal dry matter or faecal water output.

Fungal endophytes (*Acremonium lolii*) affect perennial ryegrass (*Lolium perenne*). Perennial ryegrass is not a major pasture in Western Australia, however it is becoming

more common as farmers attempt to provide animals with feed over the dry summer months. It has been reported that sheep grazing ryegrass infested with *Acremonium lolii* are more prone to scouring and flystrike (Leathwick and Atkinson, 1995). The mechanisms whereby *Acremonium lolii* might lead to scouring are not clear. It has been postulated that fungal endophytes may alter gastrointestinal function by changing the balance of local hormone actions, resulting in decreased gastrointestinal tract transit time (Wesselink *et al.*, 1995).

It is unlikely that perennial ryegrass endophytes play a significant role in scouring in adult sheep in Western Australia as scouring is a widespread problem despite the fact that perennial ryegrass is not common in non-irrigated agricultural areas. In addition, Larsen (1997) noted that in south-western Victoria the main manifestation of PRG endophyte infestation, ryegrass staggers, occurs in Autumn while scouring is usually observed in winter and early spring. This suggests that there is no temporal relationship between scouring and perennial ryegrass endophytes.

Grazing sheep, particularly lambs and weaners, are exposed to a variety of protozoa and bacteria infestations that may lead to diarrhoea. Protozoal infection due to infestation with coccidia (*Eimeria* spp), *Cryptosporidium* and *Giardia* are common in young lambs (less than three months of age) but rare in older animals. Diarrhoea is a clinical sign of infection due to villous atrophy and leakage of enterocyte junctions. As sheep mature they develop strong immunity to these protozoa and as such outbreaks in adult sheep are extremely rare (de Graaf *et al.*, 1999). Similarly, diarrhoea caused by bacterial infections such as salmonellosis and yersiniosis is very rare in mature sheep in Australia (Jacobson, 2006). Therefore, these conditions are not responsible for the majority of scouring observed in grazing sheep.

Nematode infection

It is widely recognised that in areas with a winter rainfall climate, scouring in adult sheep is primarily caused by the ingestion of infective trichostrongylid larvae from green pasture (Jacobson, 2006). It has long been recognised that strategic use of anthelmintics will reduce the severity of scouring in sheep (Morley *et al.*, 1976, Watts *et al.*, 1978, Allerton *et al.*, 1998). Larsen *et al.* (1994) reported that adult sheep that were not dosed with a slow-release anthelmintic capsule were 12-16 times more likely to develop severe dag than sheep that received a capsule. Likewise, Gogolewski *et al.* (1997) also found that treatment with anthelmintic capsules greatly reduced the severity

of scouring in grazing sheep. While slow-release capsules appear to be highly effective at controlling scouring, suppressive anthelmintic treatment in the form of regular drenching is less effective (Pullman *et al.*, 1991). Therefore, it would appear that scouring in adult sheep can mainly be attributed to the presence of third and fourth stage larvae (Larsen *et al.*, 1999).

As sheep mature they acquire strong resistance to internal parasites and are generally immune by 12 months of age (Stear *et al.*, 1999c). This immunity can break down in times of stress or low nutrition. In ewes, the peri-parturient relaxation in immunity in late gestation and lactation is also recognised as a period where the host is significantly more susceptible to parasite infection. This is thought to be due to a re-partitioning of nutrients towards the growing fetus and to the mammary gland, giving a diminished immune response (Kahn, 2003). As a consequence, greater numbers of incoming larvae are able to establish and the egg production of adult females is increased due to a relaxation of the immunological suppression of female fecundity (Barger, 1993).

Scouring in lambs and in adults with a weak immune response is generally thought to be caused by a high worm burden leading directly to damage of the abomasum and intestines and consequently diarrhoea. Infection with abomasal parasites (*Teladorsagia* spp) can lead to a stretching of the abomasal glands and a reduction in acid-secreting cells as the nematodes colonise the abomasal mucosa (Fox, 1997). Heavy infection with intestinal parasites such as *T. colubriformis* and *T. vitrinus* leads to severe villous atrophy, decreased villous: crypt ratios and goblet cell hyperplasia (Pullman *et al.*, 1989) as well as the sloughing of enterocytes into the intestinal lumen due to increased turnover of the GI mucosa (Holmes, 1985).

Despite the physical damage caused by parasites colonising the GI tract, malabsorption is not normally a consequence of parasite infection in sheep due to the compensatory absorptive ability of the lower gastrointestinal tract (Poppi *et al.*, 1986, Bown *et al.*, 1991, Coop and Holmes, 1996, Coop and Sykes, 2002, Sykes and Greer, 2003). Therefore, it seems unlikely that scouring in adult sheep is due to reduced nutrient absorption caused by high numbers of worms in the abomasal and small intestine mucosa. As a result, sheep that are more resistant to parasites and consequently have lower worm burdens and WEC are still likely to be prone to scouring.

Larsen *et al.* (1994) found no differences in worm numbers between sheep with severe dag and sheep with little or no dag, suggesting that the magnitude of the worm burden

was unrelated to the severity of scouring. However, it was also noted in this study that the ingestion of parasite larvae was a necessary cause for the onset of scouring. Therefore, the most plausible explanation was that scouring was due to some form of hypersensitive or allergic-type immune response to ingested third and fourth stage larvae. Consistent with this hypothesis, higher numbers of tissue eosinophils in the fundus, jejunum and ileum and altered lymphocyte populations were observed in sheep with severe dag compared to unaffected sheep (Larsen *et al.*, 1999).

Therefore, it appears that in adult sheep it is the immune response of the host that is most responsible for scouring and not the pathophysiological effects of the parasite.

2.5 Immunobiology of nematode infection in sheep

In this section, I will describe how sheep mount an immune response to GIN and the cellular and humoral mechanisms involved. The possibility that this immune response can lead to scouring is also discussed.

2.5.1 Acquisition of immunity

Resistance to nematode parasites in sheep is not innate and is acquired at 6-12 months of age, after being exposed to infective larvae on pasture (Stear *et al.*, 1999c). Unlike results from murine studies, where the host's first ('primary') infections are expelled rapidly, repeated challenge with larvae is needed for the expulsion of a primary adult nematode population in ruminants (Balic *et al.*, 2000). Barger *et al.* (1985) found that adult nematodes began to be expelled by lambs after six weeks of a primary infection of *H. contortus,* consisting of a weekly dose of 2400 or 4800 L_3. However at lower rates of weekly infection (600 or 1200 L_3) there was no expulsion of adults, suggesting that a threshold of immunological stimulation needs to be reached before rejection of adult nematodes will take place. Similarly, Dobson *et al.* (1990c) found that lambs began to expel adult *T. colubriformis* after 7 weeks of primary infection at dose rates of 2000, 1124 or 632 L_3/week, but this process was delayed by about 5 weeks at dose rates of 200 L_3/week. The acquisition of immunity depends on the age of the sheep as well as the level of larval challenge. Dobson *et al.* (1990b) found no difference in resistance to *T. colubriformis* in lambs either raised indoors, or on pastures contaminated with nematode larvae up until 20 weeks of age. After 20 weeks of age sheep exposed to larvae on pasture had an increased ability to resist infection than sheep raised in worm-free conditions. Repeated infection with larvae is also required for resistance to *T. circumcincta* (Seaton *et al.*, 1989). The exception is *Nematodirus* species such as *N.*

battus, where sheep are able to expel the vast majority (>80%) of an adult worm burden derived from a primary L_3 challenge within three weeks (Winter *et al.*, 1997).

Differences in resistance to larval and adult stages

While immunity to the larval stages of *T. circumcincta* seems to appear concurrently with resistance to the adult stages (Seaton *et al.*, 1989), immunity to the larval stages is established more rapidly than to adults in *H. contortus* (Barger *et al.*, 1985) and *T. colubriformis* (Dobson *et al.*, 1990c). This suggests that there are stage-specific antigens that elicit independent immune responses from the host. Emery *et al.* (1992a) found that sheep immunised with repeated infections of *T. colubriformis* L_3 which were allowed to develop to adults, were highly immune to a challenge of *T. colubriformis* adults that were surgically implanted into the duodenum. However this immunity towards adult *T. colubriformis* was significantly reduced when the immunising infections were terminated after seven or ten days, indicating that adult worms produce specific antigens not produced by larval stages. Further work by these authors showed that sheep immunised by surgical implantation of adult *T. colubriformis* were then able to reject a challenge with larvae ten days later, but not for 7-10 days after the challenge was given, indicating that the immune response was stimulated by antigens associated with adult nematodes (Emery *et al.*, 1992b). It is well known that the larval stage of *T. colubriformis* can elicit an immune response in immunised sheep despite the absence of adult worms, and the challenge can be rejected by the host in a matter of hours (McClure *et al.*, 1992, Wagland *et al.*, 1996, Harrison *et al.*, 1999).

Differences in resistance to different species

Despite the evidence of a specific, antigen-related immune response, the effector mechanism(s) appear to be non-selective to some degree. Worms from different species are also expelled when they reside in the same location as the species that immunity has been developed against. This was demonstrated by Emery *et al.* (1993b) who reported that sheep had significant resistance to the intestinal parasites *T. colubriformis* and *N. spathiger* after being immunised with repeated infections of *T. colubriformis* L_3. This heterologous resistance did not apply to the abomasal parasites *T. circumcincta* and *H. contortus.* It is apparent that non-specific immune mechanisms operate towards species that reside in the same location in the gut or further downstream than the target species. Therefore, sheep that are immune only to abomasal parasites may also have a degree of immunity to intestinal parasites, but not the reverse. This was demonstrated by Stewart

(1955), who reported that sheep that were immune to *H. contortus* were also able to expel *T. colubriformis* during a concurrent infection. However, sheep immune only to *T. colubriformis* were not able to expel an infection with the abomasal parasite. Similarly, Dobson *et al.* (1992) found that immunising sheep with *T. circumcincta* also conferred some protection towards *T. colubriformis.* In contrast, sheep immunised with *T. colubriformis* have no protection to infection with *H. contortus* or *T. circumcincta* (Barnes and Dobson, 1993).

The immunity conferred in the small intestine by exposure to *T. circumcincta* suggests that the immune response in adult sheep may be greater in mixed infections. This is due to the immune response in the small intestine being a product of the local, specific immune response as well as some non-specific immunity generated higher up in the gut. Therefore, the pathology of worm infection may be more severe in mixed infections. If clinical signs such as scouring are related to immunopathology, then they would be expected to be more pronounced when there is a heightened immune response.

In summary, sheep acquire good resistance to nematodes once they reach 6-12 months of age and have been exposed to infective larvae on pasture. Different larval stages and adult worms produce specific antigens that elicit independent immune mechanisms from the host. Immunity is specific to different species of nematodes, but immune mechanisms can act non-selectively against parasites that reside either in the same location or downstream in the gut.

2.5.2 Manifestations of immunity

Hypobiosis (arrested development)

It is well known that the abomasal parasites *H. contortus* and *T. circumcincta* can arrest their development at the fourth larval stage (hypobiosis), reducing their metabolism and remaining buried in the mucosa of the abomasum (Eysker, 1993). The reasons leading to hypobiosis are not clear, but have been broadly grouped into two categories – arrested development due to environmental factors and inhibited development due to the immune response of the host (Gibbs, 1986). The former category refers to an evolutionary adaptation whereby the parasite arrests its development until seasonal conditions are at an optimum for egg hatching and subsequent larval survival (Eysker, 1993). In winter rainfall areas, this means that *T. circumcincta* larvae can remain at the fourth larval stage in the abomasum during the dry summer, undetected by the host.

They then resume development at the onset of winter rain and begin laying eggs once again.

There is also considerable evidence that hypobiosis is an immunologically-mediated phenomenon. Dunsmore (1961) first noted that suppressing the host's immune response with corticosteroids decreased the proportion of arrested larvae. Smith (2007) obtained a similar result, with significantly lower percentages (14% vs. 0.001%) of inhibited *T. circumcincta* fourth stage larvae (L_4) in sheep treated with corticosteroids compared to untreated sheep, 23 days after infection. Interestingly, Smith (2007) noted that the percentage of inhibited L_4 in untreated sheep was much higher (88%) ten days after infection, indicating that under the conditions of his experiment inhibited development was a short-lived phenomenon. Stear *et al.* (1995) reported that numbers of arrested *T. circumcincta* L_4 were associated with high levels of IgA to fourth stage larvae, providing direct evidence for the involvement of the immune response with hypobiosis. Halliday *et al.* (2007) investigated the kinetics of IgA production in sheep infected with *T. circumcincta* in a study that involved cannulation of the gastric lymph duct draining the abomasum, enabling sequential measurements of the local IgA response. They found that the peak IgA response occurred 10 days after an infection with a single dose of 50,000 *T. circumcincta* L_3, whereas evidence of inhibited larval development was apparent from day five. Therefore it was concluded that IgA was probably not the main immune mechanism inhibiting larval development. Despite this, previously infected (immune) sheep had higher percentages of inhibited L_4 than parasite-naïve sheep, so there was still clear evidence for an immunologically-mediated inhibition.

Stear *et al.* (1995) also found a positive correlation between the number of arrested larvae and adult worm burden. It has been proposed that the inhibited larvae may act as a reservoir to replace adult worms lost from the host (Balic *et al.*, 2000). In other words, the higher the number of adults the more incoming larvae will arrest their development at the larval stage. Michel (1963) observed that the maintenance of an adult worm burden of *O. ostertagi* in calves was the result of resumed development of inhibited larvae, rather than newly ingested larvae. In immune sheep in the field, hypobiosis is probably due to a combination of all three of these factors, namely the host immune response, and adaptations of the parasite population due to seasonal conditions and density-dependent population regulations.

Reduced fecundity of adult females

Like hypobiosis, changes in fecundity in adult female nematodes can be explained by both host immune responses and adaptations by the parasite population. It is clear that nematode parasite populations in sheep are governed by density-dependent regulation mechanisms (Barger, 1987, Wakelin, 1987, Dobson *et al.*, 1990a, Stear *et al.*, 1999b). That is, egg production is decreased when there are a large number of adults. Stear *et al.* (1999b) have estimated that in *T. circumcincta,* egg production increases up to a population of about 5000 adult nematodes after which it decreases rapidly. From an evolutionary point of view, it is logical for parasites to reduce their fecundity as their population increases because there are only finite resources within the host.

Dobson *et al.* (1990a) have speculated that this reduction in fecundity may be due to an acquired host immune response and that a threshold of immunological stimulation must first be reached. This would explain why a large worm burden must be present before fecundity is affected. Consistent with this, Bisset *et al.* (1996) found significantly lower eggs per adult female in genetically-resistant Romney sheep infected with *T. colubriformis*, compared to susceptible sheep. Resistant sheep also had significantly lower total worm burdens and higher levels of parasite-specific IgG_1 and IgM, suggesting that fecundity is regulated by the host's immune response. Similarly, Gruner *et al.* (2004) found negative correlations between worm fecundity and total worm burden (-0.6 for *T. circumcincta*; -0.29 for *T. colubriformis*) in a study involving genetically resistant and susceptible hosts. Resistant sheep had lower worm burdens than susceptible sheep and fewer eggs per adult female for *T. circumcincta,* but not for *T. colubriformis*, indicating the complex interaction of mixed-species infections.

How the immune system acts to reduce the fecundity of adult females is not clear, but Stear *et al.* (1995) have noted a significant association between the length of adult female *T. circumcincta* (which is then strongly correlated with worm fecundity) and local IgA activity against *T. circumcincta* L_4. This finding suggests reduced fecundity in adults can be linked to damage to the immature larval stages. Emery *et al.* (1992b) have also noticed damaged cuticles and loss of eggs in adult *T. colubriformis* transplanted directly into immune hosts. Therefore, it appears the host's immune response can reduce the fecundity of nematodes at all stages of their life-cycle.

Failure of larvae to establish

In ruminants, the major feature of acquired immunity to nematodes is the ability to prevent newly ingested larvae from establishing (Balic *et al.*, 2000). Multiple larval infections are needed to establish this ability, which has been termed 'rapid expulsion' (Miller, 1984). It was first noted in work with guinea pigs that larval infections given to immune hosts could be expelled within a matter of hours (Rothwell, 1989). Russell and Castro (1979) reported that 81% of a *Trichinella spiralis* larval infection given to immune rats was expelled within just fifteen minutes. The remainder of the larvae were embedded in the mucosal layer of the duodenum and expelled after 4-8 days. Similar mechanisms have been shown to operate in sheep. As sheep become immune to challenge with *T. colubriformis*, increasing numbers of L_3 can be observed in the faeces (Chiejina and Sewell, 1974). McClure *et al.* (1992) found that immune sheep challenged with 30,000 *T. colubriformis* L_3 expelled most of that larvae within 24 hours and the remainder of the challenge was rejected within 14 days. Subsequently, Wagland *et al.* (1996) reported that immune sheep could expel a challenge of 30,000 *T. colubriformis* L_3 within two hours. This was confirmed by Harrison *et al.* (1999), who found that immune sheep challenged with 40,000 *T. colubriformis* L_3 could expel the majority, or in several cases the entire challenge within two hours. Rapid expulsion of larvae also occurs in sheep infected with *T. circumcincta,* although the kinetics may be somewhat different than with expulsion of *T. colubriformis.* Seaton *et al.* (1989) reported that sheep immunised with twelve weeks of continuous *T. circumcincta* infection were able to expel effectively further challenges with larvae, as opposed to sheep immunised with only four or eight weeks of infection, which still did not have the ability to expel incoming larvae. However, as sheep were not necropsied until thirteen days after challenge it was not possible to determine how rapidly incoming larvae were rejected. Balic *et al.* (2003) found that immune sheep infected with *T. circumcincta* L_3 still had significant numbers of larvae present in abomasal tissue three days after challenge, but the majority of larvae were rejected within five days of challenge and no larvae were recovered after ten days. However, *In vitro* experiments have shown that rapid expulsion of *T. circumcincta* larvae can take place within a time frame similar to *T. colubriformis.* Jackson *et al.* (2004) incubated exsheathed *T. circumcincta* L_3 with strips of abomasum tissue from freshly necropsied sheep. They found that in immune sheep 95% of the larvae failed to adhere to the tissue within three hours of incubation, whereas in parasite-naïve sheep 80% of the larvae were found within the tissue after the same time-period.

The rapid nature of these responses suggests that immediate hypersensitive-type immune responses are a feature of parasite-resistant sheep. In humans, hypersensitivity reactions in the gut can lead to diarrhoea (Farthing, 2003). Therefore, the rejection of larvae is likely to be the prime cause of immune-mediated scouring. It is not clear why some sheep that have low WEC do not scour. It is possible that they have a low WEC due to their major immune response being suppression of female worm fecundity, or inhibition of the fourth larval stage. These responses will lead to reduced WEC, but as worms are not actually being rejected, diarrhoea may not be a consequence. This question will be addressed in this thesis.

2.5.3 Mechanisms involved in immunity

Lymphocytes

Infection with nematode parasites stimulates antigens in the immune system, whereby antigenic fragments are bound to major histocompatibility complex (MHC) proteins on the surface of antigen-presenting-cells (APC) such as macrophages and dendritic cells. These antigen-MHC complexes are then presented to T-cells (Acheson and Luccioli, 2004). T-cells can be classed as T-helper (T_H) or cytotoxic (T_C) cells. T_H cells secrete cytokines and stimulate the production of antibody-secreting plasma cells (B cells), while T_C cells are directly involved in the phagocytosis of tumours and virally infected cells (Figure 2.1). Immunity to nematode parasites involves the production of specific immunoglobulins and granulocytes (Acheson and Luccioli, 2004). As a result, the production of T_H cells and B cells is the primary lymphocyte response in parasite-infected sheep.

Smith *et al.* (1984) reported an increase in lymphocytes in gastric lymph of sheep within 24 hours of challenge with 50,000 *T. circumcincta* L_3. This response peaked at 5 days after infection and was followed by a peak in IgA-producing plasma cells, indicating large numbers of T_H lymphocytes had been produced. Pfeffer *et al.* (1996) found that sheep infected with *T. axei* had an increased T_H/T_C T-cell ratio within seven days of the start of infection, and this ratio was maintained with repeated infections. This indicates that nematode parasites stimulate specific antibody production by the immune system, rather than a generalised phagocytosis. Shen *et al.* (2000) have also noted a significant increase in T_H cells in immune sheep following challenge with *T. colubriformis.* Gill *et al.* (1993) have reported that depletion of T_H cells reduces the resistance of sheep to *H.*

contortus. Conversely, depletion of T_C cells through treatment with specific mAbs enhances the resistance of sheep to *T. colubriformis* (McClure *et al.*, 1995).

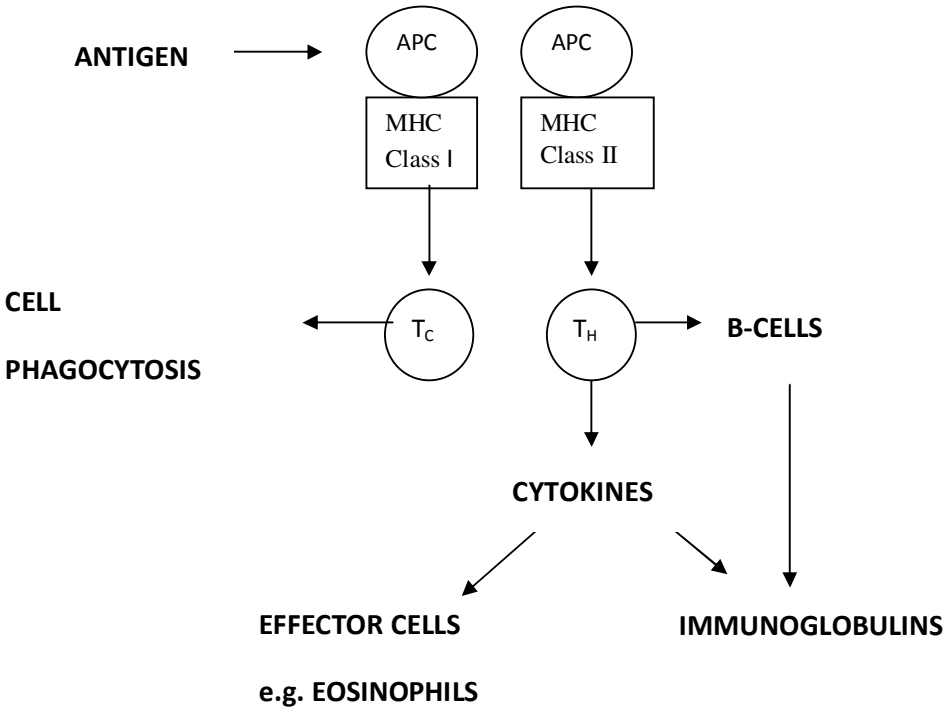

Figure 2.1 Schematic diagram of lymphocyte response to antigens. Antigens bind to either class I or class II MHC molecules on the surface of antigen presenting cells (APC). Class I molecules stimulate production of cytotoxic T-cells (T_C) and class II molecules stimulate production of T-helper cells (T_H). The primary lymphocyte response in nematode parasite infection in sheep is a T_H response.

There seems to be little lymphocyte infiltration into gastric lymph or intestinal tissue when incoming larvae are expelled before reaching their tissue niche, i.e. rapid expulsion (McClure *et al.*, 1992, Balic *et al.*, 2002). Larsen *et al.* (1999) reported that significantly increased T_H/T_C T-cell ratios in tissue sections from the pylorus and upper jejunum were a feature of adult sheep with high-dag scores. Therefore, it could be postulated that immune-mediated scouring only occurs when larvae reach their tissue niche, and is not a consequence of rapid expulsion.

Cytokines

The nature of resistance to infectious diseases is characterised by the pattern of cytokine gene expression produced during infection. Cytokines are a set of small, signalling proteins similar to hormones and neurotransmitters. They have autocrine, paracrine and endocrine effects and are responsible for recruiting and activating cells involved in the adaptive immune response (Finkelman *et al.*, 1997). Cytokines are secreted by T_H cells. Generally, infectious agents stimulate a T_H cell response that can be grouped into two categories based on the pattern of cytokine gene expression, Th1 or Th2. Cytokines produced by the Th1 response include Interleukin (IL)-1, IL-2, tumour necrosis factor (TNF-∝) and interferon-gamma (INF-γ), while cytokines produced by the Th2 response include IL-4, IL-5, IL-10 and IL-13 (Finkelman *et al.*, 1997). Intracellular pathogens such as bacteria and viruses normally stimulate a Th1 response, while nematode parasites stimulate a Th2 response (Miller and Horohov, 2006). The cytokines associated with Th2 responses are associated with allergic immune responses typically seen in disorders such as asthma and food allergy. Characteristics of this response include fluid secretion, smooth muscle contraction and production of granulocytes (Artis, 2006).

The pattern of cytokine expression in sheep infected with GIN has been the subject of several recent studies that indicate a strong Th2 immune response, although Th1 cytokines are also upregulated. McClure *et al.* (1995) reported that treating sheep with monoclonal antibodies to ovine INF-γ significantly enhanced their resistance to challenge with *T. colubriformis*. It was postulated that this was due to Th2 immune responses being favoured by the absence of INF-γ production. Gill *et al.* (2000) found that sheep genetically resistant to *H. contortus* had increased production of IL-5 and decreased production of INF-γ in both abomasal and mesenteric lymph node lymphocytes. Consistent with these observations, Pernthaner *et al.* (2005a) reported that parasite-resistant Romney sheep had higher levels of IL-5 and IL-13 mRNA expression compared to susceptible sheep following challenge with *T. colubriformis.* However in this study TNF-∝ mRNA expression was also increased in resistant sheep, indicating that the Th1/Th2 dichotomy is not absolute, and there was no difference in levels of IL-4, IL-10 and INF-γ mRNA expression. Further studies by this group of researchers indicated that genes coding IL-5 and IL-13 were strongly upregulated in resistant sheep, particularly following repeated infections of *T. colubriformis* (Pernthaner *et al.*, 2006). Genes coding for INF-γ and TNF-∝ were upregulated to a

lesser extent and mainly in primary infections, indicating that there is a shift towards Th2 responses as sheep build up acquired immunity to GIN. Consistent with this, Meeusen *et al.* (2005) noted that there was no difference in cytokine mRNA expression in sheep challenged with a primary *H. contortus* infection, but in immune sheep IL-4, IL-5 and IL-13 mRNA expression was strongly upregulated. In this study INF-γ gene expression was also increased in immunised sheep. Craig *et al.* (2007) have investigated cytokine gene expression in naïve or immune sheep challenged with *T. circumcincta* and found higher expression of IL-4, IL-5, IL-6, IL-10 and IL-13 mRNA in immune sheep, with no difference in expression of IL-2 and INF-γ mRNA. So it appears that the pattern of cytokine expression in sheep responding to GIN challenge is strongly skewed towards a Th2 response.

Antibodies

Parasite infection in sheep leads to a marked increase in antibody production. This is true systemically with parasite-specific antibody titres increased in efferent lymph (Smith *et al.*, 1984) and serum (Shaw *et al.*, 1998, Sykes *et al.*, 2007) as well as locally with increased production of antibodies in tissue and mucus at the site of infection (McClure *et al.*, 1992, Pfeffer *et al.*, 1996)

IgA

The most intensively investigated antibody isotype in sheep has been IgA. IgA is produced in the Peyer's patches in the ileum and possibly also in the lamina propria throughout the small intestine (Miller, 1996b). It is the dominant isotype at the mucosal surface in mammals and as such would be expected to play a major role in immunity towards nematodes that colonise the mucosa. Increased levels of parasite-specific IgA have been detected in tissue (Stear *et al.*, 1995) mucus (Sinski *et al.*, 1995) and serum (Douch *et al.*, 1994) of parasitised sheep. The function of IgA is not certain but it may play several roles. First, there is a strong negative correlation between worm length and IgA levels in gastric lymph of sheep infected with *T. circumcincta,* and it has been postulated that mucosal IgA interferes with the worms' feeding behaviour (Smith, 1988). Fecundity is strongly associated with worm length (Stear *et al.*, 1999c) and so IgA also acts to regulate the egg laying potential of adult females. As discussed previously, IgA has also been implicated in host-mediated arrested development of *T. circumcincta* L_4. Henderson and Stear (2006) reported that the kinetics of IgA production and numbers of circulating eosinophils was remarkably similar in lambs

infected with *T. circumcincta*, suggesting that IgA interacts with eosinophils to facilitate worm rejection. Beraldi *et al.* (2008) noted a significant negative correlation between serum concentrations of parasite-specific IgA and worm burdens in *T. circumcincta*-infected lambs. This shows that IgA plays a significant role in worm rejection.

IgG

There has been less research into the role that IgG plays in parasitised sheep, although levels of parasite-specific IgG in serum are significantly increased following challenge with *H. contortus* (Gill *et al.*, 1993, Schallig *et al.*, 1995), *T. circumcincta* (Sinski *et al.*, 1995) and *T. colubriformis* (Sutherland *et al.*, 1999). Several antigens from *T. colubriformis* L_3 are recognised by IgG from immune sheep (Kiel *et al.*, 2007, Harrison *et al.*, 2008). Results from human medical research indicate that IgG plays a significant role in Th2-mediated allergic immune responses. Hanson *et al.* (1998) reported that rats allergic to ovalbumin had high levels of IgG in serum, as well as high numbers of eosinophils and mast cells in intestinal tissue and often developed diarrhoea. Mast cells, which are commonly implicated in allergic and hypersensitive immune responses, have a high-affinity IgG receptor that can lead to mast cell degranulation with appropriate antigenic stimulation (Tkaczyk *et al.*, 2001). It is interesting to note that most parasite-specific IgG measured in sheep is IgG_1 rather than IgG_2 (McClure, 2009, Beasley et al., 2010). This is consistent with a Th2, hypersensitive response being the major method of immunity in sheep as IgG_1 is associated with Th2 responses and IgG_2 is associated with Th1 responses (Kanai et al., 2007). Thus it appears that IgG may also play a major role in the pathogenesis of immune-mediated inflammation and scouring in sheep.

IgE

Results from early studies involving parasitic nematodes of rodents and man have demonstrated that elevation of IgE levels is a characteristic immune response to nematode infection (Jarrett and Miller, 1982). IgE is typically implicated in 'allergic' type immune response such as human asthma (Prussin and Metcalfe, 2006). The production of IgE in response to seemingly harmless dietary and environmental antigens has led to an association of IgE with atopic immune responses. However, IgE is thought to have evolved in mammals as a defence against parasites and, as such, IgE production plays a major role in parasite rejection, but can also lead to associated symptoms such as inflammation, itching, mucus secretion and diarrhoea (Sutton and

Gould, 1993). Even though the antigens associated with parasitic nematodes would not normally be considered allergenic, they are highly allergenic in the context of nematode infection because of the Th2 dominance of the immune response (McReynolds *et al.*, 1993). IgE production is stimulated by the Th2 cytokines IL-4 and IL-5 and downregulated by the production of INF-γ (Miller, 1996b). IgE is responsible for a large amount of the cell-mediated cytotoxicity against GIN. *In vitro*, IgE has been shown to stimulate eosinophils and mast cells through high-affinity Fcε receptors (Sutton and Gould, 1993) and is implicated in the mucosal permeability and epithelial cell secretion associated with immune responses towards GIN (Miller, 1996b). Increased motility of smooth muscle and intestinal fluidity, both mediated by IgE, have been associated with parasite expulsion in rodents (Baird and O'Malley, 1993). The production of IgE in immune sheep may therefore be a large contributor to the pathogenesis of immune-mediated scouring.

In calves infected with *O. ostertagi*, IgE levels in serum and lymph are inversely correlated with worm burdens during primary infections or acute challenges (Thatcher *et al.*, 1989) but are positively correlated with worm burdens during heavy infections (Baker and Gershwin, 1993). The conflicting results may be due to different infection regimes or increased sequestration of IgE by mast cells during heavy infections. mAbs to ovine IgE have recently become available and the results from numerous researchers suggest that IgE plays a major role in worm rejection in immune sheep. Parasite-specific IgE levels are increased in lymph from immune sheep following challenge with *T. circumcincta* L_3 (Huntley *et al.*, 1998), and increased levels of parasite-specific IgE in serum and increased numbers of IgE-bearing cells are present in the GI mucosa of immune sheep following challenge with *T. colubriformis* L_3 (Harrison *et al.*, 1999). There is also a clear trend for sheep bred for parasite resistance to have higher levels of IgE, following nematode challenge, than susceptible sheep. Bendixsen *et al.* (2004) and Pernthaner *et al.* (2005b) both found higher levels of parasite-specific IgE in gut tissue homogenates and lymph, respectively, from resistant lambs compared to susceptible lambs following challenge with *T. colubriformis* L_3. Pettit *et al.* (2005) found higher levels of IgE-bearing lymphocytes in resistant sheep naturally infected with *T. circumcincta* compared to susceptible sheep. In addition, Clarke *et al.* (2001) have reported a significant association between resistance to *T. colubriformis* and a polymorphism on the ovine IgE gene. Therefore, it is clear that increased production of IgE in sheep is associated with lower GIN numbers.

While IgE is an effective defence mechanism against GIN, the allergic-type signs it produces are likely to also have detrimental effects for the host. The role of IgE in immune-mediated pathology has been demonstrated in humans, where symptoms of asthma have been reduced by treatment with anti-IgE mAbs (Noga *et al.*, 2006). IgE is also implicated in immune-mediated diarrhoea in humans (Farthing, 2003) and piglets (Sun *et al.*, 2008). In sheep, serum IgE levels have been linked with faecal softening in following challenge with *T. colubriformis* L_3 (Shaw *et al.*, 1998). Shaw *et al.* (1999) have also noted a positive genetic correlation between serum IgE levels and dag score in grazing Romney sheep. Thus it appears likely that production of IgE and the associated inflammation and cell-mediated cytotoxicity may be largely responsible for immune-mediated scouring.

Effector cells

Immunity to nematode parasites relies on a combination of humoral and cellular responses (Meeusen, 1999). Increased production of antibodies, lymphocytes and cytokines are responsible for promoting the growth of granulocytes (neutrophils, eosinophils and mast cells) in the GI mucosa, which ultimately facilitate the removal of the parasites. Granulocytes are so called because of the presence of large granules in their cytoplasm. Upon activation by cytokines or antibody-antigen complexes, granulocytes undergo a process termed degranulation whereby the contents of these granules are released into the extracellular space surrounding the granulocyte. The contents of the granules include biologically active substances such as histamine, therefore they have potent pharmacological effects in surrounding tissue.

The most abundant granulocytes in blood and tissue are normally neutrophils. Neutrophils are involved in the acute phase of inflammation and normally migrate in large numbers into damaged tissue sites. Neutrophil increase rapidly in the mucosa following challenge with nematode L_3 (Meeusen, 1999) and there are some reports that worm burdens are negatively correlated with neutrophil numbers in serum (Rowe *et al.*, 2008). However, neutrophils have not been extensively studied in parasitised-sheep. The granulocytes most associated with Th2 immune responses are eosinophils and mast cells. They have been widely studied and play a major role in the immune response and immunopathology of nematode infection in sheep.

Eosinophils

Eosinophils are small granulocytes (12-15 μm in size) that are produced in the bone marrow before migrating to the bloodstream and tissue. They are closely associated with parasite infection and allergic and inflammatory conditions such as asthma and inflammatory bowel disorder (Prussin and Metcalfe, 2006, Rothenberg, 2004) Consistent with their role in allergy and hypersensitive immune response, eosinophil differentiation from myeloid precursor cells and subsequent activation is stimulated by the Th2 cytokines IL-3 and in particular IL-5 (Yamaguchi *et al.*, 1988, Balic *et al.*, 2000, Prussin and Metcalfe, 2006). Eosinophils possess high-affinity receptors for IgE, IgG and IgA, and binding of these antibodies to their respective receptor results in degranulation. The relative importance of each of these antibody sub-classes is currently under debate. Due to the association of eosinophils with allergy and parasite infection, it has been suggested that IgE plays a major role in eosinophil degranulation (Gounni *et al.*, 1994). However recent research in humans suggests that IgA is a more potent stimulator of eosinophil degranulation (Pleass *et al.*, 2007) and, in sheep, there is a strong correlation between IgA responses and numbers of circulating eosinophils following infection with *T. circumcincta* (Henderson and Stear, 2006).

Following degranulation, eosinophils release pre-formed and newly synthesised mediators. Pre-formed mediators are stored in the cytoplasmic granules of the cell and include major basic protein, eosinophil cationic protein, and eosinophil peroxidases and neurotoxins (Prussin and Metcalfe, 2006). These mediators have a range of pharmacological effects that include stimulating degranulation of other granulocytes, as well as smooth muscle contraction (Jones, 1993). These mediators all have toxic affects against GIN *in vitro* (Kilon and Nutman, 2004). Newly formed lipid mediators are generated by the metabolism of arachidonic acid, which is liberated from phospholipids in cell membranes that are disrupted during cell degranulation (Samuelsson, 1983, Shaw *et al.*, 1985). Two main classes of mediator are formed, the leukotrienes and prostaglandins (Figure 2.2). Both of these mediators are potent vasodilators and also induce contraction of nonvascular smooth muscle. Their role will be discussed more fully later in this section.

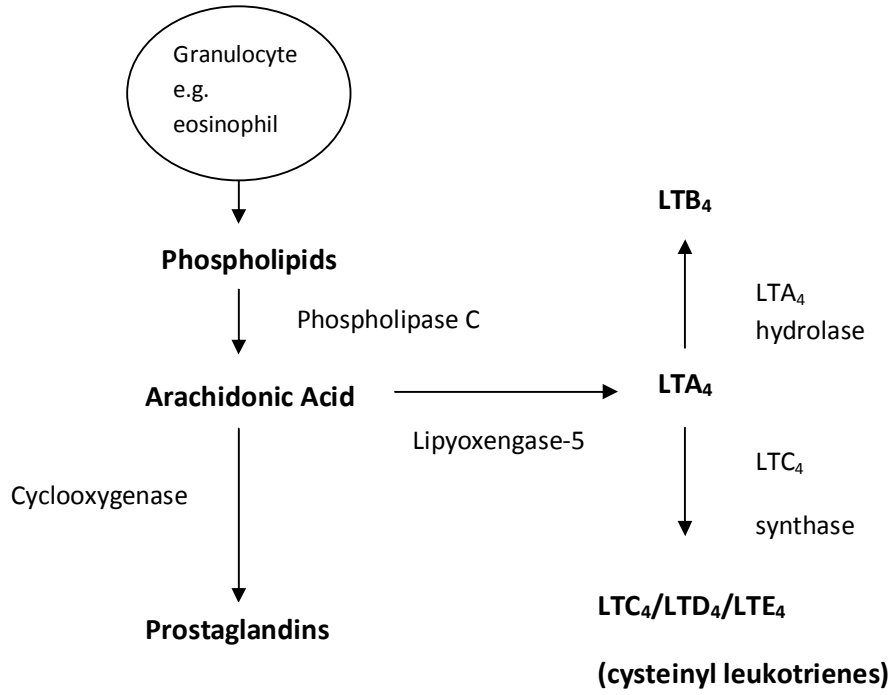

Figure 2.2 Formation of lipid mediators from granulocytes. After appropriate stimulation, the phospholipids in the cell membrane are disrupted to yield arachidonic acid. This is then metabolised by two independent pathways, catalysed by the enzymes lipyoxengase-5 and cyclooxygenase, to form either leukotrienes or prostaglandins.

While increased numbers of eosinophils are a characteristic feature of GIN infection, the precise role that they play is unclear and has been the subject of much debate. The close association of eosinophils with parasitic larvae in histological sections, together with the toxic properties of granule-derived mediators, has led a long-held view that eosinophils are intimately associated with killing extracellular parasites (Jacobsen *et al.*, 2007). Eosinophils are also accepted to be closely involved in the immunopathology that is associated with allergic conditions such as asthma (Venkataraman *et al.*, 2006). However, results from murine models suggested that eosinophils play little role in immunity to parasites. Sher *et al.* (1990) and Dent *et al.* (1997) have reported that eliminating production of eosinophils through treatment with anti-IL-5 mAbs had no effect on the ability of mice to resist an infection with *Schistosoma mansoni.* Thus it was hypothesised that eosinophils are not associated with immunity and have mainly detrimental effects on the host (Wardlaw *et al.*, 1995).

Eosinophil function is far more complex than originally thought. In addition to producing toxic mediators, it is now realised that eosinophils can regulate both Th1 and

Th2 immune responses by modulating T-cell functions and releasing a variety of cytokines including IL-4, IL-12 and IL-13 (Jacobsen *et al.*, 2007). Eosinophils are therefore able to stimulate the activation of other granulocytes such as mast cells. These regulatory features have shed new light on the role that eosinophils play in immunity, and it is now thought that eosinophils have a significant role in parasite rejection, while having some detrimental side effects for the host (Bruschi *et al.*, 2008).

Large numbers of eosinophils have consistently been a feature in the pathology of GIN infection in sheep (Rothwell, 1989) but, as in studies with rodents and humans, their precise role has been difficult to elucidate. Significant increases in numbers of eosinophils (Kimambo *et al.*, 1988) as well as cells expressing IL-5 mRNA (Bao *et al.*, 1996) have been detected in immune sheep compared to parasite-naïve controls. This demonstrates that eosinophils are a feature of acquired immunity, and there is now overwhelming evidence to suggest that in sheep they have a role in parasite rejection. Dawkins *et al.* (1989) and Buddle *et al.* (1992) have both noted increased peripheral eosinophilia in sheep bred for low worm egg counts. Doligalska *et al.* (1999) and Stear *et al.* (2002) have reported significant, negative correlations between circulating eosinophil numbers and WEC in sheep infected with *T. circumcincta*. Tissue eosinophil numbers and eosinophil potentiating activity are also associated with low worm egg counts (Douch *et al.*, 1986, Stevenson *et al.*, 1994, Bisset *et al.*, 1996). *In vitro,* eosinophils are associated with larval migration inhibition (LMI) activity in mucus (Douch *et al.*, 1984) and can kill *H. contortus* larvae in the presence of specific antibody for a larval surface antigen (Rainbird *et al.*, 1998).

The close association of eosinophils and diarrhoea in human studies (Bueno and Fioramonti, 2002) and the increased numbers of eosinophils in sheep with high-dag scores (Larsen *et al.*, 1994) suggests that eosinophils are intimately associated with immune-mediated scouring. Shaw *et al.* (1998) also noted a positive but non-significant correlation between eosinophils and faecal consistency score in sheep infected with *T. colubriformis.* Higher numbers of eosinophils in sheep bred for worm-resistance (Bisset *et al.*, 1996) may help to explain the higher dag scores observed in resistant sheep.

Mast cells / Globule leukocytes

Nematode infection in the GI tract is invariably accompanied by the production of mucosal mast cells and globule leukocytes (Rothwell, 1989, Miller, 1996a). Globule

leukocytes refer to mast cells that have undergone degranulation. Mucosal mast cells are recruited from the bone marrow by Th2 cytokines such as IL-4 (Miller, 1996a). Like eosinophils, mast cells can undergo degranulation to release potent-preformed mediators such as histamine, as well as newly-synthesised arachidonic acid metabolites (Wood, 2006). Mast cells possess a high affinity receptor for IgE, and are involved in the pathogenesis of many allergic type responses such as asthma, inflammatory bowel disease, food allergies and enteritis (Bradding *et al.*, 2006, Carroll *et al.*, 2002, Prussin and Metcalfe, 2006, Wood, 2006). The binding of antigen-specific IgE to mast cells is so strong it is essentially irreversible, so mast cells are normally coated in IgE. Antigenic stimulation of the membrane-bound IgE leads to cell degranulation. Therefore, the rapid expulsion of ingested nematode L_3 in immune sheep can be attributed mainly to an IgE-mast cell response in the gut mucosa. The function of mediators derived from mast cell granules, such as chymases and sheep mast cell proteinase, is to open up the tight junctions between enterocytes in the mucosal epithelium. This leads to increased leakage of macromolecules into the intestinal lumen (Scudamore *et al.*, 1995, Miller, 1996a). In addition, lipid mediators such as leukotrienes and prostaglandins can cause increased secretion of water and electrolytes and smooth muscle contraction. This micro-environment prevents incoming worm larvae from reaching their tissue niche, but may have side effects for the host in terms of increased leakage of plasma protein into the gut and possibly diarrhoea (Miller, 1996b).

The role of mast cells in the rejection of GIN from immune sheep has been extensively studied. Numbers of mast cells in the lamina propria are increased in immunised sheep following challenge with nematode L_3, indicating their role in acquired immunity (Stevenson *et al.*, 1994, Sykes *et al.*, 2007). However, simply quantifying numbers of mast cells at post-mortem examination can be misleading as this does not reflect their activation status (Balic *et al.*, 2000). More accurate measures of mast cell activity can be gained by quantifying the numbers of globule leukocytes and sheep mast cell proteinase, as these represent mast cells that have been degranulated by antigenic challenge. The products of mast cell degranulation are clearly involved in worm rejection, as evidenced by the negative correlations between globule leukocytes and worm burdens (Meeusen *et al.*, 2005, Douch *et al.*, 1986, Stankiewicz *et al.*, 1995, Sykes *et al.*, 2007). Numbers of globule leukocytes are also higher in genetically nematode-resistant sheep than susceptible sheep (Bisset *et al.*, 1996, Gruner *et al.*, 2004). It has been shown that immune sheep release sheep mast cell proteinase within two hours of L_3 challenge, but measurements of sheep mast cell proteinase have not

been sensitive enough to correlate with worm counts (Huntley *et al.*, 1987, Jones *et al.*, 1992). The rapid nature of mast-cell responses is emphasised by the work of Bendixsen *et al.* (1995) who reported that histamine was released by isolated mucosal mast cells from immune sheep within thirty minutes of stimulation with *T. colubriformis* antigen and leukotrienes were released within three hours.

Mast cells/globule leukocytes clearly play a major role in protective immunity against GIN in sheep but they also contribute to the pathogenesis of immune-mediated diarrhoea in pigs (Ahrens *et al.*, 2003, Sun *et al.*, 2008) and humans (Farthing, 2003, Wood, 2006). Therefore, it may be hypothesised that mast cells and the mediators they release can lead to immune-mediated scouring in sheep. However, Larsen *et al.* (1994) found no difference in mast cell or globule leukocyte numbers between sheep with high or low-dag scores. Scouring in immune sheep is associated with rejection of immature larval stages (Larsen *et al.*, 1999, Jacobson, 2006). Therefore, it would be expected that the immune mechanisms responsible are either rapid expulsion (larvae fail to reach the mucosa), or removal of developing larvae from the mucosa before they become adults.

Balic *et al.* (2003) and Meeusen *et al.* (2005) reported that when sheep rapidly expelled a larval challenge there was no infiltration of granulocytes into the lamina propria. McClure *et al.* (1992) noted that there were very few signs of immunopathology, e.g. villous atrophy when immune sheep rejected a larval challenge within two hours. Therefore, it seems that larvae need to penetrate the mucosa and develop to at least the fourth larval stage before immune-mediated scouring can occur. On this evidence, rapid expulsion, mediated by mast cells, is probably not the main cause of immune-mediated scouring. This is supported by the aforementioned observation that changed lymphocyte ratios area a feature of sheep with high-dag scores, and lymphocyte infiltration is normally observed when larvae reach the lamina propria.

However, the products of mast cell degranulation, such as histamine and prostaglandins, have not been determined in sheep with high and low-dag scores. It cannot be discounted that mediators released from mast cells during the rapid expulsion phase contribute to diarrhoea. This could be in the form of increased contraction of smooth muscle and peristalsis, which may not cause post-mortem signs of immunopathology such as villous atrophy. Indeed, McClure *et al.* (1992) noted a large infiltration of globule leukocytes into the mucosal epithelium in immune sheep within two hours of challenge with *T. colubriformis*, accompanied by leakage of parasite-specific IgG into intestinal fluid. These authors hypothesised that mediators released from mast cells

could be responsible for the leakage of antibodies by opening up blood vessels surrounding the intestinal wall. Therefore, mast cells and their associated mediators still may play a role in the pathogenesis of scouring, either in the rapid expulsion phase or later on when they are recruited into mucosal tissue.

Mediators

Histamine and serotonin

The biogenic amines, histamine and serotonin (5-hydroxytryptamine, 5-HT), are stored in the granules of mast cells in the GI tract and, as such, are the first mediators released during mast cell granulation. As mentioned above, histamine release is extremely rapid, with mast cells from immune sheep able to release histamine within thirty minutes of incubation with *T. colubriformis* antigen (Bendixsen *et al.*, 1995). Histamine acts on specific receptors in smooth muscle and intestinal cells and induces contraction of non-vascular smooth muscle, vasodilation of capillaries and separation of endothelial cells (Wood, 2006). Serotonin acts on G-protein coupled receptors in smooth muscle and endothelial cells to stimulate a second-messenger cascade, also resulting in vasodilation and fluid secretion (Hansen and Skadhauge, 1995). Histamine release is a characteristic of the pathology of immune-mediated diarrhoea and food allergies (Wood, 2006, Farthing, 2003, Sun *et al.*, 2008).

Exogenous injections of histamine into the skin of sheep results in plasma leakage within ten minutes (Colditz, 1991). This reaction has some genetic component, with sheep selected for resistance to flystrike having increased plasma leakage in response to histamine injection than genetically-susceptible sheep (Colditz *et al.*, 1992). Douch *et al.* (1984) showed that histamine was produced in intestinal fluids of sheep during challenge with nematode larvae. Steel *et al.* (1990) reported that both histamine and serotonin were released in sheep following challenge with *T. colubriformis* L_3, with the maximum histamine response in immune sheep occurring six days after challenge. This suggests that mast cell degranulation is still actively occurring long after the rapid expulsion of incoming larvae, and is probably directed towards L_5 or adult nematodes. Similarly, increased histamine levels were detected in intestinal fluid from immune sheep by Jones *et al.* (1990) and Jones and Emery (1991) peaking at three and six days after challenge respectively. Jones *et al.* (1990) noted a negative correlation between duodenal histamine levels and WEC ($r=-0.46$, $P<0.05$) indicating that the actions of histamine are involved in worm rejection. Interestingly, the correlation between

histamine and WEC was significantly higher than the correlation between histamine and total worm counts, suggesting that the actions of histamine may affect worm fecundity more strongly than worm survival. Liu *et al.* (2007) also noted increased histamine levels in mesenteric lymph node and jejunum tissue from sheep infected with *T. colubriformis* and *T. circumcincta* compared to worm-free sheep. These authors also detected a trend for histamine concentrations to be negatively correlated with WEC (r=-0.32, p=0.09). Therefore, histamine is directly involved in rejection of worms but is also likely to be involved in the pathogenesis of immune-mediated scouring.

Leukotrienes and prostaglandins

The lipid mediators derived from the metabolism of arachidonic acid, leukotrienes and prostaglandins, have many potent effects. Leukotrienes act on a family of G-protein coupled receptors that are present on endothelial and epithelial cells as well as eosinophils and mast cells (Evans, 2003). They are able to stimulate the production of cytokines from mast cells as well as having direct effects on endothelial tissue, namely vasodilation, and cause contraction of nonvascular smooth muscle (Bueno and Fioramonti, 2002). Prostaglandins act in a similar manner, binding to G-protein coupled receptors and stimulating vasodilation and contraction of smooth muscle (Montuschi and Barnes, 2002). PGE_2 plays a key role in the electrolyte balance in the gut, raising cyclic AMP (cAMP) levels and leading to increased secretion of chloride ions from epithelial cells (Pyne *et al.*, 1997, Graness *et al.*, 1997). The release of leukotrienes and prostaglandins from degranulated eosinophils and mast cells is a central mechanism in the pathophysiology of asthma (Bradding *et al.*, 2006) and immune-mediated diarrhoea in humans (Ucar *et al.*, 1998, Farthing, 2003, Crentsil, 2005). Therefore, their release during worm infection of sheep probably contributes to immune-mediated scouring.

Moqbel *et al.* (1987) demonstrated that leukotriene concentrations were elevated in intestinal mucus during expulsion of *T. spiralis* from immune guinea pigs. Numbers of eosinophils and mast cells were also increased in intestinal tissue sections. Therefore it appeared that the recruitment of granulocytes into the intestinal mucosa resulted in an increase in lipid mediators and consequent smooth muscle contraction, fluid secretion and vascular permeability. This prevented larvae from being able to establish themselves in their mucosal niche. Evidence of the direct role that leukotrienes play in parasite expulsion in murine models was provided by Machado *et al.* (2005). These authors found that mice that lacked the Lipoxygenase-5 pathway and, consequently,

could not produce leukotrienes, had significantly higher WEC and total worm burdens than normal mice. Leukotriene levels are also elevated in immune sheep challenged with *T. colubriformis* (Jones *et al.*, 1990, Jones *et al.*, 1994). Parasite-resistant sheep have significantly higher concentrations of leukotrienes in GI mucus than susceptible sheep, when naturally infected by grazing pastures infected with *T. colubriformis* and *T. circumcincta* (Gray *et al.*, 1992). *In vitro*, leukotrienes are closely involved with inhibiting larval migration (Douch *et al.*, 1996). Therefore, it is clear that they are a key component of immunity and larval rejection.

There is less information on the role that prostaglandins play in the immune response to GIN. *In vitro,* PGE_1 incubated with *Nippostrongylus brasilensis* causes irreversible damage to the worms and prevents them re-establishing in rats (Richards *et al.*, 1977). Levels of $PGF_{1\alpha}$ are increased in immune sheep following challenge with *T. colubriformis* (Jones and Emery, 1991) but whether or not prostaglandins play an active role in worm rejection is unclear. Douch *et al.* (1983) reported that mucus taken from immune sheep inhibited the *in vitro* migration of *T. colubriformis* L_3 on agar plates by 93%, while PGE_1 or PGE_2 added directly to the plate had no effect on larval migration. Emery and McClure (1995) found that dosing sheep with indomethacin, which prevents prostaglandin production by inhibiting the cyclooxyengase pathway, had no effect on WEC or total worm burdens in sheep infected with *T. colubriformis.* There is clearly a need for more research to determine the role that prostaglandins play in immunity to nematodes and whether they contribute to immune-mediated scouring.

Bradykinin

Bradykinin is a peptide formed by the cleavage of high molecular-weight kinogen. The kinogen is synthesised by hepatocytes and released into the bloodstream, where it is activated by kininogenases during tissue injury and acute inflammation. It is also released from mast cells during activation by IgE (Bhoola *et al.*, 1992). Bradykinin is an extremely potent vasodilator and can increase vascular permeability, cause pain and induce smooth muscle contraction (Bhoola *et al.*, 1992, Campbell *et al.*, 1993). Bradykinin is an important inflammatory mediator in the pathogenesis of asthma (Soler *et al.*, 1990, Farmer *et al.*, 1992) and immune-mediated diarrhoea (Bueno and Fioramonti, 2002, Farthing, 2003)

Bradykinin binds to two specific G-protein coupled receptors that are widely expressed in gastrointestinal smooth muscle and in the lamina propria and mucosal epithelium

(Manning *et al.*, 1982). It acts in several ways. First, it binds directly to receptors in nonvascular smooth muscle cells and leads to an increase in intracellular Ca^{2+} concentration, resulting in contraction of smooth muscle (Marsh and Hill, 1994, Siragy *et al.*, 1994). Second, bradykinin stimulates arachidonic acid metabolism in granulocytes. Mast cells and eosinophils both express bradykinin receptors and undergo degranulation when stimulated by bradykinin (Bandeira-Melo *et al.*, 1999, Bueno and Fioramonti, 2002). Production of cysteinyl leukotrienes and PGE_2 are increased in bronchial lavage fluid in sheep following allergenic stimulation, but are significantly decreased following treatment with specific bradykinin-receptor antagonists (Abraham *et al.*, 1991). Finally, bradykinin increases vascular permeability leading to increased migration of leukocytes and plasma protein leakage (Colditz, 1991, Renne *et al.*, 2005). All of these mechanisms may aid in removal of ingested nematode larvae.

Most work on bradykinin has focused on the immunopathology that it provokes in allergic disorders such as asthma. There has been little research into the protective role that bradykinin may play in GIN infection. The increased fluid secretion, altered gut motility and metabolism of arachidonic acid associated with bradykinin production are consistent with the immune response of adult sheep towards GIN. Bradykinin is also closely associated with eosinophilia in inflammation of the airway in guinea pigs (Farmer *et al.*, 1992) and is a potent chemo attractant of eosinophils in humans suffering allergic rhinitis (Turner *et al.*, 2001). Therefore, it may be closely involved in the pathology of immune-mediated scouring.

Overall, the literature suggests that inflammatory mediators play a crucial role in immunity to GIN infection in sheep. However, there is also evidence, mainly from human medical studies, that they also are implicated in immune-mediated diarrhoea. This provides support for the hypothesis that inflammatory mediators will be responsible for rejection of worms from the gut but may also lead to scouring.

2.5.4 Summary of immunobiology and relation to immune-mediated scouring

Repeated challenge of the immune system with infective parasitic larvae leads to a strong acquired immunity. This immunity rapidly expels further larval challenges and/or arrests the development of worms at the fourth larval stage. The immune response is skewed towards a Th2 cytokine response, whereby T-lymphocytes release cytokines such as IL-4, IL-5 and IL-13. These cytokines induce antibody class-

switching to IgE and stimulate the production of granulocytes. IgE can bind to sensitised mast cells to facilitate the rapid removal of incoming larvae in less than two hours. Larvae that penetrate the mucosa are expelled within days.

The degranulation of mast cells releases pre-formed mediators, histamine and serotonin, while degranulation of eosinophils releases basic proteins. Leukotrienes and prostaglandins can be synthesised from the membrane phospholipids of both cell types. Bradykinin can be released from mast cells or produced from the cleavage of circulating high molecular weight kinogen. These mediators can cause nonvascular smooth muscle to contract by raising the intracellular Ca^{2+} concentration or they can stimulate fluid secretion from endothelial cells by the production of second messengers such as cAMP. This increases peristalsis, increases the flow rate of digesta and decreases absorption of electrolytes from the intestinal lumen. It is an effective immune response as it prevents larvae from reaching their tissue niche or expels them from the mucosa before they establish to adults. A possible side-effect is increased scouring and the pathology described here bears many similarities to immune-mediated diarrhoea and other inflammatory disorders such as asthma in humans.

2.6 Conclusions and research hypotheses

Breeding worm-resistant sheep is a sustainable and long-term solution to the problem of increasing anthelmintic resistance in sheep production. The most significant benefit is reduced re-infection rates due to low WEC affecting the epidemiology of the parasite population. However, scouring will not be reduced in sheep with low WEC and may be increased in some environments. This is because in adult sheep scouring is related to immunopathology, rather than the direct effects of the parasites. Scouring is a major problem in sheep production as the build up of faecal material around the breech pre-disposes sheep to flystrike. Scouring is not, however, an inevitable consequence of worm-resistance. Some highly worm-resistant sheep do not suffer diarrhoea. As such, there is the potential to select worm-resistant sheep that do not scour. As scouring is mainly caused by the ingestion of worm larvae, research is clearly required into the mechanisms of immune-mediated scouring in worm-resistant sheep.

The general hypothesis in this thesis is that the inflammatory mechanisms that result in larval rejection and consequently low WEC in parasite-resistant sheep also lead to significant softening of the faeces.

Four specific research hypotheses will be tested in this thesis –

i) When parasite-resistant sheep are challenged with a trickle dose of *T. colubriformis* and *T. circumcincta* in an animal house, they will resist the challenge but have lower faecal dry matter than sheep of a similar genotype that are kept in identical conditions and are not challenged.

ii) Parasite-resistant sheep with high-dag scores under field conditions will have lower faecal dry matter when challenged with larvae, compared to sheep with low-dag scores.

iii) Inflammatory mediators, granulocytes, serum antibodies and interleukin-5 will all be negatively correlated with both worm numbers and faecal dry matter in parasite-resistant sheep during larval challenge

v) A mixed challenge with both *T. colubriformis* and *T. circumcincta* larvae will result in lower faecal dry matter than with a single challenge with either nematode species.

Chapter 3 - General Materials and Methods

3.1 Experimental design

In order to test the general hypothesis, an experimental model was developed to mimic immune-mediated scouring in a controlled animal house environment. Three experiments were conducted, all of which followed the model shown in Figure 3.1. Infective nematode larvae were administered daily to parasite-resistant sheep and changes in faecal dry matter were monitored. After six weeks (three and a half weeks in experiment three) of larval challenge, sheep were euthanised and total worm counts determined. Tissue and mucus samples were taken from the abomasum and small intestine, and the numbers of inflammatory cells and concentrations of inflammatory mediators quantified.

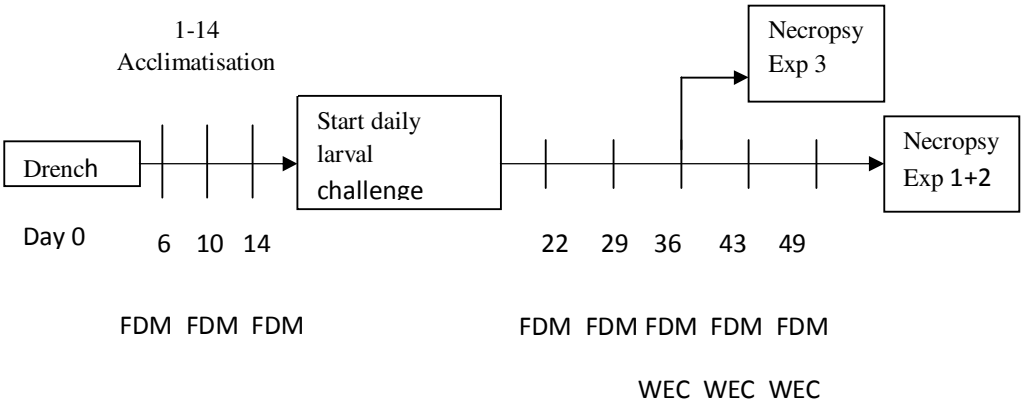

Figure 3.1. Experimental design. FDM indicates faecal dry matter was measured on this day. WEC indicates that worm egg counts were performed on this day.

More details of the experimental design for each experiment will be given in the experimental chapters. All experiments were approved by the Animal Ethics Committee of the University of Western Australia.

3.2 Animals

The rams were selected from the parasite-resistant, Rylington Merino line based at the Mount Barker research station owned by the Department of Agriculture & Food Western Australia. Sheep from this line have been bred for worm resistance since 1987. Rams were used in these experiments as ewes from this flock are retained for breeding and males are normally culled rather then castrated. Worm resistance is determined by best linear unbiased prediction (BLUP) estimated breeding values (EBV) for WEC at hogget age following a natural, moderately- high, parasite challenge consisting mainly of *Trichostrongylus* spp. and *T. circumcincta*. Selection is based on an index that places 50% selection pressure on WEC, 20% to reduce scouring (dag score) and 30% on production traits (Karlsson and Greeff, 2006).

The rams used for this research were selected on the basis of EBV for WEC, as well as a phenotypic indicator of scouring (dag score) taken at hogget age. Before each experiment the rams were drenched (Q-drench, Jurox, active ingredients – 40 g/L levamisole, 37.5 g/L closantel, 25 g/L albendazole and 1 g/L abamectin), and then transported to the Large Animal Facility or Shenton Park research station at the University of Western Australia where they were housed indoors in individual pens. The rams were fed a daily diet consisting of 800 g oaten chaff, 400 g lupins and 25 g mineral supplements (Siromin^{TM}). Water was supplied *ad libitum.* Food refusals were measured daily. Generally, sheep always ate their whole ration. No sheep at any stage ate less than 80% of their ration, and no sheep left food refusals for more than two days in a row.

3.3 Worm egg counts and faecal dry matter

Worm egg counts were performed using the modified McMaster technique where one observed egg equals 50 eggs per gram of wet faeces (Whitlock, 1948). For determination of faecal dry matter, at least 10 g of faeces was collected from the rectum. The wet weight of the faeces was recorded. The faeces was then placed in an oven at 90°C for at least 48 hours and then weighed again. The faeces was repeatedly weighed until no further change in weight was noted. The dry weight was then divided by the wet weight to determine the percentage of dry matter.

3.4 Post-mortem procedures

Sheep were euthanised with an overdose of pentobarbitone (Lethabarb, Virbac animal health Australia, 1mL/2kg bodyweight) given intravenously. Sheep were then immediately eviscerated. The abomasum was tied off at the pyloric junction and removed. The first five metres of the small intestine was carefully separated from the mesentery and also removed. The organs were then opened and the contents collected in separate buckets. After collection of mucus and tissue samples for histopathology (see below), the mucosal surface of the organs was vigorously washed with water and the washings added to the contents in the bucket. The small intestine was split lengthwise along its entire length and the mucosal surface scraped with the handle of a pair of scissors and these scrapings added to the bucket. These were then passed through a 150 μm sieve, placed in individual plastic containers and water added to give a final volume of 1L (small intestine) or 2L (abomasum). Formalin was then added to give a final concentration of 5% formalin and the containers stored at 4 °C until worms were counted. The small intestine was then discarded; the abomasum was retained for digestion to remove larval stages (see below).

3.4.1 Mucus and tissue collection

Small tissue samples (approximately 1-2 cm^2) were taken from the fundic region of the abomasum and small intestine, approximately 30-40 cm distal to the pylorus. These were then affixed to a small piece of cardboard with the mucosal surface exposed and placed into 4% buffered formaldehyde solution (approximately 10x sample volume) for histology. The small intestine sample was cut open and laid flat before fixation. The time between evisceration and fixing of tissues was no more than ten minutes. Mucus was collected by gently scraping the surface of the abomasum and the first metre of the small intestine with a glass microscope slide. Mucus was split into 3-4 aliquots, placed into plastic 1.5 ml cryovial tubes and snap frozen in liquid nitrogen. Tubes were then placed into -80 °C freezers for storage prior to mediator and protein assay.

3.4.2 Abomasum digest and worm counts

The cold abomasum was weighed and then added to a solution containing 0.83 % pepsin (w/v) (Sigma, Australia) in 0.6 % hydrochloric acid. Three hundred ml of solution was used for each 100 g of tissue. This was then incubated at 38 °C for 3-3.5 hours. The solution was shaken every 30 minutes. The abomasum was then discarded

and the solution passed through a 45 µm sieve and collected in a plastic container. Formalin was then added to give a concentration of 5% formalin and then containers stored at 4°C.

On the day of the worm counts, the formalin was removed from the samples by passing the contents through a 45 µm sieve. The contents of the sieve were then hosed back into the container and made up with water to the original volume of the sample (1L for small intestine, 2L for abomasum, various for abomasum digest). A 10 ml aliquot was then taken from the container and worms counted under a Nikon SMZ800 stereomicroscope with a Microlight 150 fibreoptic light guide. Worms were differentiated into early fourth stage larvae, developed fourth stage larvae and male and female adults. If no worms were found in an aliquot, a second aliquot was taken and counted again. The total number of worms from each organ was then calculated by multiplying the number of worms in the 10 ml aliquot by a tenth of the total volume. The total number of worms from the abomasum was calculated by adding the number from the contents and the number from the digest.

3.5 Assay of inflammatory mediators

3.5.1 Cysteinyl leukotrienes and prostaglandin E_2

Mucus samples were weighed and then homogenised in 5x the sample weight (w/v) of 2:1 methanol/ 0.1M phosphate buffered saline (pH 7.5). The mucus was then centrifuged at 1500 g for 15 minutes at 4 °C. The supernatants were carefully poured into new tubes and the pellet discarded. The supernatant was acidified (pH 4) with 0.1M acetic acid. Mediators were isolated with solid phase extraction as follows. 300 mg C18 SPE cartridges (Alltech Associates Australia) were activated by rinsing with 5 ml tetrahydrofuran, 5 ml methanol and 5 ml millipore water. 500 µl of supernatant was then added to the cartridge, which was then washed with 5 ml water and 5 ml of petroleum ether. Leukotrienes were eluted with 3 ml of methanol, while prostaglandins were eluted with 3 ml of ethyl acetate containing 1% methanol. The eluting solvents were then evaporated to dryness with compressed air and the residues resuspended in EIA buffer supplied with the ELISA kits (see below). The percentage recovery of extraction was determined by 'spiking' several samples with a known amount of cysteinyl leukotriene or PGE_2 standard and including these in the assay alongside unspiked samples. The recoveries from the spiked samples were averaged to determine

the percentage recovery. In the final calculations of mediator concentrations, values were adjusted to reflect the percentage recoveries.

Concentrations of cysteinyl leukotrienes were determined using an ELISA kit (product number 520501, Cayman Chemical, Ann Arbor, USA) according to the manufacturers instructions. Because of the rapid metabolism of PGE_2 in tissue, I elected to instead measure PGE_2 metabolites as an indirect measure of PGE_2 production in mucus. This was done using an ELISA kit (product number 514531, Cayman Chemical, Ann Arbor, USA) according to the manufacturers instructions. This kit converts all metabolites to a stable derivative that is then directly quantified by ELISA.

Both the prostaglandin and leukotriene assays are based on the competition of mediators in the samples and an acetylcholinesterase-eicosanoid conjugate (tracer) for a limited amount of specific eicosanoid antiserum. Briefly, 50 µl of samples or standards, 50 µl of the tracer and 50 µl of the antiserum were added to each well of a 96-well ELISA plate pre-coated in mouse anti-rabbit IgG antibody and incubated for 18 hours at 20 °C. The plate was then washed five times to remove unbound reagents and 200 µl of Ellman's reagent was added to each well. Ellman's reagent reacts with the tracer to produce 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm. Thus, the intensity of absorbance is inversely proportional to the concentration of mediators in the sample or standard. The standard curve was prepared under the same conditions as the samples and consisted of eicosanoid standard serially diluted in EIA buffer to yield eight concentrations ranging from 7.8 pg/ml to 1000 pg/ml for cysteinyl leukotrienes, and 0.39 pg/ml to 50 pg/ml for PGE_2 in EIA buffer. Absorbances were read at 412 nm on a Multiskan spectrum plate-reader version 2.1. Results were calculated by fitting a four-parameter logistic curve, using spreadsheets developed by Cayman Chemical and included in the assay kit. The limit of detection was 13 pg/ml for the leukotriene kit, and 2 pg/ml for the prostaglandin E_2 metabolite kit.

3.5.2 Bradykinin

For determination of bradykinin, frozen samples were homogenised in an equal amount of 1% trifluroacetic acid containing a protease inhibitor cocktail (Sigma #80345; 20 µl per 1 ml trifluroacetic acid). Samples were then centrifuged at 1500 g for 15 min before the supernatant was added to a SPE C18 column that had been rinsed with successive washes of 1% trifluroacetic acid and a 60:39:1 mixture of acetonitrile, water and 1% trifluroacetic acid. The same acetonitrile mixture was used to elute the kinins, after the

columns had been washed with 1% trifluroacetic acid. The solvents were then evaporated with a gentle stream of nitrogen. The resulting residues were re-suspended in EIA buffer and assayed using a commercial ELISA kit (Bachem, USA, product number S-1135). The standard curve ranged from 8-500 pg/ml. The concentration of bradykinin in several of the samples from the control group was below the minium detection limit of the assay – these were assigned the minimum value of the standard curve of 8 pg/ml.

This is a competitive ELISA based on the competition of bradykinin in standards or samples and biotinylated tracer for a limited amount of specific antiserum. Briefly, 50 µl of standard or sample was added together with 25 µl antiserum and 25 µl tracer to each well of a 96-well ELISA plate pre-coated in mouse ant-rabbit IgG. The plate was incubated at room temperature for two hours, washed five times with EIA buffer and 100 µl of streptavidin-horseradish peroxidase added to each well. After further incubation at room temperature for one hour, the plate was washed five times and 100 µl of TMB solution added to each well. The plate was then incubated at room temperature for 40 minutes. Instead of terminating the reaction, the developing colour was read at 650 nm on a Multiskan spectrum plate-reader version 2.1. This procedure is recommended by the kit manufacturers for inexperienced users of the kit, in order to avoid losing the top of the standard curve. Results were calculated by fitting a four-parameter logistic curve, using a spreadsheet supplied by the kit manufacturers.

3.6 Protein assay

The total protein concentration of mucus samples was estimated using a modified version of the Lowry method similar to Hartree (1972). The copper reagent for the assay was prepared by dissolving 20 g sodium carbonate in 260 ml water, 0.4 g 5 x hydrated copper sulfate in 20 ml water and 0.2 g sodium potassium tartrate in 20 ml water. These three solutions were then well mixed to produce the reagent. A 500 ml solution of Lowry concentrate was prepared by mixing 300 ml of the copper reagent with 100 ml of 1% SDS and 100 ml of 1M sodium hydroxide.

Frozen mucus samples were thawed and homogenised in an equal amount (w/v) of cold 0.1M phosphate buffered saline (pH 7.5). They were then centrifuged at 1500g for twenty minutes and the supernatants retained. A protein standard curve was prepared by serially diluting bovine serum albumin in PBS. 400 µl of sample supernatant or standard was then added to 400 µl Lowry concentrate, mixed and incubated at 20 °C for

ten minutes. 200 µl of 0.2 N Folin & Ciocalteu's phenol reagent (Sigma, Australia) was then added. The solution was then rapidly vortexed and incubated at 20 °C for thirty minutes. Absorbances were read at 750 nm in plastic cuvettes, using PBS as a reference.

3.7 Histology

Formaldehyde-fixed sections were embedded in paraffin wax blocks, sectioned at 5 µm and stained with either haematoxylin and eosin (eosinophils and globule leukocytes) or toluidine blue (mast cells). Granulocytes are not uniformly distributed throughout the lamina propria. Eosinophils are generally found near the muscularis mucosa, while globule leukocytes are located more towards the epithelium. In addition, the thickness of the lamina propria can vary greatly between animals and also between different parts of a tissue section from the same animal. For this reason, quantifying cells by counting the number in randomly selected fields of view can be misleading. Therefore, for quantification of inflammatory cells, 5-10 locations on each tissue section were selected at random. At each location, the full thickness of the lamina propria was scanned from the base of the muscularis mucosa to the mucosal epithelium. Eosinophils were identified by their characteristic eosinophilic granules and double-lobed nucleus. Globule leukocytes were identified by the large eosinophilic globules in the cytoplasm. Care was taken to distinguish mast cells from mucin-producing cells that stained a similar colour with toluidine blue. Mast cells were identified by their distinct granules. Scanning was done using an Olympus CH2 microscope at 400x magnification. The area of tissue scanned varied with the thickness of the tissue sample and was calculated using a stage micrometer. Numbers of cells were expressed per mm^2 of tissue.

3.8 Immunoglobulin ELISA

Monoclonal antibodies against ovine immunoglobulin light chain (i.e. total antibody), IgG_1, IgG_2 and IgM were kindly supplied by Dr K. Beh, formerly of CSIRO livestock industries, Armidale, Australia. Monoclonal antibodies against ovine IgA and IgE were kindly supplied by Dr S.J. McClure, CSIRO livestock industries, Armidale, Australia. The antigens used were *T. colubriformis* L_3 soluble protein extract and *T. circumcincta* L_3 excretory/secretory product, prepared according to the methods described in Emery *et al.* (1991). Preliminary assays showed that only very small amounts of IgG_2 were present in serum. Therefore, only IgG_1 was measured in all animals and the results reported. No IgA or IgE activity against *T. circumcincta* antigen was detected using the

available monoclonal antibodies. Therefore, *T. circumcincta*-specific IgA was measured using a commercially available rabbit anti-sheep IgA polyclonal antibody (Bethyl laboratories, USA, cat # A130-108A). With no commercial antibodies available against ovine IgE, it was unfortunately not possible to measure *T. circumcincta*-specific IgE.

Serum samples were thawed on the day of the assay. ELISA plates (Immunolon 1B, Denmark) were coated overnight at 4°C with 100 µl of antigen (0.25 µg/ml, diluted in carbonate/bicarbonate coating buffer, 0.05M, pH 9.6). Plates were washed four times with phosphate buffered saline (pH 7.3) containing 0.05 % Tween 20 (PBST), and then 100 µl of serum diluted in PBST (see Table 3.1) was added to duplicate wells. Plates were then incubated for one hour at 37°C before being washed four times in PBST. 100 µl of the various anti-sheep monoclonal antibodies were then added, after being diluted in high salt diluent (HSD – 0.45M NaCl, 10% foetal calf serum, 1% Tween 20) as shown in Table 3.1. After incubation at 37°C for one hour, and four washes in PBST, 100 µl of sheep anti-mouse immunoglobulin conjugated to horseradish peroxidase (Chemicon Australia, cat # AP326P, 1:100 in HSD) was added to each well. Plates were incubated for one hour at 37°C, washed four times in PBST and 100 µl of TMB substrate (ELISA systems, Australia) added to the wells. The reaction was stopped by the addition of 100 µl 1M H_2SO_4 and absorbances read at 412 nm on a multiskan plate reader. Foetal lamb serum was used as a negative control – its absorbance was deemed to represent non-specific binding and thus this value was subtracted from the absorbance values of all serum samples.

Preliminary assays were performed to identify high-reading sera. These were the used as a positive control on each plate. For comparison of the results, the positive control was assigned a value of 100 ELISA units. The absorbances of the unknown samples were then expressed in ELISA units relative to the absorbance of the positive control. As the same positive control was run on each plate in each assay inter-assay variation was thus accounted for.

Table 3.1. Monoclonal antibody and sera dilutions used for immunoglobulin ELISA
*Rabbit anti-sheep polyclonal antibody (Bethyl)

Monoclonal	Antigen	Antibody Dilution	Sample Dilution
Light Chain (Beh, 1988)	*T. colubriformis*	1:5000	1:80
	T. circumcincta	1:5000	1:80
IgG_1 (Beh, 1987)	*T. colubriformis*	1:5000	1:80
	T. circumcincta	1:5000	1:20
IgM (Beh, 1988)	*T. colubriformis*	1:1000	1:20
	T. circumcincta	1:1000	1:40
IgA (Beh, 1988)	*T. colubriformis*	1:2	1:20
	T. circumcincta	1:100*	1:20
IgE (Bendixsen *et al.*, 2004)	*T. colubriformis*	1:2	1:20

3.9 Interleukin-5 ELISA

IL-5 in serum was estimated using monoclonal antibodies against rat IL-5 that have been validated for use in sheep (Doligalska *et al.*, 1999, Sykes *et al.*, 2007). ELISA plates (Immunolon 4, Denmark) were coated overnight at 4°C with 50 µl of mouse anti-rat IL-5 monoclonal antibody (TRFK-5, Abcam, USA), diluted 1:1000 in 0.1M PBS (pH 7.5). Plates were then washed five times in PBST and 50 µl serum samples, diluted 1:20 in PBST, were added in duplicate. Plates were incubated overnight at 4°C and then washed five times in PBST before the addition of 50 µl of mouse anti-rat IL-5 biotintylated monoclonal antibody (TRFK-4, Abcam, USA), diluted 1:100 in HSD. Plates were incubated for one hour at room temperature, washed five times in PBST and

100 µl of streptavidin conjugated to horseradish peroxidase (Pharmagen, Australia, 1:5000 in 1% BSA in PBS) was added to each well. Plates were incubated at room temperature for one hour, washed five times in PBST and 100 µl of TMB substrate was added. Plates were then shaken on a plate shaker for thirty minutes before the reaction was stopped with 100 µl of 1M H_2SO_4 per well and absorbances read at 412 nm. The concentration of IL-5 was expressed in ELISA units as described above, with foetal lamb serum used as a negative control.

Chapter 4 – Challenging sheep with nematode larvae results in an inflammatory immune response and lowers faecal dry matter

4.1 Introduction

Controlling nematodes by improving the genetic resistance of the host appears an attractive, long-term solution to the increasing threat of anthelmintic resistance. Researchers have demonstrated the feasibility of a breeding program aimed at breeding worm-resistant sheep (Woolaston and Windon, 2001, Karlsson and Greeff, 2006, Bisset *et al.*, 1996). However, in areas with a Mediterranean, winter-rainfall climate there is an increased tendency for resistant sheep to scour as a result of nematode infection (Karlsson *et al.*, 2004). Larsen *et al.* (1994) showed that scouring in sheep with low worm egg counts (WEC) during the winter rainfall season is most likely due to a hypersensitive immune response to ingested larvae ('immune-mediated scouring'). Scouring was an "over-response" for worm immunity as other sheep within the same flock had low WEC without exhibiting scouring (Larsen *et al.*, 1994). Increased numbers of eosinophils, a key indicator of inflammation and hypersensitive immune responses (Rothwell, 1989) were detected in the intestinal mucosa of scouring individuals and the phenomenon appears to have some genetic component (Larsen *et al.*, 1994).

The inflammatory response is central to effector mechanisms against gastrointestinal nematode parasites (Emery, 1996). Following ingestion of worm larvae, inflammatory changes are observed in the intestinal and abomasal mucosa, including the accumulation of eosinophils, mast cells and globule leukocytes (Jones *et al.*, 1994). These cells are able to release potent inflammatory mediators such as the prostaglandins and the cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4). These lipid mediators may aid in removing both larvae and possibly adult worms from the intestinal tract, as well as stimulating fluid secretion and smooth muscle contraction. It is possible that some sheep bred for worm resistance have an increased, inflammatory-immune response to worm larvae compared to un-selected sheep, leading to increased scouring. My aim is to study immune-mediated scouring but, before doing so, need to develop a reliable experimental model where the condition can be reproduced under controlled conditions in the animal house. Relying on seasonal conditions in the paddock to generate

experimental animals is too variable and an inefficient use of resources. I hypothesised that immune-mediated scouring could be satisfactorily mimicked in the animal house by administering an artificial infection of worm larvae to parasite-resistant sheep. Specifically, I hypothesised that a trickle infection of worm larvae would decrease faecal dry matter in penned, worm-resistant sheep compared to uninfected sheep of the same genotype kept under identical conditions. Furthermore, I expected that there would be higher numbers of inflammatory cells and increased concentrations of prostaglandin E_2 and cysteinyl leukotrienes in the intestinal and abomasal mucosa at post-mortem examination.

4.2 Materials and methods

4.2.1 Experimental design

Six, twenty-month-old parasite-resistant Merino rams were drenched and then received a dose of 6000 nematode L_3 weekly for six weeks. Three additional parasite-resistant rams were kept worm free to act as controls. Faecal samples were taken regularly before and after infection started to determine faecal moisture. After six weeks all rams were euthanised and total worm counts determined. Tissue samples were taken from the abomasum and small intestine and the numbers of eosinophils, mast cells and globule leukocytes were quantified. Mucus samples were also taken from the abomasum and small intestine and concentrations of cysteinyl leukotrienes and prostaglandin E_2 were determined.

The rams used all had high-dag scores but low EBV for WEC (Table 4.1). They were therefore assumed to be susceptible to immune-mediated scouring. I used sheep that I thought would be susceptible to increase the chance of reproducing scouring in an animal house.

Table 4.1. Mean EBV for worm egg count (WEC) and mean dag scores in winter and spring 2006 for rams used in experiment one and flock averages. Dag scores are on a subjective 1-5 scale where 1 is no dag and 5 is severe dag.

	WEC EBV	Winter dag Score[1]	Spring dag Score
Experimental rams (n=9)	-96.1	3.3	2.5
Flock average (n=60)	-87.4	2.1	2.2

[1] Then crutched

4.2.2 Challenge regime

Trichostrongylus colubriformis and *Teladorsagia circumcincta* L_3 were cultured at the Animal Health Laboratories of the Department of Agriculture & Food Western Australia. After a two-week period of acclimatisation in the animal house, six rams were selected at random for larval challenge. The remaining three rams acted as un-challenged controls. Only three control animals were used as we anticipated that there would be little deviation from baseline faecal moisture in the absence of nematode infection and any other intestinal inflammation (e.g. coccidia). In addition we thought we would be able to detect differences in post-mortem histopathology with a small control group as long as they were maintained worm-free. The dose consisted of 6000 L_3 (1:1, *T. colubriformis* and *T. circumcincta*) per week, given daily in equal proportions. This dose is comparable to the larval availability on winter pastures grazed by sheep from the parasite-resistant Rylington line (Williams *et al.*, 2009) and would certainly be sufficient to elicit an immune response from the host (D.G. Palmer, pers. comm.). The larvae were suspended in water and administered orally using a drench gun throughout the six weeks of the trial.

4.2.3 Sampling procedures

Faecal samples were taken from rams upon arrival at the animal house (approximately 48 hours after drenching) and WEC performed to ensure sheep were worm-free. During the acclimatisation period, three faecal samples were taken from each sheep at regular intervals four days apart and faecal dry matter was determined as described in Chapter 3. These three values were averaged to determine a baseline, pre-challenge value of

faecal moisture content for each sheep. Following the commencement of larval dosing, faecal samples were taken weekly for dry matter determination. Three weeks after dosing commenced, weekly faecal samples were also taken for WEC. Six weeks after larval dosing commenced, all nine rams were euthanised. Tissue and mucus samples were taken at post-mortem examination, and total worm counts were determined as described in Chapter 3.

4.2.4 Histology and ELISA

Tissue samples were processed using standard histological processes and the numbers of eosinophils, mast cells and globule leukocytes counted as described. The concentrations of cysteinyl leukotrienes and prostaglandin E_2 metabolites in abomasal and small intestinal mucus were determined using commercial ELISA kits. The percentage recovery of mediators from solid-phase extraction was 81% for leukotrienes and 77% for PGE_2. The coefficient of variation of extraction was less than 10%. Concentrations of mediators were expressed per mg of protein. Full details of the techniques are provided in Chapter 3.

4.2.5 Statistical analysis

The pattern of faecal dry matter over the course of the experiment was tested with a mixed model analysis in SAS (version 9, SAS Institute Inc, Cary, NC, USA), with treatment group and week of challenge fitted as fixed factors and the individual sheep as the random model. The baseline faecal dry matter (i.e. the mean of the three pre-challenge faecal dry matter measurements) was used as the value for each sheep at day 0. Differences of least squares means were used to examine differences between the control and challenged groups at each time-point. Student's t-tests were used to determine differences in mediator concentrations in mucus and mean cell counts in tissue samples between control and challenged groups. Within the challenged group, the difference in mediator concentrations and cell counts between each organ was compared with a paired t-test. The number of adult *T. circumcincta* and *T. colubriformis* were also compared in the challenged sheep with a students t-test, after transformation ($\log_{10}$ (n+1)) to normalise variances. T-tests were conducted using Genstat 5 for Windows (Second Edition, Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Histogram and fitted-value plots of residuals were used to check for assumptions of normal distribution and homogeneity of variances. The level for significance was $P<0.05$.

4.3 Results

4.3.1 Worm infection

WEC in the challenged group were low throughout the experiment and, although WEC in one ram reached 500 eggs per gram of wet faeces (epg) the week before slaughter, the remaining WEC were low (<100epg) with 2 rams having zero epg over the six weeks. WEC in the control rams were all negative. The resistance of the rams to worms was confirmed by the low total worm counts at slaughter. Only adult worms were found – no immature worms were found for either species despite the sheep receiving a dose of L_3 the day before slaughter. One challenged ram had no worms of either species (Table 4.2). There were higher numbers of *T. circumcincta* than *T. colubriformis* ($P<0.05$; Table 4.2). Control rams were worm-free.

Table 4. 2. Total worm burdens in challenged rams (geometric means with 95% confidence intervals). Numbers followed by a different subscript are significantly different. ($P<0.05$)

Species (stage)	Mean number found in challenged rams
T. circumcincta (L_4)	0
T. circumcincta (adult)	198 (0.1, 1725.1)^{A}
T. colubriformis (L_4)	0
T. colubriformis (adult)	5 (0.7, 35.7)^{B}

4.3.2 Faecal dry matter

The percentage of faecal dry matter was consistently lower in the challenged group throughout the experiment (Figure 4.1). There was an interaction between treatment group and week of challenge ($P<0.001$). One week after challenge began, faecal dry matter in the challenged sheep was lower ($P<0.05$) than at baseline values and also lower ($P<0.005$) than faecal dry matter in the unchallenged controls. After one week faecal dry matter did not significantly change in the challenged group but it remained lower ($P<0.05$) than in the control group at each sampling. 36 days after challenge began, faecal dry matter in the control group was higher ($P<0.05$) than the baseline value. Three weeks after infection started, two rams in the infection group had faecal

dry matter values of 13-14% and their faeces were noticeably fluid until slaughter. Faeces in the control group remained firm throughout.

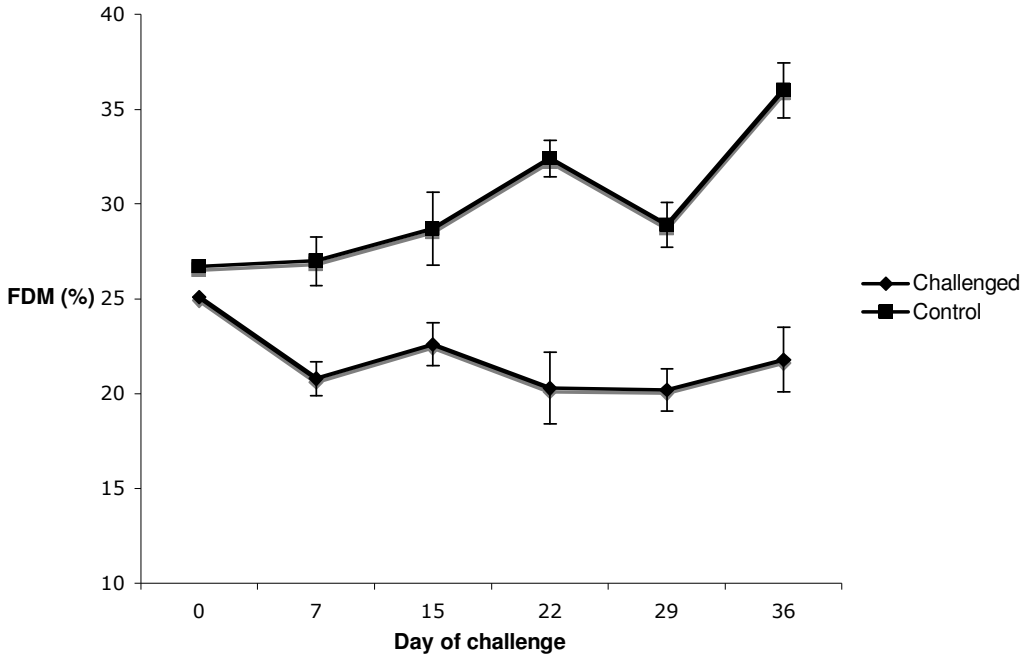

Figure 4.1. Faecal dry matter percentage (FDM%, means ±s.e.) in control (n=3) and challenged (n=6) rams following start of challenge at day 0.

4.3.3 Inflammatory mediators

Concentrations of leukotrienes and prostaglandin E_2 in mucus were higher ($P<0.05$) in the challenged group than in the control group in both the abomasum and small intestine (Table 4.3). Within the challenged group, concentrations of leukotrienes were higher ($P<0.05$) in the small intestine than in the abomasum. Concentrations of PGE_2 in the small intestine were higher than in the abomasum ($P = 0.096$).

Table 4.3. Concentrations (pg/mg protein) of cysteinyl leukotrienes (CLT) and prostaglandin E_2 (PGE_2) in mucus from the abomasum and small intestine of control rams (n=3) and challenged rams (n=6). Within columns, different subscripts denote different values ($P<0.05$) within the abomasum and small intestine. *indicates that for CLT, concentrations in the challenged group were higher ($P<0.05$) in the small intestine than the abomasum.

		CLT	PGE_2
Abomasum	Control	90 ± 18.2^{A}	97 ± 33.9^{A}
	Challenged	$431 \pm 63.6^{*B}$	250 ± 43.6^{B}
Small Intestine	Control	122 ± 26.2^{A}	65 ± 13^{A}
	Challenged	$813 \pm 89.4^{*B}$	480 ± 120.9^{B}

4.3.4 Histology

Mean counts of eosinophils and mast cells were higher ($P<0.05$) in abomasal and intestinal tissues from challenged rams than in control rams (Table 4.4). Degranulation of mast cells was common but this was not quantified. Globule leukocytes were higher in challenged rams but this was not significant in the abomasum ($P=0.7$) or small intestine ($P=0.19$; Table 4.4).

Table 4.4. Inflammatory cell counts (mean number of cells / mm^2 of tissue from 10 fields ± s.e. of group mean) in small intestine and abomasum of control and challenged rams. Values within rows followed by a different subscript are different ($P<0.05$).

		Control	Challenged
Eosinophils	Abomasum	23 ± 3.2^A	45 ± 9^B
	Small Intestine	17 ± 2.8^A	55 ± 10^B
Globule Leukocytes	Abomasum	15 ± 5.3	20 ± 10.5
	Small Intestine	9 ± 4.6	21 ± 7.6
Mast Cells	Abomasum	23 ± 4.8^A	63 ± 15.7^B
	Small Intestine	37 ± 10.4^A	140 ± 21.6^B

4.4 Discussion

The results support my hypothesis that nematode infection causes an inflammatory response in the intestinal tract and this manifests as increased moisture in the faeces. The results also show that immune-mediated scouring can be mimicked in the animal house as all six rams responded to the trickle L_3 infection over six weeks. Although only two animals could be considered to be clinically scouring, all six challenged rams had wetter faeces than the controls. The fact that all six challenged rams had wetter faeces can be attributed to the fact that they all had high-dag scores in the field. They were therefore considered susceptible to immune-mediated scouring. In addition, the small numbers of animals used in this trial were sufficient to detect significant differences between control and infected animals for inflammatory cells and associated lipid mediators.

Leukotrienes and prostaglandins are potent mediators of the immune system, causing increased fluid and electrolyte secretion and contraction of nonvascular smooth muscle. Their involvement in asthma (Montuschi and Barnes, 2002) and inflammatory bowel disease in humans (Bueno and Fioramonti, 2002) has been well documented. Exogenous injections of PGE_2 in previously healthy pigs have been shown to impair

intestinal transport and lead to diarrhoea, which was caused by increased mucosal secretion and reduced absorption of water and electrolytes (De Saedeleer *et al.*, 1992). This is consistent with the pathology of immune-mediated diarrhoea due to parasitic infection in humans (Farthing, 2003).

The release of leukotrienes and PGE_2 is probably induced by IgE binding to mast cells, and the consequent metabolism of liberated arachidonic acid. Leukotriene C_4 is also released in large amounts by eosinophils (Shaw *et al.*, 1985). Larsen *et al.* (1994) reported increased eosinophilic infiltration in the jejunum and ileum as the major difference between scouring and non-scouring Merino sheep. Researchers have reported increased levels of IgE in both sheep serum and lymph following parasite infection, with parasite-resistant sheep displaying increased levels compared to unselected sheep (Bendixsen *et al.*, 2004, Pernthaner *et al.*, 2005b). Shaw *et al.* (1999) also noted a positive genetic correlation between IgE and dag score in Romney sheep, suggesting that the inflammatory mechanisms that lead to reduced worm burdens can also lead to scouring.

Gray *et al.* (1992) and Jones *et al.* (1994) have also demonstrated that levels of leukotrienes increase in parasite-resistant sheep compared to control animals following nematode infection, and numbers of eosinophils and mast cells also increase (Bisset *et al.*, 1996). The results from this current experiment provide further support that elevated levels of inflammatory mediators generated following ingestion of worm larvae are causally linked to increased scouring in resistant sheep. However, this will need to be validated further in studies involving sheep that are both susceptible and not susceptible to immune-mediated scouring.

The similarity of numbers of globule leukocytes between control and infected rams is puzzling, considering the large difference in mast cell numbers between the two groups. It may be that the staining techniques used in this study were insufficient to estimate the number of globule leukocytes accurately or that turnover of globule leukocytes in immune sheep was too rapid. Degranulation assays have been used to quantify the release of sheep mast cell proteinase from isolated mast cells incubated with soluble protein extract from nematode L_3 (Huntley *et al.*, 1987, Jones *et al.*, 1992), but have not been sensitive enough to establish a correlation with worm counts. However, this assay may be useful in further studies on immune-mediated scouring.

The pattern of faecal dry matter detected in this pen trial is interesting, given field observations of scouring in Merino sheep. Under field conditions in a Mediterranean environment, signs of clinical scouring usually appear from approximately two months after the break of season. The exact conditions that lead to severe scouring are likely to be complex and difficult to replicate experimentally in pens. They may include the length of time and dose of larval infection that challenges the primed immune system (i.e. a sheep continuously grazing throughout the day and thus constantly ingesting larvae) and a large, undefined component from lush green pasture. The significant decrease in faecal dry matter, after one week of this regime of L_3 challenge, strongly implies that infective larvae induce conditions associated with diarrhoea in the absence of significant worm infection. This is also supported in field studies where albendazole capsules, that killed ingested nematode larvae, ameliorated or prevented scouring (Larsen *et al.*, 1994).

This increased fluid secretion is likely to be a powerful method of rejecting worms by the sheep. The fact that no immature worms or larvae were found at post-mortem examination, despite receiving a dose of L_3 the day before slaughter, supports this concept. Assuming no experimental error, it can only be concluded that the sheep had been expelling their entire worm challenges for at least several days before slaughter. Wagland *et al.* (1996) and Harrison *et al.* (1999) reported that immune sheep expelled an entire challenge of 20,000 *T. colubriformis* L_3 within two hours of challenge and worms residing in the same location were also expelled (Emery *et al.*, 1993b).

This rapid expulsion phase is likely to be due to mast cell degranulation, and the release of stored mediators such as histamine as well as arachidonic acid metabolites. McClure *et al.* (1992) reported that immune sheep that rapidly expelled a challenge of *T. colubriformis* had large numbers of globule leukocytes in the mucosal epithelium, as well as high concentrations of parasite-specific IgG in intestinal mucus. Release of mediators such as leukotrienes leads to marked vasodilation of capillaries. This vasodilation most likely increases leakage of plasma proteins, including immunoglobulins, into the mucus and intestinal lumen. Whether or not IgG physically interacts with nematode larvae and facilitates their removal is unclear. It may be that the increased plasma leakage, mucus production and smooth muscle contraction are the mechanisms responsible for physically trapping larvae and flushing from the intestinal lumen, and high antibody levels in mucus can simply be ascribed to enteric plasma loss into the lumen.

The increased concentrations of mediators in the small intestine, compared to the abomasum, may indicate that the sheep were more effective at expelling *T. colubriformis* than they were at expelling *T. circumcincta*. It is also possible that the lower mediator concentrations may be because of rapid destruction of the lipid compounds due to the reduced pH in the abomasum, rather than decreased synthesis in the abomasal mucosa. However, the higher numbers of *T. circumcincta* at post-mortem examination support the hypothesis that there is an increased immune response in the small intestine. Whether *T. colubriformis* or *T. circumcincta* L_3 are more or less responsible for the induction of immune-mediated scouring in winter rainfall regions of Australia needs further investigation. The unique contribution of this experiment is to provide an experimental regime to decrease faecal dry matter in pens, relieving the dependence on limited, seasonal investigations in the field. While it is difficult to get sheep to clinically scour in an animal house, this experimental regime provides a satisfactory replication of faecal softening that should allow me to investigate the causal relationships between larval challenge, the inflammatory immune response and diarrhoea. These relationships will be addressed in the following chapter.

Chapter 5 – Relationships between faecal dry matter, worm burdens, inflammatory cells and mediators in parasite-reistsant sheep

5.1 Introduction

Breeding sheep to be naturally resistant to parasites is a long-term and sustainable solution to the threat of anthelmintic resistance (Karlsson and Greeff, 2006). However, in areas with a Mediterranean, winter-rainfall climate, sheep that are highly resistant to parasite infection may be more prone to immune-mediated scouring. The major parasitic nematodes of sheep in these climatic zones are the abomasal nematode *Teladorsagia circumcincta* and the intestinal nematode *Trichostrongylus colubriformis.* Infection with these parasites leads to scouring (diarrhoea), which is a major management and animal welfare problem due to the build up of faecal material on wool which leads to flystrike (Morley *et al.*, 1976). It has long been considered that diarrhoea was a symptom of a heavy adult worm burden. However, it is now apparent that most parasite-related diarrhoea is due to a hypersensitive immune response to ingested larvae – 'immune-mediated scouring' (Larsen *et al.*, 1999). As a result, breeding sheep on the basis of low worm egg counts (WEC) may bring about a moderate increase in scouring (Bisset *et al.*, 1996, Karlsson *et al.*, 2004).

The immune response of sheep to nematode parasites is heavily mediated by Th2 cytokines such as IL-4, IL-5 and IL-13 (Miller and Horohov, 2006). These cytokines lead to the recruitment of mast cells and eosinophils into the abomasal and intestinal mucosa (Rothwell, 1989). These cells release potent inflammatory mediators, such as histamine, and also arachidonic acid metabolites such as leukotrienes and prostaglandins (Prussin and Metcalfe, 2006). These mediators, as well as potent vasodilators such as bradykinin, probably act to remove worm larvae by causing leakage of plasma protein into the abomasum and intestinal lumen, and contracting non-vascular smooth muscle. A side-effect of this immune response may be diarrhoea.

In this chapter I explore further the mechanisms associated with low worm egg count or 'immune-mediated' scouring in sheep. In chapter four I was able to reproduce faecal softening in sheep that was associated with immunity to an indoor trickle infection of nematode larvae. I found that concentrations of leukotrienes and prostaglandin E_2 (PGE_2) were higher in parasite-infected Merino rams than uninfected controls, and was

accompanied by a concurrent decrease in faecal dry matter and egg counts. In this chapter, I investigated the pattern of faecal dry matter in penned, parasite-resistant Merino rams judged as being susceptible or otherwise to immune-mediated scouring, when challenged with both *T. circumcincta* and *T. colubriformis* L_3. I also quantified numbers of granulocytes and the production of inflammatory mediators (cysteinyl leukotrienes, prostaglandin E_2 and bradykinin) in abomasal and intestinal mucus. It was postulated that rams susceptible to immune-mediated scouring would have reduced faecal dry matter compared to rams that are not susceptible. In addition, I expected that in all challenged sheep there would be negative correlations between granulocytes, inflammatory mediators and both faecal dry matter and worm burdens at post-mortem examination.

5.2 Materials and methods

5.2.1 Experimental design

Fifty Merino rams aged twenty-months were selected from the parasite-resistant Rylington Merino flock (Chapter 3). The rams were selected on the basis of EBV for WEC and field dag scores such that there were ten rams in each of the following four groups – 1) high WEC, high-dag score, 2) high WEC, low-dag score, 3) low WEC, high-dag score 4) low WEC, low-dag score. A fifth group of ten rams acted as an unchallenged control group – its EBV and dag scores were average for the flock (Table 5.1).

The rams were allowed to acclimatise to the animal house for two weeks. The rams in groups 1-4 were then dosed daily with *T. colubriformis* and *T. circumcincta* larvae for six weeks. The control group was not challenged with larvae. After 6 weeks, all rams were necropsied for total worm counts. Tissue and mucus samples were taken from the abomasum and duodenum and analysed for numbers of inflammatory cells and concentrations of cysteinyl leukotrienes, PGE_2 and bradykinin.

Table 5.1. Mean EBV for worm egg count (WEC) and mean dag scores in winter and spring 2006 for rams used in experiment two and flock averages. Dag scores are on a subjective 1-5 scale where 1 is no dag and 5 is severe dag.

	High FEC EBV		Low FEC EBV			Flock
	High dag (n=10)	Low dag (n=10)	High dag (n=10)	Low dag (n=10)	Control (n=10)	Average (n=233)
FEC EBV	-78.3	-80.0	-96.5	-98.5	-91.7	-89.4
Winter dag	2.7	1.5	2.3	1.2	1.7	2.1
Spring dag	3.3	1.2	3.6	1.3	2.1	2.4

5.2.2 Challenge regime

Trichostrongylus colubriformis and *Teladorsagia circumcincta* L_3 were kindly provided by Phillip Stein, Novartis Animal Health, Australia. After the acclimatisation period, rams in groups 1-4 were dosed daily with 500 *T. colubriformis* and 500 *T. circumcincta* L_3. This dose rate was shown in Chapter 4 to elicit both a significant immune response, and also decreased faecal dry matter, in rams from the Rylington Merino flock The larvae were suspended in water and delivered orally using a drench gun.

5.2.3 Sampling procedures

Faecal samples were taken from rams upon arrival at the animal house and WEC performed to ensure sheep were worm-free. During the acclimatisation period, three faecal samples were taken from each sheep at regular intervals four days apart and faecal dry matter was determined as described in Chapter 3. These three values were averaged to determine a baseline, pre-infection value of faecal moisture content for each sheep. Following the commencement of larval dosing, faecal samples were taken weekly for dry matter determination. Three weeks after dosing commenced, weekly faecal samples were also taken for WEC. Six weeks after larval dosing commenced, all fifty rams were euthanised. Tissue and mucus samples were taken at post-mortem examination, and total worm counts were determined as described in Chapter 3.

5.2.4 Histology and ELISA

Tissue samples were processed using standard histological processes and the numbers of eosinophils, mast cells and globule leukocytes counted as described. It was not possible to measure inflammatory mediator concentrations in all fifty rams due to a lack of funds. Therefore, rams were selected randomly from the treatment groups 1-5 for mediator analysis as shown in Table 5.2. The concentrations of cysteinyl leukotrienes, prostaglandin E_2 metabolites and bradykinin in abomasal and small intestinal mucus were determined using commercial ELISA kits. Percentage recoveries from extraction were 77% for leukotrienes, 75% for PGE_2 and 76% for bradykinin. The coefficient of variation of extraction was less than 12%. More than one plate was needed to analyse all the samples. The inter-assay co-efficient of variation between the plates was <15% for all three assay kits. Concentrations of mediators were expressed per mg of protein. Full details of the techniques are provided in Chapter 3.

Table 5.2. The number of sheep in each treatment group that were used for analysis of inflammatory mediators. Treatment groups refer to high or low EBV for WEC (HWEC or LWEC) and high or low field dag scores (HDS or LDS).

	Mediator		
Treatment Group	PGE_2	Leukotrienes	Bradykinin
HWEC/HDS	6	7	7
HWEC/LDS	6	6	7
LWEC/HDS	6	7	7
LWEC/LDS	6	6	7
Control	6	7	7
Total	30	33	35

5.2.5 Statistical analysis

The pattern of faecal dry matter and FEC ($\log_{10}(n+1)$) in groups 1-5 over the course of the experiment was tested using a mixed model in SAS (version 9, SAS Institute Inc, Cary, NC, USA). The fixed model consisted of the main effects and interaction of treatment group (1-5) and week of challenge. The random error term was the individual sheep. Differences between groups at each time-point were examined using differences of least squares means. Total worm burdens ($\log_{10}(n+1)$) were analysed using a two-way ANOVA with FEC EBV and field dag score as main effects. Separate analyses were conducted for each nematode species and for immature and adult worms. To examine the relationships between larval challenge and inflammatory cells and mediators, the challenged sheep were divided into distinct groups based on their total worm burden at post-mortem examination and also their level of faecal dry matter just before necropsy in relation to the median value. Thus, sheep were classified as having a low total worm count (<1200 worms) or high total worm count (>1200 worms) and also low faecal dry matter (<27%) or high faecal dry matter (>27%). One-way ANOVA was then used to test for differences between these groups and also the unchallenged control sheep. A separate ANOVA was used to test for differences between sheep with high

and low worm counts and high and low faecal dry matter. A separate analysis was carried out for each cell type and each mediator. Where appropriate, post-hoc testing was carried out with Fisher's least squares differences test. In the forty challenged sheep, correlations were also sought between the post-mortem data (i.e. granulocytes and mediators) and worm burdens (log_{10} (n+1) transformed numbers of immature and adult *T. colubriformis* and *T. circumcincta*), FEC and faecal dry matter. General linear models (GLM) were constructed with FEC EBV and dag score as fixed effects in the model with the post-mortem parameter (e.g. abomasal mast cells) fitted as a continuous predictor variable (covariate). The model included the interactions between the covariate and the main effects, with the dependent variable being either FDM, FEC or worm burden. For faecal dry matter and FEC, the value used was the final measurement obtained before necropsy. Where the covariate had a significant effect, product-moment correlations were determined between the variables of interest. In the case of a significant interaction between the covariate and fixed factor, separate correlations were calculated for each group within the interacting factor. Correlations between cells and mediators in different segments of the gastrointestinal tract were calculated with Spearman's rank test. GLM and correlations were carried out using the STATISTICA program (Version 9, Statsoft Inc, Tusla, USA). Histogram and fitted-value plots of residuals were used to check for assumptions of normal distribution and homogeneity of variances. P-values of <0.05 were considered significant.

5.3 Results

5.3.1 Faecal dry matter

There were no differences in faecal dry matter (ca 30%) between any of the groups before larval dosing (Figure 5.1). During the challenge period there was no difference between the high and low WEC groups within each dag score category, so data are presented as high-dag score and low-dag score groups. The interaction between treatment group and week was significant. In both challenged groups (high-dag score and low-dag score), faecal dry matter was lower ($P<0.05$) than pre-challenge values at 14 days after infection. The opposite trend was observed in the control group where their faecal dry matter were higher on days 7 and 28 compared with day 0 ($P<0.05$). In comparison with the controls, the faecal dry matter of the high-dag score group was lower (28 vs. 35%) at 7 days after larval dosing commenced ($P<0.05$), and this significant difference persisted at each subsequent sampling (Figure 5.1). The low-dag score group had a lower faecal dry matter than the control group ($P<0.05$) on days 14,

28 and 35 after infection, but the latter 2 times were mainly due to the sharp increase in the faecal dry matter of the control group (Figure 5.1). When challenged groups were compared, the high-dag score group had lower ($P<0.05$) faecal dry matter than the low-dag score group at 14, 21 and 35 days after larval dosing commenced.

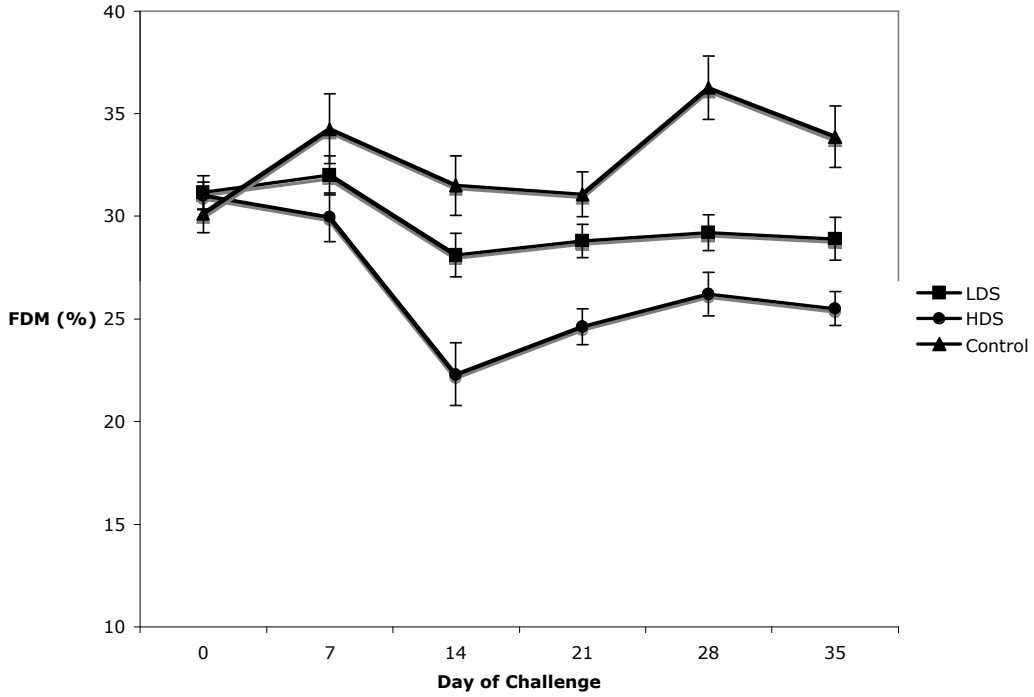

Figure 5.1. Faecal Dry matter (FDM) % (means ± s.e.m) in rams with high-dag scores (HDS, n=20) and low-dag scores (LDS, n=20) during continuous challenge with T. *colubriformis* and T. *circumcincta* L_3. Control rams (n=10) were unchallenged.

5.3.2 Faecal worm egg counts

Worm egg counts were low in all groups throughout the experiment (Table 5.3). High WEC EBV rams had consistently higher values than Low WEC EBV rams but the differences were not significant.

Table 5.3. Faecal worm egg counts (WEC - means ± s.e.m) in low and high WEC EBV and low and high-dag score Merino rams during continuous challenge with T. *colubriformis* and T. *circumcincta* L_3. Back-transformed means and s.e.m are shown.

Week of challenge	WEC		Dag	
	High	Low	High	Low
3	25 ± 7.7	12 ± 6.2	19 ± 7.5	17 ± 6.4
4	103 ± 35.3	62 ± 15.9	91 ± 34.6	69 ± 16.7
5	103 ± 23.1	36 ± 11.4	84 ± 22.7	50 ± 14.6

5.3.3 Total worm counts

Establishment of *T. colubriformis* in all challenged sheep was very low (Table 5.4). There were no significant effects of WEC EBV or dag score, although sheep with low WEC EBV tended to have less adult worms than sheep with high WEC EBV (P=0.09). Greater numbers of *T. circumcincta* were observed in the challenged sheep, and sheep with low WEC EBV had lower developed (P=0.036) and total (P=0.01) fourth stage larvae than sheep with high WEC EBV. There was also an effect of dag score, where sheep with high field dag scores had significantly less *T. circumcincta* early L_4 then sheep with low dag scores (P=0.01). There were no effects of WEC EBV or field dag score on the numbers of adult *T. circumcincta* (Table 5.4).

Table 5.4. Total worm burdens (geometric means with 95% confidence intervals) of sheep with either high or low dag scores (HDS/LDS) or high or low EBV for WEC. EL_4 refers to early fourth stage larvae and DL_4 to developed fourth stage larvae. Within the WEC EBV and dag score categories, * indicates a significant difference between worm burden for that species/stage.

	T. colubriformis			*T. circumcincta*		
	EL_4	DL_4	Adult	EL_4	DL_4	Adult
HWEC EBV	14	14	72	109	234*	518
	(10, 21)	(10, 21)	(34, 157)	(49, 244)	(117, 468)	(268,1001)
LWEC EBV	13	14	31	41	66*	404
	(9, 18)	(10, 21)	(18, 56)	(21, 78)	(28, 159)	(188, 866)
HDS	14	12	58	34*	107	348
	(10,21)	(9, 18)	(32, 107)	(17, 69)	(48, 242)	(146, 830)
LDS	12	16	39	130*	145	600
	(9, 18)	(10, 25)	(18, 85)	(64, 261)	(62, 340)	(367, 980)

5.3.4 Inflammatory cells and mediators

Challenged sheep had higher concentrations of all inflammatory cells and mediators than unchallenged sheep and these differences were significant except in the case of leukotrienes, PGE_2 in the abomasum and bradykinin in the small intestine (Tables 5.5 & 5.6). There was a trend for sheep with low total worm counts to have higher levels of mediators and more inflammatory cells than sheep with high total worm counts in both the abomasum and small intestine (Table 5.5). However, the only significant difference was that sheep with low total counts had higher concentrations of bradykinin in abomasal mucus. When sheep with low and high faecal dry matter were compared, the only significant difference was that sheep with low faecal dry matter had more eosinophils in the small intestine (Table 5.6). However, there was a slight trend for sheep with low faecal dry matter to have higher levels of mediators and cells, apart from bradykinin in the abomasum and globule leukocytes in the small intestine.

5.3.5 Correlations between traits

Eosinophils in the small intestine were negatively correlated with faecal dry matter but there was a three-way interaction with FEC EBV and dag score. Subsequent correlation analysis revealed that eosinophils were not correlated with faecal dry matter in sheep that had both low dag scores and also low FEC EBV. There was a negative relationship between eosinophils and faecal dry matter in the other three groups of challenged sheep. The overall correlation between eosinophils and faecal dry matter was -0.45 ($P=0.001$, $n=40$) but this strengthened to -0.62 ($P<0.001$, $n=30$) when the ten sheep in the low dag/low FEC EBV group were excluded (Figure 5.2). There were no significant correlations between FEC and cells or mediators but levels of leukotrienes, mast cells and eosinophils in the abomasum tended to be negatively correlated with FEC (Table 5.7). Only globule leukocytes in the small intestine were negatively correlated with numbers of immature and adult *T. colubriformis*. The majority of the significant correlations and trends were with *T. circumcincta* burdens, particularly adult worms. These correlations were all negative, i.e. sheep with high numbers of cells and/or mediators had fewer worms. These correlations were significant in the case of globule leukocytes and leukotrienes in the small intestine (Figures 5.3 and 5.4). The concentrations of bradykinin in the abomasum interacted with dag score, whereby bradykinin was significantly correlated with adult *T. circumcincta* but only in sheep with low dag scores. Finally, there was a three-way interaction between dag score, FEC EBV and concentrations of PGE_2 in the small intestine. Negative correlations were observed between adult *T. circumcincta* and PGE_2 in sheep that had either both high dag scores and high FEC EBV or both low dag scores and low FEC EBV (Figure 5.5).

Table 5.5. Concentrations of mediators in mucus (pg/mg protein) and inflammatory cells (cells/mm^2 tissue) from the abomasum and small intestine of unchallenged rams (control), challenged rams with high total worm counts (>1200 worms,) and challenged rams with low total worm counts (<1200 rams). Values presented are means ± s.e.m and values within rows followed by a different superscript are different ($P<0.05$).

	Abomasum		
	Control	High worm count	Low worm count
Prostaglandin E_2	34 ± 5.8	123 ± 28.4	150 ± 45.5
Leukotrienes	289 ± 54.1	473 ± 84.2	560 ± 150.9
Bradykinin	39 ± 13.5^{A}	71 ± 9.3^{A}	160 ± 34.3^{B}
Mucosal mast cells	33 ± 5.3^{A}	57 ± 9.2^{AB}	86 ± 15.4^{B}
Globule leukocytes	13 ± 2.2^{A}	38 ± 5.2^{B}	47 ± 6.3^{B}
Eosinophils	14 ± 2.4^{A}	40 ± 3.8^{B}	46 ± 12.3^{B}
	Small Intestine		
	Control	High worm count	Low worm count
Prostaglandin E_2	48 ± 16.9^{A}	160 ± 19.3^{B}	163 ± 24.8^{B}
Leukotrienes	567 ± 159.2	917 ± 154.8	1016 ± 139.2
Bradykinin	43 ± 16.2	96 ± 18.1	143 ± 35.4
Mucosal mast cells	52 ± 6.4^{A}	109 ± 11.3^{B}	134 ± 18.1^{B}
Globule leukocytes	25 ± 4.2^{A}	44 ± 6.1^{B}	57 ± 6.4^{B}
Eosinophils	30 ± 4.8^{A}	92 ± 10.4^{B}	87 ± 8.9^{B}

Table 5.6. Concentrations of mediators in mucus (pg/mg protein) and inflammatory cells (cells/mm^2 tissue) from the abomasum and small intestine of unchallenged rams (control), challenged rams with high faecal dry matter (FDM, >27%) and challenged rams with low faecal dry matter (<27% rams). Values presented are means ± s.e.m and values within rows followed by a different superscript are different (P<0.05).

	Abomasum		
	Control	High FDM	Low FDM
Prostaglandin E_2	34 ± 5.8	133 ± 25.2	141 ± 54.6
Leukotrienes	289 ± 54.1	389 ± 83.9	620 ± 136.8
Bradykinin	39 ± 13.5^{A}	134 ± 41.8^{B}	83 ± 10.5^{AB}
Mucosal mast cells	33 ± 5.3^{A}	64 ± 11.5^{AB}	79 ± 13.5^{B}
Globule leukocytes	13 ± 2.2^{A}	36 ± 4.6^{B}	49 ± 6.1^{B}
Eosinophils	14 ± 2.4^{A}	40 ± 7.5^{B}	46 ± 10.3^{B}
	Small Intestine		
	Control	High FDM	Low FDM
Prostaglandin E_2	48 ± 16.9^{A}	160 ± 15.8^{B}	175 ± 27.7^{B}
Leukotrienes	567 ± 159.2	849 ± 95.6	1118 ± 153.8
Bradykinin	43 ± 16.2	105 ± 20.7	125 ± 29.8
Mucosal mast cells	52 ± 6.4^{A}	113 ± 12.5^{B}	129 ± 18.1^{B}
Globule leukocytes	25 ± 4.2^{A}	51 ± 6.6^{B}	50 ± 5.1^{B}
Eosinophils	30 ± 4.8^{A}	76 ± 8.2^{B}	103 ± 9.8^{C}

Table 5.7. Summary of general linear models showing variables and interactions that contributed to significant (P<0.05) or a trend (<0.1) in variation of faecal dry matter (FDM), WEC and worm burden. Significant P values are in bold and italicised.

Model	Covariate	n	F-ratio	β covariate coefficient (±s.e)	P-value
FDM	EOS (SI)*Dag*EBV	40	7.96		***0.01***
WEC	EOS (A)	40	3.04	-0.27 ± 0.15	0.09
	GL (A)	40	4.65	-0.44 ± 0.19	0.06
	LT (A)	26	2.81	-0.32 ± 0.19	0.098
Tric L_4	GL (SI)	40	5.4	-0.36 ± 0.15	***0.026***
Tric Adult	GL (SI)	40	6.46	-0.38 ± 0.14	***0.016***
Tela L_4	GL (SI)	40	5.33	-0.33 ± 0.14	***0.024***
	LT (SI)	26	2.37	-0.3 ± 0.18	0.09
Tela Adult	MC (A)	40	3.91	-0.32 ± 0.16	0.056
	MC (SI)	40	2.93	-0.28 ± 0.16	0.09
	GL (A)	40	2.97	-0.3 ± 0.17	0.089
	GL (SI)	40	4.45	-0.33 ± 0.15	***0.042***
	EOS (A)	40	4.1	-0.33 ± 0.16	0.051
	LT (SI)	26	9.1	-0.57 ± 0.19	***0.006***
	BK (SI)	28	3.39	-0.36 ± 0.19	0.07
	PGE (SI)*Dag*EBV	24	8.88		***0.002***
	BK (A) *Dag	28	6.84		***0.01***

Tric refers to *T. colubriformis* and *Tela* refers to *T. circumcincta*. MC – mast cells, GL – globule leukocytes, EOS – eosinophils, PGE – Prostaglandin E_2, LT – leukotrienes, BK – bradykinin. 'A' refers to cells/mediators from abomasum and 'SI' to small intestine. EBV refers to WEC EBV and dag to field dag scores (See Table 5.1).

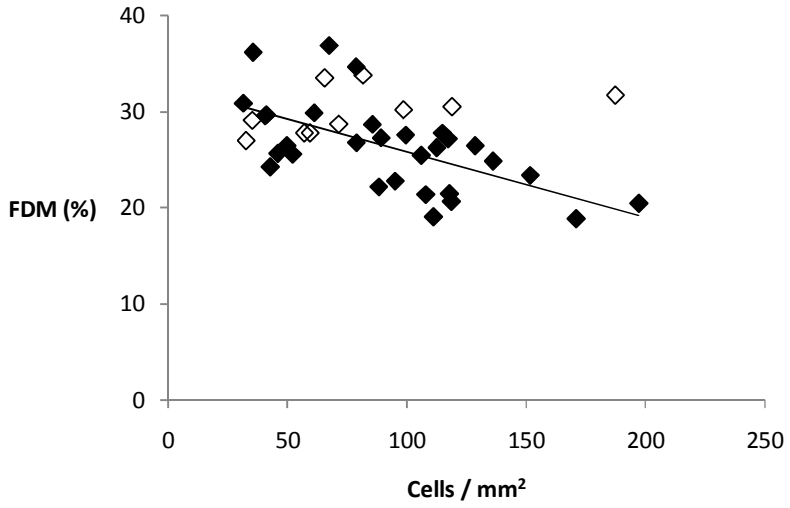

Figure 5.2. Relationship between faecal dry matter (FDM) and eosinophils in small intestinal tissue from sheep with low dag scores and also low FEC EBV (◊, n=10, r=0.45, P=0.3) and sheep with high dag scores or low dag scores but high FEC EBV (♦, n=30, r = -0.62, P<0.001).

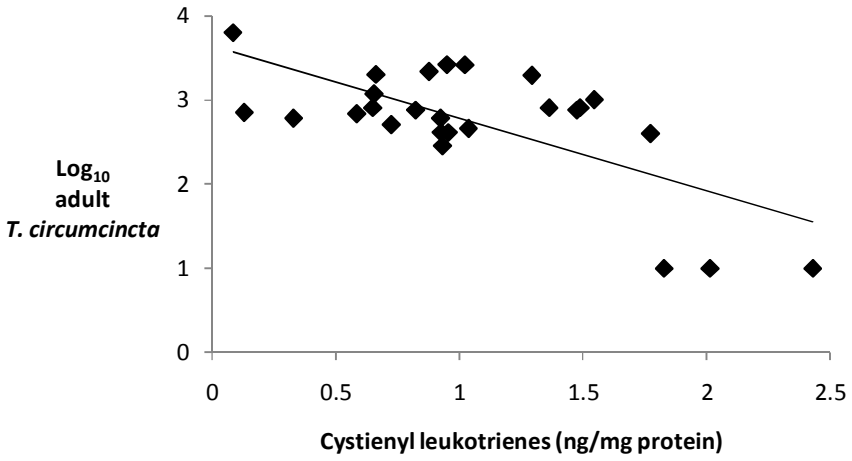

Figure 5.3. Relationship between concentration of cystienyl leukotrienes in small intestinal mucus and numbers of adult *T. circumcincta.* r = -0.56, P=0.003, n=26.

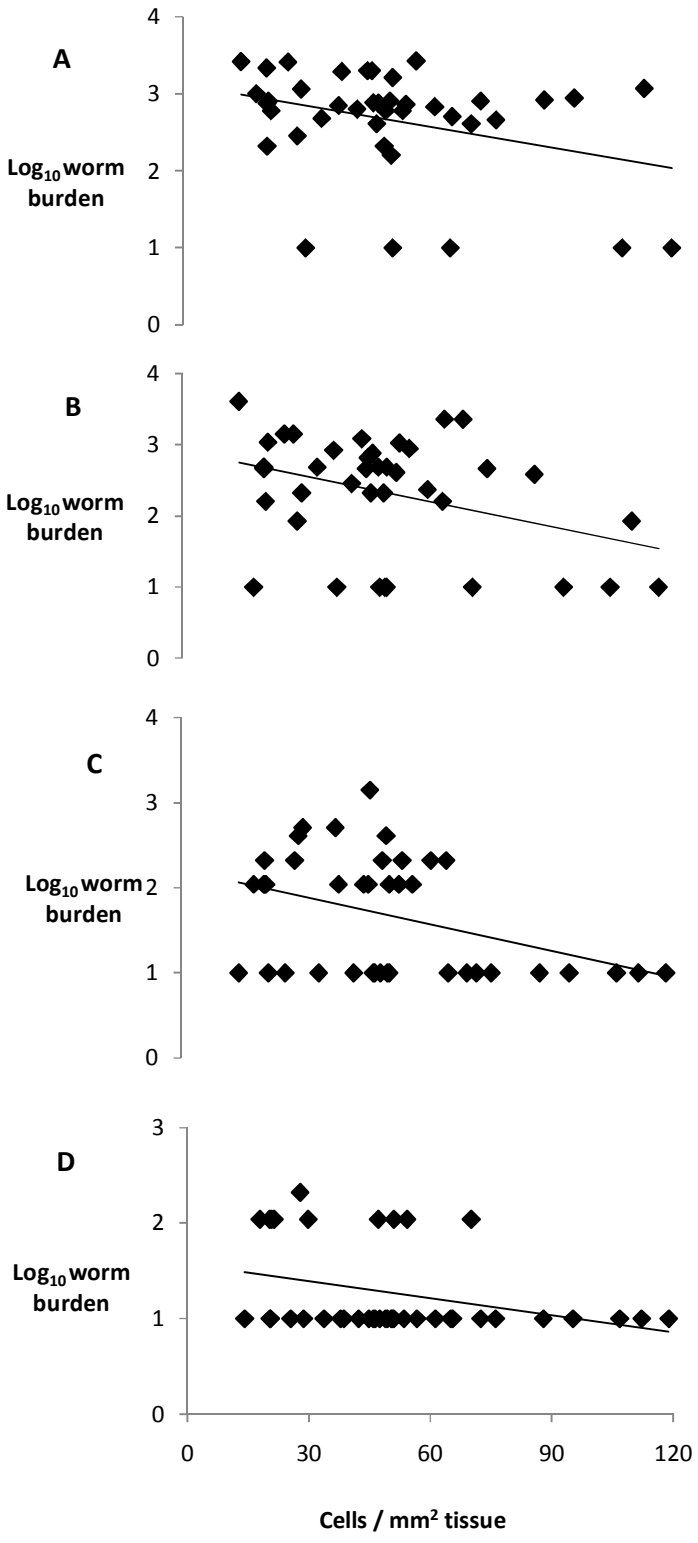

Figure 5.4. Relationships between globule leukocytes in small intestinal tissue and A) adult *T. circumcincta,* r = -0.35, P=0.03 B) immature *T. circumcincta,* r = -0.38, P=0.017 C) adult *T. colubriformis,* r = -0.4, P=0.011 and D) immature *T. colubriformis,* r = -0.33, P=0.037. For all relationships n=40.

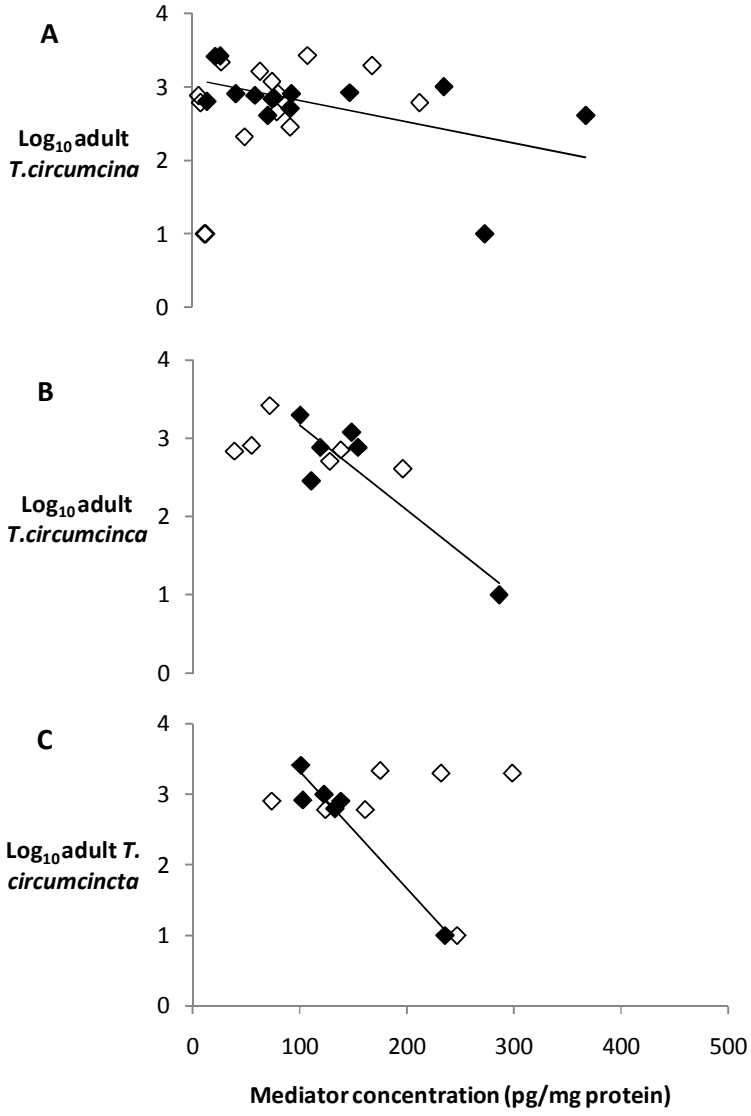

Figure 5.5. Relationships between numbers of adult *T. circumcincta* and concentrations of A) bradykinin in abomasal mucus from sheep with low dag scores (♦, n=14, r = -0.55, P=0.044) and sheep with high dag scores (◊, n=14, r= 0.41, P=0.18) B) PGE_2 in small intestinal mucus from sheep with high FEC EBV and high dag scores (♦, n=6, r = -0.9, P=0.016) or high FEC EBV and low dag scores (◊, n=6, r = -0.54, P=0.26) and C) PGE_2 in small intestinal mucus from sheep with low FEC EBV and low dag scores (♦, n=6, r = -0.97, P=0.001) and low FEC EBV and high dag scores (◊, n=6, r = -0.12, P=0.79).

Correlations between cells and mediators

There were no correlations between the concentrations of PGE_2 and leukotrienes in the abomasum and the corresponding concentration in the small intestine (data not shown). However, concentrations of bradykinin in the abomasum were correlated (r = 0.56, P=0.006) with concentrations of bradykinin in the small intestine. For all three inflammatory cell types, the numbers of cells in the abomasum were positively correlated with the corresponding numbers of cells in the small intestine (Table 5.8). Numbers of globule leukocytes were positively correlated (P<0.001) with mast cells in both the abomasum and small intestine. There was a positive correlation (P<0.05) between the numbers of globule leukocytes and eosinophils in the abomasum.

Table 5.8. Correlations (Spearmans, n=40) between inflammatory cells in the abomasum and small intestine. MC – mast cells, GL – globule leukocytes, EOS - eosinophils ^P<0.1, *P<0.05, **P<0.01

		Abomasum			Small intestine		
		MC	GL	EOS	MC	GL	EOS
Abomasum	MC	-	0.56**	0.13	0.27^	0.14	0.15
	GL	0.56**	-	0.34*	0.34*	0.35*	0.19
	EOS	0.13	0.34*	-	0.17	0.3^	0.36*
Small intestine	MC	0.27^	0.34*	0.17	-	0.49**	0.03
	GL	0.14	0.35*	0.3^	0.49**	-	0.01
	EOS	0.15	0.19	0.36*	0.03	0.01	-

Concentrations of bradykinin in the abomasum was positively correlated (P<0.05) with numbers of mast cells in the abomasum, and also globule leukocytes in both the abomasum and small intestine (Table 5.9). Concentrations of leukotrienes in the small intestine were positively correlated (P<0.05) with numbers of globule leukocytes in the abomasum. There were no other significant correlations, although there was a trend

(P<0.1) for concentrations of PGE_2 in the small intestine to be positively correlated with numbers of mast cells and globule leukocytes in the abomasum.

Table 5.9. Correlations (Spearman) between inflammatory mediators in the abomasum small intestine. Prostaglandin E_2 (PGE_2) – n=24, leukotrienes (LT) - n=26, bradykinin (BK) – n=28. MC – mast cells, GL – globule leukocytes, EOS – eosinophils ^P<0.1, *P<0.05, **P<0.01

		Abomasum			Small intestine		
		PGE_2	LT	BK	PGE_2	LT	BK
Abomasum	MC	0.05	0.1	0.43*	0.35^	-0.02	-0.04
	GL	0.21	0.37^	0.4*	0.34^	0.41*	0.21
	EOS	-0.02	0.08	0.14	-0.01	-0.001	0.23
Small intestine	MC	0.02	0.27	0.18	-0.08	0.07	0.05
	GL	0.04	0.2	0.41*	-0.1	0.15	0.34^
	EOS	-0.06	-0.1	0.28	-0.1	-0.2	0.09

5.4 Discussion

Rams were able to effectively resist the larval challenge, as evidenced by the small total worm counts, with numbers of *T. colubriformis* particularly low. However, this immunity to the larval challenge may also result in more fluid faeces. Inflammatory cells and mediators (except prostaglandin E_2) were associated with low WEC and/or worm numbers. Eosinophils in the small intestine were negatively correlated with faecal dry matter, and there was a general trend for inflammatory mediators to be higher in sheep with low faecal dry matter, but this did not reach significance. Therefore, my hypothesis was partially supported by these results.

I found that when parasite-resistant sheep were challenged with nematode larvae, faecal dry matter was significantly lower than in unchallenged sheep and was lowest in those sheep deemed susceptible to immune-mediated scouring on the basis of dag scores. This supports the first part of my hypothesis. The lower *T. circumcincta* burdens in sheep with high dag scores indicate that scouring in immune sheep is most probably due to how sheep react to the larval challenge, rather than the size of their adult worm burden. These results show that dag scores observed in immune sheep in the field are related to a reduction in faecal dry matter, at least partly caused by challenge with nematode larvae.

Sheep with low faecal dry matter had significantly higher numbers of eosinophils in the small intestine than sheep with high faecal dry matter. Eosinophils in the small intestine were not correlated with worm numbers. However, eosinophils in the abomasum were negatively correlated with WEC and researchers in previous studies have shown that high numbers of eosinophils are associated with low worm burdens (Douch *et al.*, 1986, Stevenson *et al.*, 1994, Bisset *et al.*, 1996). Recent opinion in human medical studies is that eosinophils are involved in the rejection of intestinal parasites, but may also have detrimental consequences (such as diarrhoea) for the host (Jacobsen *et al.*, 2007, Bruschi *et al.*, 2008). Larsen *et al.* (1994) also found higher numbers of eosinophils in the small intestine of Merino sheep with high-dag scores. This suggests that eosinophils are involved in the rejection of nematode larvae, but can also lead to diarrhoea as a side-effect.

I found a consistent trend for inflammatory mediators and mast cells/globule leukocytes to be negatively correlated with both WEC and total numbers of *T. circumcincta.* Only globule leukocytes in the small intestine were correlated with numbers of *T.*

colubriformis, but this is probably as most sheep had very low numbers with several sheep being completely free of all stages. Thus, there was probably not enough variation for meaningful correlations to be calculated. To my knowledge, this is the first measurement of bradykinin production in sheep in response to nematode larval challenge and the results suggest that it is significantly involved in larval rejection. Sheep with low total worm counts had significantly more bradykinin in abomasal mucous, and there was a strong negative correlation between bradykinin in intestinal mucous and numbers of *T. circumcincta.* Increased levels of leukotrienes were significantly correlated with low numbers of *T. circumcincta*. Other researchers have demonstrated that leukotrienes are increased in sheep bred for low WEC compared to susceptible animals (Gray *et al.*, 1992, Jones *et al.*, 1994). In addition, leukotrienes are associated with increased larval migration inhibitory activity in mucus (Douch *et al.*, 1996; D.L. Emery, unpublished data). The negative correlations between globule leukocytes and numbers of *T. circumcincta* and *T. colubriformis* are evidence of their close association with larval rejection and are consistent with previous work (Douch *et al.*, 1986, Stankiewicz *et al.*, 1995, Meeusen *et al.*, 2005, Sykes *et al.*, 2007). PGE_2 may also have some role in this process as evidenced by the negative correlations with *T. circumcincta* burdens in the current experiment. Therefore, the inflammation associated with larval challenge is a major mechanism of worm rejection.

Although not significant, there was also evidence for a trend whereby inflammatory mediators were negatively correlated with faecal dry matter. Sheep with low faecal dry matter had numerically higher levels of all mediators than sheep with high faecal dry matter, with the exception of bradykinin in the abomasum. However, high between-animal variation in the results of the assays meant that standard errors were high and statistical significance was not reached. This experiment could perhaps be expanded to include more animals. Alternatively, the use of antagonists specific for mediator receptors could be used to see if faecal softening or larval establishment is affected when mediator production is inhibited. This approach has clearly demonstrated the involvement of leukotrienes, prostaglandins and bradykinin in the pathology of asthma (Abraham *et al.*, 1991, Farmer *et al.*, 1992). The mediators that I measured in this experiment are all potent vasodilators and stimulate contraction of non-vascular smooth muscle, and they have been implicated in immune-mediated diarrhoea in humans (Farthing, 2003, Nielsen *et al.*, 1988). Therefore, inflammatory mediators released from granulocytes may be involved in the pathogenesis of immune-mediated scouring.

The positive correlation between bradykinin and globule leukocytes in both the abomasum and small intestine clearly demonstrates that bradykinin is released during the degranulation of mast cells and probably contributes to the rapid expulsion of incoming larvae. In addition to being released by mast cells, bradykinin can also stimulate degranulation itself by binding to two distinct receptors on both mast cells and eosinophils (Bandeira-Melo *et al.*, 1999). The positive correlations between bradykinin, and also inflammatory cells in the different segments of the gut indicates that *T. circumcincta* and *T. colubriformis* both elicit a similar type of immune response from the host. It is also interesting to note the positive correlation in the abomasum between globule leukocytes and eosinophils. This is in contrast to Balic *et al.* (2006) who found a negative correlation between eosinophils and globule leukocytes in the abomasum of sheep challenged with *H. contortus*. The difference in these results may be due to the worm species – *H. contortus* lives mainly in the lumen of the gastric pit while *T. circumcincta* penetrates and resides deeper in the mucosa. This may influence the dynamics of the cellular immune response. Alternatively, the difference may be due to the larval challenge regime. In my experiment sheep were under daily challenge for six weeks while in that of Balic *et al.* (2006) they were necropsied 24 or 48 hours after a single challenge dose. The regime used in my experiment more closely resembles the natural challenge faced by sheep in the field. The results here indicate that the inflammatory cells and mediators that are produced in response to larval challenge ultimately play complementary roles in immunity and worm rejection.

All sheep had very low numbers of *T. colubriformis*, but sheep with high EBV for WEC had significantly higher numbers of *T. circumcincta* L_4 than those sheep with low EBV for WEC. There was also a trend for sheep with a low EBV for WEC to have a low WEC following challenge, especially by week 5, consistent with a faster possible depression of worm fecundity. This confirms that EBV calculated for low WEC are repeatable following artificial larval challenge. The higher establishment of *T. circumcincta*, compared to *T. colubriformis,* observed in this experiment is similar to my results in Chapter 4. This indicates that two independent immune mechanisms may be operating towards the two species. This could be important in designing breeding programs for parasite resistance, and warrants further investigation. My results suggest that sheep in the parasite-resistant Rylington flock are all highly immune to *T. colubriformis*, and the majority of variation in WEC seems to derive from resistance to *T. circumcincta.*

Sheep with low EBV for WEC and also low field dag scores maintained these characteristics during this experiment. That is, they had higher faecal dry matter than sheep with high-dag scores but also lower worm burdens than sheep with high EBV for WEC. This is an encouraging result, as it confirms that sheep can be bred to be resistant to worms and not prone to scouring. Total worm numbers were similar between sheep in the low WEC EBV group, regardless of their field dag scores. This shows that sheep with low WEC and low-dag scores regulate their worm burdens in a similar way to sheep with high-dag scores. Therefore, it could be postulated that scouring in sheep with low WEC is an allergic response that is completely inappropriate, and unnecessary, for worm expulsion. However, it has been consistently shown that, in winter-rainfall areas, sheep selectively bred for low WEC have an increase in dags (Bisset *et al.*, 1997, Karlsson *et al.*, 2004)

It is likely then that the immune response that results in low WEC is also associated with scouring, and the magnitude of the immune response determines both the level of resistance (low WEC) and the severity of scouring (dag scores). The reason(s) why some sheep are able to have an effective immune response, without suffering scouring, are not clear. The interaction I observed between intestinal eosinophil numbers, dag scores and WEC EBV suggests that there is an underlying physiological difference in the sheep that have both low WEC and low dag scores. In other words, they have a similar eosinophil response to sheep with high dag scores but this does not lead to diarrhoea, perhaps due to a better capacity to reabsorb water and soluble nutrients in the colon. This does not explain why the sheep with low dag scores and low WEC EBV were different to those that also had low dag scores but high WEC EBV, especially given that in my experiment intestinal eosinophils had no relationship with worm burdens. Despite this complexity, it appears that there is some physiological difference that can be exploited by selective breeding. The interaction observed between bradykinin and dag scores, whereby bradykinin was only correlated with numbers of *T. circumcincta* in sheep with low dag scores, may also suggest some fundamental difference between scouring and non-scouring sheep. The encouraging result from this chapter is that both EBV for low WEC, and low-dag scores, appear to be repeatable and will allow phenotypic selection of non-scouring resistant animals until other methods become available to identify susceptible sheep.

Chapter 6 – Serum antibodies and interleukin-5 are increased in sheep during larval challenge but are not correlated with faecal dry matter

6.1 Introduction

In Chapters 4 and 5, I established that a trickle larval challenge decreases faecal dry matter in parasite-resistant sheep, in the absence of significant worm establishment. This suggests that the inflammatory response to incoming larvae is responsible for scouring in resistant sheep. However, some sheep were able to resist the larval challenge without a significant softening of the faeces. There is a need to further investigate the mechanisms of immune-mediated scouring, so sheep can be identified that are resistant to nematodes and not prone to scouring.

In this chapter I describe correlations between serum immunoglobulins and interleukin-5 levels and worm burdens and faecal dry matter in parasite-resistant sheep. Interleukin-5 is the major cytokine involved in eosinophil recruitment and proliferation (Prussin and Metcalfe, 2006) and in Chapter 5 I found that sheep with low faecal dry matter had increased numbers of eosinophils in the small intestine. Antigen-specific immunoglobulins are a hallmark of acquired immunity to parasite infections (Miller and Horohov, 2006). Quantifying the levels of these parameters and their relationship with faecal dry matter will increase our understanding of the mechanisms involved in immune-mediated scouring in sheep. In addition, identifying parameters in serum samples that are correlated with diarrhoea offers the possibility of non-invasive methods of identifying parasite-resistant sheep that are prone to scouring. My hypothesis is that serum antibody and interleukin-5 levels will be negatively correlated with both faecal dry matter and worm numbers at post-mortem examination.

6.2 Materials and methods

6.2.1 Experimental design

The experimental design is the same as in Chapter 5. All fifty rams used in the experiment described in Chapter 5 were blood sampled before larval challenge began. They were blood sampled again two and four weeks after larval challenge commenced. Blood was collected by venipuncture of the jugular vein and collected into EDTA tubes.

Within two hours of collection, blood was centrifuged and the serum collected and stored at -20ºC. A breakdown in the freezer meant that only a single pre-challenge and post-challenge sample from each ram could be analysed. The levels of ovine IgG_1, IgM, IgA, IgE and total antibody (light chain) were determined against both *T. colubriformis* and *T. circumcincta* antigen using sandwich ELISA as described in Chapter 3. IL-5 was also measured using sandwich ELISA as described in Chapter 3. As mentioned in Chapter 3, IgE specific for *T. circumcincta* antigen was not determined as no reaction was observed using the available monoclonal antibody. Concentrations of immunoglobulins and IL-5 were expressed as ELISA units relative to a positive control (high-reading sera) run on every plate (see Chapter 3).

6.2.2 Statistical analysis

A paired t-test (Microsoft Excel 2007) was used to examine the difference in means between the pre-larval challenge and post-larval challenge for levels of immunoglobulins and IL-5. A separate test was conducted for the ten control sheep and the forty challenged sheep. GLM were used to test for associations between the serum parameters and faecal dry matter, WEC and worm burdens. WEC EBV and dag score were fitted as fixed effects in the model while the serum parameter (e.g. IL-5) was fitted as a continuous predictor variable (covariate). The model included the interactions between the covariate and the main effects, with the dependent variable being either FDM, WEC or the post-mortem data (Immature stages (total L_4) and adult worms for *T. colubriformis* and *T. circumcincta*). Only data from the forty challenged sheep was used in the models. For FDM and WEC, the value taken on the same day as post-challenge blood sampling and also the value from seven days later were tested as separate variables. Where the covariate had a significant effect, product-moment correlations were determined between the variables of interest. In the case of a significant interaction between the covariate and fixed factor, separate correlations were calculated for each group within the interacting factor. WEC and worm burden data was transformed ($\log_{10}$ n+10) prior to analysis. ELISA units generally did not require transformation with the exception of *T. colubriformis*-specific IgA which was skewed and also subjected to a $\log_{10}$ transformation prior to analysis. GLM and correlations were carried out using the STATISTICA program (Version 9, Statsoft Inc, Tusla, USA). P-values of <0.05 were considered significant.

6.3 Results

6.3.1 Larval challenge and serum antibody concentrations

In the control (unchallenged) sheep, there were no differences in serum antibody concentrations between the pre-challenge and post-challenge period. In the challenged sheep, concentrations were significantly higher in the post-challenge period for all antibodies except light chain specific for *T. colubriformis* (Tables 6.1 & 6.2). Similarly, IL-5 was higher in the post-challenge period for those sheep that received the larval challenge while in the control group there was no change (Table 6.3).

Table 6.1. ELISA units (means ± s.e.m) for serum antibodies against *T. colubriformis* in Merino rams either challenged with a mixture of *T. colubriformis* and *T. circumcincta* (n=40), or unchallenged controls (n=10). P-value (paired t-test) is comparing pre- and post- challenge values (four weeks) within control and challenged categories.

		ELISA Units		
		Pre-challenge	Post-challenge	P-value
Light Chain	Control	54 ± 2.7	66 ± 9.6	NS
	Challenged	58 ± 3.2	64 ± 3.5	NS
IgG_1	Control	69 ± 8	72 ± 6.7	NS
	Challenged	76 ± 4.4	95 ± 4.3	0.001
IgM	Control	49 ± 6.4	56 ± 5.3	NS
	Challenged	56 ± 3.5	88 ± 4.9	<0.0001
IgA	Control	11 ± 2.5	10 ± 1.7	NS
	Challenged	13 ± 2.6	27 ± 5.7	0.017
IgE	Control	77 ± 8.4	74 ± 9.4	NS
	Challenged	66 ± 4.5	87 ± 4.7	0.001

Table 6.2. ELISA units (means ± s.e.m) for serum antibodies against *T. circumcincta* in Merino rams either challenged with a mixture of *T. colubriformis* and *T. circumcincta* (n=40), or unchallenged controls (n=10). P-value (paired t-test) is comparing pre- and post- challenge values (four weeks) within control and challenged categories.

		ELISA Units		
		Pre-challenge	Post-challenge	P-value
Light Chain	Control	52 ± 5.1	55 ± 5.8	NS
	Challenged	52 ± 2.5	68 ± 2.8	<0.0001
IgG_1	Control	72 ± 8.9	71 ± 6.7	NS
	Challenged	63 ± 4.1	81 ± 2.9	<0.0001
IgM	Control	47 ± 3.2	53 ± 2.9	NS
	Challenged	48 ± 2.6	59 ± 3.1	0.001
IgA	Control	141 ± 18.5	142 ± 11.6	NS
	Challenged	123 ± 9.5	152 ± 10.2	0.005

Table 6.3. ELISA units (mean ± s.e.m) for serum IL-5 in Merino rams challenged with a mixture of *T. colubriformis* and *T. circumcincta* (n=40), or unchallenged controls (n=10). P-value (paired t-test) is comparing pre- and post- challenge values (four weeks) within control and challenged categories

		ELISA Units		
		Pre-challenge	Post-challenge	P-value
IL-5	Control	72 ± 11.7	87 ± 8.2	NS
	Challenged	59 ± 5.1	89 ± 6.4	<0.0001

6.3.2 Associations between serum parameters and FDM, WEC and worm burden

There were no significant correlations between faecal dry matter or WEC and any serum parameter measured (data not shown). There were several significant relationships between serum antibodies and numbers of immature and adult *T. circumcincta* (Table 6.4) but there was no association with the concentration of IL-5. The majority of these relationships were negative, i.e. sheep with less worms had higher concentrations of antibody in serum (Figures 6.1 and 6.2). However, there was a positive association between *T. colubriformis*-specific IgG_1 and numbers of immature *T. circumcincta* (Figure 6.3). There was a significant interaction between *T. colubriformis*-specific IgA and WEC EBV in the analysis of both adult and immature *T. circumcincta.* Subsequent correlation analysis showed that there was a negative association between IgA and numbers of *T. circumcincta* but only in the sheep selected as having low EBV for WEC (Figure 6.2). Also, *T. colubriformis*-specific IgM interacted with dag score – IgM was negatively correlated with numbers of adult *T. circumcincta* in sheep with high dag scores but not in those with low dag scores (Figure 6.4). No significant relationships or trends were observed in the analysis of *T. colubriformis* burdens (data not shown).

Table 6.4. Summary of general linear models showing serum parameters and interactions that contributed to significant (P<0.05) or a trend (<0.1) in variation of immature and adult *T. circumcincta* worm burden. Significant P values are in bold and italicised. *Tric* refers to antibody specific for *T. colubriformis* and *Tela* refers to antibody specific for *T. circumcincta*. Dag refers to field dag score and WEC refers to EBV for WEC. AB refers to total antibody.

Model	Covariate	F –ratio	β covariate coefficient (± s.e.)	P-value
Immature	*Tric*_IgG_1	5.01	0.31 ± 0.15	***0.044***
	*Tric*_IgA* WEC	4.53		***0.041***
Adult	*Tric*_AB	2.97	-0.29 ± 0.17	0.094
	*Tela*_AB	4.69	-0.34 ± 0.15	***0.038***
	*Tela*_IgG_1	17.14	-0.57 ± 0.13	***<0.001***
	*Tela*_IgM	5.98	-0.4 ± 0.16	***0.02***
	*Tela*_IgA	1.62	-0.31 ± 0.17	0.075
	*Tric*_IgM*Dag	4.58		***0.04***
	*Tric*_IgA*WEC	12.64		***<0.001***

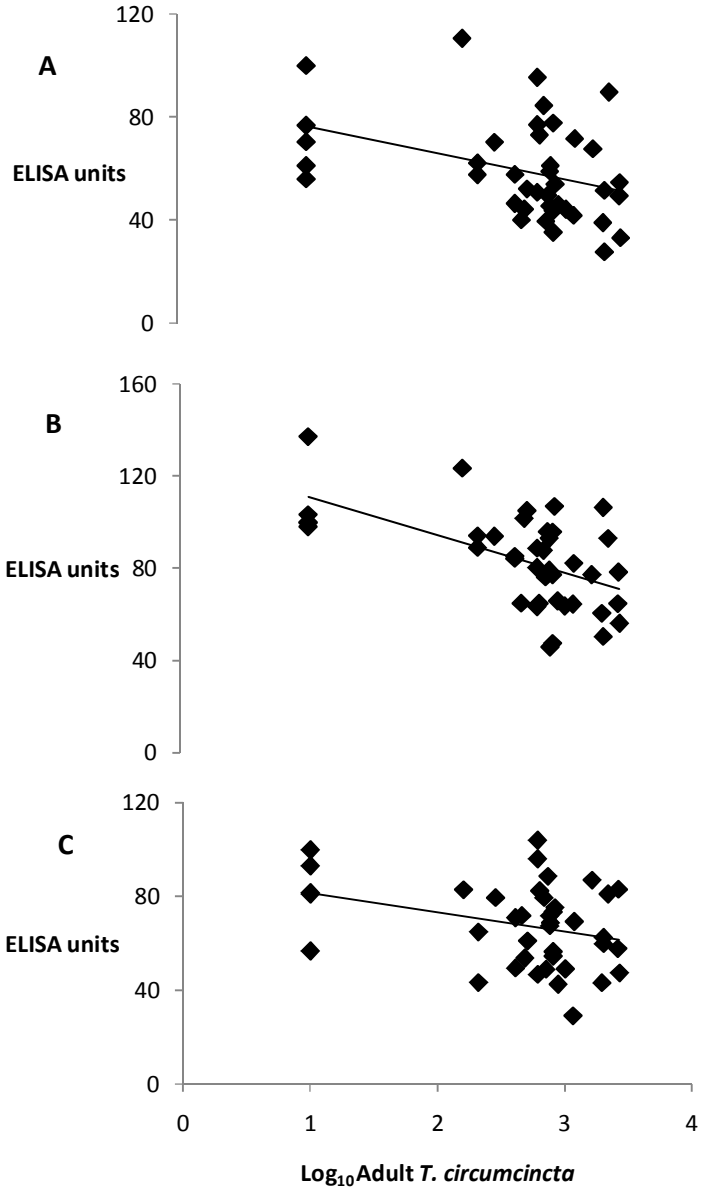

Figure 6.1. Scatter plot of significant relationships between $\log_{10}$ transformed numbers of adult *T. circumcincta* (n=40) and concentrations of *T. circumcincta* specific A) total antibody (r = -0.33, P=0.04), B) IgG_1 (r = -0.56, P<0.001) and C) IgM (r = -0.38, P=0.016).

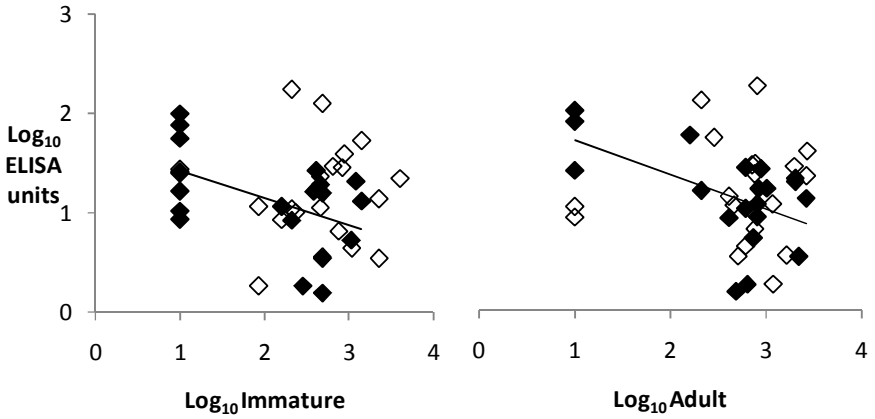

Figure 6.2 Scatter plot of relationships between $\log_{10}$ transformed numbers of immature and adult *T. circumcincta* and $\log_{10}$ *T. colubriformis*-specific IgA in sheep with low EBV for WEC (♦, n=20) and high EBV for WEC (◊, n=20). For low EBV sheep, r = -0.5 (P=0.024) for immature and r = -0.54 (P=0.042) for adult worms. For high WEC EBV sheep, r = 0.02 (P=0.95) for immature and r = 0.05 (P=0.9) for adult worms.

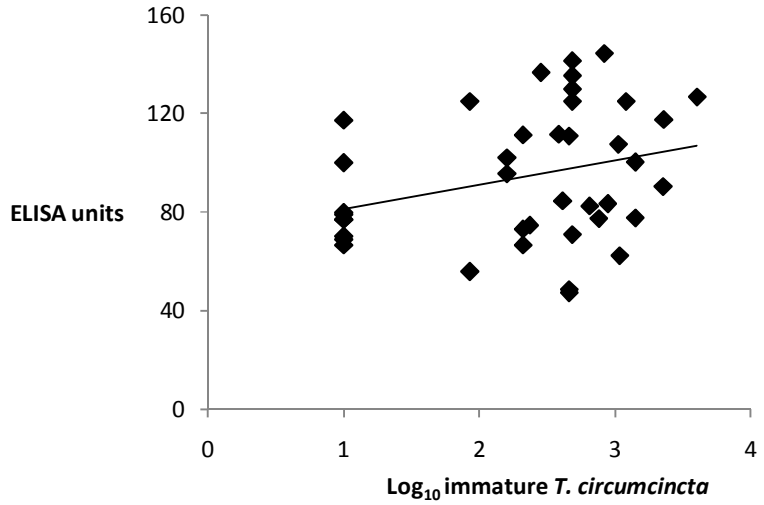

Figure 6.3. Scatter plot of relationship between *T. colubriformis*-specific IgG_1 and $\log_{10}$ transformed numbers of immature *T. circumcincta*. r = 0.31, P=0.049, n=40.

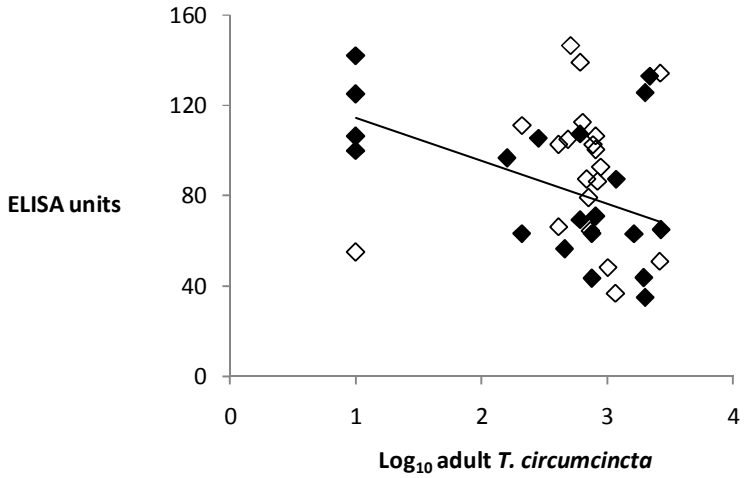

Figure 6.4. Scatter plot of relationship between *T. colubriformis*-specific IgM and $\log_{10}$ transformed numbers of adult *T. circumcincta* in sheep with high dag scores (♦, r = -0.5, P=0.02, n=20) and sheep with low dag scores (◊, r = 0.1, P=0.61, n=20).

6.4 Discussion

Concentrations of interluekin-5 and all parasite-specific antibodies were increased by larval challenge except *T. colubriformis*-specific light chain. Therefore, the production of antibodies that are specific for larval antigens is likely to be a critical component of acquired immunity to parasites. There were negative correlations between adult *T. circumcincta* and all antibody classes, suggestive of some causal involvement in worm rejection. However, there was no clear trend for negative correlations between antibody levels and *T. colubriformis* and immature *T. circumcincta,* so this hypothesis can only be partially supported. There were no correlations between faecal dry matter and antibody levels, so this part of my hypothesis is also rejected. Therefore, antibody levels in serum are likely to be poor indicators of sheep that are susceptible to immune-mediated scouring.

There is strong evidence that the antibodies I measured are involved in immunity to *T. circumcincta*, evidenced by negative, significant correlations, but there was less evidence of a relationship with *T. colubriformis.* However, this was probably because all sheep had low numbers of *T. colubriformis*. Therefore, there was probably not enough variation to calculate meaningful correlations. This is similar to my results in

Chapter 5, where there were much stronger correlations between inflammatory mediators and numbers of *T. circumcincta* than there were numbers of *T. colubriformis.* The association of antibodies, particularly IgA, with reduced worm growth and fecundity of *T. circumcincta* has also been demonstrated by Stear *et al.* (1999b) and Beraldi *et al.* (2008). In the present study the association was much stronger between IgA and *T. circumcincta* burdens then it was for WEC, so it may also be that IgA facilitates rejection of developing larvae as well as limiting fecundity. The IgA results have to be interpreted with caution as the secretory component of IgA , which is produced at the mucosal level and not detectable in serum probably constitutes the majority of IgA produced during parasite infection (Snoeck *et al*., 2006) Nevertheless, the interactions between IgA and WEC EBV suggest selecting for low WEC is indirectly selecting for an enhanced IgA response, as only those sheep with very low WEC EBV had IgA levels that correlated with worm burden. Overall, the results demonstrate that antigen-specific antibodies are possibly involved in rejection of nematodes from the gut.

The interaction between IgM and dag score was puzzling. It could be postulated that sheep with high dag scores produce more IgM and this is involved in immunity to *T. circumcincta* and also perhaps possibly involved in the pathogenesis of scouring. There was no relationship between IgM and faecal dry matter so this unlikely. However, this result may indicate that there is some underlying difference between scouring and non-scouring sheep that manifests itself in immunity to parasites.

Correlations between antibodies and numbers of *T. circumcincta* were much stronger with adult worms than with immature worms, despite the fact that L_3 antigens were used in the ELISA. This suggests that even though larval and adult nematodes produce different, stage-specific antigens (Emery *et al.*, 1992b) there are numerous common antigens between adult worms and L_3. There also appear to be common antigens between *T. colubriformis* and *T. circumcincta,* as evidenced by significant correlations between numbers of *T. circumcincta* and *T. colubriformis*-specific antibodies. Bisset *et al.* (1996) have also noted significant correlations between numbers of *H. contortus, T. colubriformis* and *T. circumcincta* and antibodies specific for the other two species. This suggests that the most common parasitic nematodes of sheep have considerable homogeneity in the antigens that they produce, and breeding sheep to be parasite-resistant on the basis of exposure to one nematode should confer considerable cross-resistance to other nematode parasites.

An unexpected result was the positive correlation between IgG_1 and immature *T. circumcincta*. IgG_1 is involved in larval rejection of *T. colubriformis* (Harrison *et al,*. 2008) and binds to mucosal mast cells (Emery, 1996) and so it could be postulated that sheep with high levels of IgG_1 would have less larvae. It is likely that *T. circumcincta* L_4 induce a different antibody response than *T. colubriformis* due to their location in the gastric glands. Antibodies may only exert a regulatory effect when larvae emerge into the lumen. The two week gap between blood sampling and post-mortem examination makes it difficult to draw conclusions about the exact kinetics of the antibody response and its effect on worm numbers. However, it could be speculated that as *T. circumcincta* L_4 emerge from the gastric glands they stimulate a marked IgG_1 response that arrests further development. Hence, the positive correlation between IgG_1 and immature *T. circumcincta* and the negative correlation between IgG_1 and adult *T. circumcincta.* Repeated blood samplings during the challenge period could add more weight to the results, unfortunately this was not possible in the current trials.

The results of this experiment suggest that blood sampling is not feasible as a method of identifying sheep likely to suffer immune-mediated diarrhoea. However, my results may have been different if I had been able to analyse the blood samples taken two weeks after challenge began, because they would have coincided with the maximum divergence in faecal dry matter. Four weeks after challenge began, there was less variation in faecal dry matter between animals (Chapter 5). Identifying sheep that are less likely to suffer from dags, but are still immune to parasite infection, is now a major focus for sheep breeders in Australia, New Zealand and the United Kingdom. Recently, researchers in human medicine have focused on non-invasive identification of intestinal inflammation through detection of the calprotectin protein in faeces. However, antibodies against human calprotectin show no cross-reactivity when used with sheep faeces (A.R. Williams, unpublished data). Skin-testing, similar to allergy-testing in humans, may also have value in identifying sheep that are likely to scour but attempts to measure the dermal eosinophil response to injected antigens have produced equivocal results (Larsen *et al.*, 1999). Gene microarray studies may offer the chance to identify parasite-resistant animals at the genetic level that are not susceptible to scouring, and develop markers for selective breeding. However, currently it seems that breeding for both low WEC and also including phenotypic measures of scouring (dags, faecal consistency score) in a selection index is the most appropriate strategy for parasite control.

Chapter 7 - Faecal dry matter, inflammatory cells and antibodies in parasite-resistant sheep challenged with either *Trichostrongylus colubriformis* or *Teladorsagia circumcincta*

7.1 Introduction

Breeding sheep to be resistant to nematode parasites is a long-term and sustainable parasite-control method. However, in some environments there is an increased propensity for parasite-resistant sheep to scour (Karlsson *et al.*, 2004). I established in Chapter 5 that dag scores observed in immune, parasite-resistant Merino sheep are due in part to a reduction in faecal dry matter caused by challenge with nematode larvae. Faecal dry matter was reduced despite the presence of a significant worm infection. Eosinophils in gut tissue, a hallmark of inflammation associated with Th2 immune responses (Meeusen, 1999) were negatively correlated with faecal dry matter. This suggests that in resistant sheep it is the rejection of larvae that results in scouring.

The results in Chapter 5 raised several questions that will be addressed in this chapter. First, in the experiment in Chapter 5, sheep were challenged with a mixture of nematode species, namely *T. colubriformis* and *T. circumcincta*. While this is representative of the larval challenge that sheep face while grazing pasture in the southern half of Australia, it means that the effects each species has on the pathology of immune-mediated scouring are confounded. While both *T. colubriformis* and *T. circumcincta* have similar life-cycles and effects on the host, the magnitude of the immune response towards each species in a mixed infection can vary markedly (Gruner *et al.*, 2004). My results in Chapters 4 and 5 suggest that sheep from the Rylington Merino line are more immune to *T. colubriformis* than they are to *T. circumcincta*. Therefore, the relative contribution of each species to immune-mediated scouring needs to be quantified.

Challenge with *T. circumcincta* leads to increased fluid secretion and increased flow rate of digesta at the abomasum. However, most of this can be re-absorbed in the distal parts of the small intestine (Wilson and Field, 1983). Challenge with *T. colubriformis* increases the flow of protein and minerals past the terminal ileum (Bown *et al.*, 1991) and, thereby, reducing the osmotic potential and capacity of the colon to absorb water. This suggests that *T. colubriformis* may be more important than *T. circumcincta* in the pathology of immune-mediated scouring. Consistent with this, in Chapter 5 I found that

eosinophils in the small intestine, but not in the abomasum, were negatively correlated with faecal dry matter.

Scouring in the field is normally observed where sheep are facing a mixed challenge of *T. colubriformis* and *T. circumcincta*. It is not known whether a mixed challenge of both species leads to more severe scouring than challenge with either species alone, and, if this is the case, whether this is due to additive effects of infection in both the abomasum and small intestine, or whether the mixed infection has synergistic effects. The effects of mixed nematode challenge in immune sheep have not been closely studied. Studies that have focused on the interactions between *Teladorsagia* and *Trichostrongylus* on sheep growth rate and/or worm establishment have been conducted with young lambs that have not yet acquired immunity (Sykes *et al.*, 1988, Jackson *et al.*, 1992). Therefore, there is little information on whether clinical signs such as immune-mediated scouring are exacerbated during a mixed challenge. There is evidence of some cross-reactivity in immunity towards different nematode species (Emery *et al.*, 1993b, Stewart, 1955), suggesting that immunopathology may be greater during mixed infections. This is because part of the immune response towards the other species also acts non-specifically towards the first species. Therefore, it is feasible that immune-mediated scouring will be more severe during a mixed species challenge, due to a heightened immune response leading to greater immunopathology.

In this chapter I will investigate the effects of challenging parasite-resistant sheep with either *T. colubriformis* or *T. circumcincta*, or both species, on faecal dry matter, levels of parasite-specific antibodies and interleukin-5 in serum and numbers of inflammatory cells in the abomasum and small intestine. My hypothesis is that faecal dry matter will be lowest in those sheep that receive the mixed challenge, followed by those that receive only *T. colubriformis*, than by those that receive only *T. circumcincta*. In addition, I hypothesise that numbers of inflammatory cells and levels of parasite-specific antibodies and IL-5 in serum will be higher in sheep that receive the mixed challenge, compared to those that are challenged with only one species.

7.2 Materials and Methods

7.2.1 Experimental design

Twenty Merino rams, aged two years, were selected from the parasite-resistant Rylington Merino flock (Chapter 3). All rams had low WEC but high-dag scores, i.e.

they were assumed to be prone to immune-mediated scouring (Table 7.1). Five rams were then randomly allocated to one of four treatment groups –

1) Challenged with *T. colubriformis* only
2) Challenged with *T. circumcincta* only
3) Challenged with both *T. colubriformis* and *T. circumcincta*
4) No challenge (control)

Table 7.1. WEC EBV and dag scores in spring (hogget age) for Merino rams used in this experiment. Dag scores are on a subjective 1-5 scale where 1 is no dag and 5 is severe dag.

	WEC EBV	Spring dag
Experimental rams (n=20)	-96	4.3
Flock average (n=192)	-91.5	2.3

Rams were allowed to acclimatise to the animal house for two weeks. Rams in groups 1-3 were then dosed daily with larvae for 24 days. Rams in group 4 received no larvae. 24 days was chosen as the challenge period as this is where the maximum divergence in faecal dry matter was observed between challenged and control rams in Chapters 4 and 5. After 24 days, all rams were necropsied and tissue samples taken from the abomasum, small intestine and colon for histology.

7.2.2 Challenge regime

After the acclimatisation period, rams in group one received 1000 *T. colubriformis* L_3 per day, rams in group two received 1000 *T. circumcincta* L_3 per day and rams in group three received 1000 L_3 of each species per day. The larvae were suspended in water and administered orally using a plastic syringe.

7.2.3 Sampling procedures

Faecal samples were taken from rams upon arrival at the animal house and WEC performed to ensure sheep were worm-free. During the acclimatisation period, three

faecal samples were taken from each sheep at regular intervals four days apart and faecal dry matter was determined as described in Chapter 3. These three values were averaged to determine a baseline, pre-infection value of faecal moisture content for each sheep. Following the commencement of larval dosing, faecal samples were taken weekly for determination of faecal dry matter. Serum was collected from each ram prior to larval dosing, and then at 10 and 20 days after dosing commenced. Serum was collected as described in Chapter 6 and was stored at -20ºC.

After 24 days, faecal samples were collected for WEC and then all twenty rams were necrospied. Tissue samples were taken from the abomasum, small intestine and spiral colon. Total worm counts were determined as described in Chapter 3.

7.2.4 Histology and ELISA

The numbers of eosinophils, mast cells and globule leukocytes in tissue sections were quantified. The concentrations of immunoglobulins in serum specific to *T. colubriformis* and *T. circumcincta* L_3 antigen, as well as IL-5, were determined by sandwich ELISA. Full details of the techniques are provided in Chapter 3.

7.2.5 Statistical analysis

The pattern of faecal dry matter over the course of the experiment was tested with a mixed model analysis in SAS (version 9, SAS Institute Inc, Cary, NC, USA), with treatment group and week of challenge fitted as fixed factors and the individual sheep as the random model. The baseline faecal dry matter (i.e. the mean of the three pre-challenge faecal dry matter measurements) was used as the value for each sheep at day 0. Differences of least squares means were used to examine differences between the control and challenged groups at each time-point. One-way ANOVA (SPSS version 17.0) was used to determine differences between treatment groups in numbers of inflammatory cells in the abomasum and small intestine, with post-hoc testing carried out with the Tukey test. For concentrations of serum antibodies and IL-5, a mixed model analysis (SPSS version 17.0) was performed with week of challenge as the within-subject factor and treatment group as the between subject factor. Generally, data conformed to assumptions of normal distribution and did not require transformation. An exception was the levels of *T. colubriformis*-specific IgA, which was skewed due to a number of zero values (i.e. less than the absorbance of the negative control). A $\log_{10}$ (n+1) transformation was effective at overcoming this skewness, and results are thus presented as $\log_{10}$ ELISA units. For all analyses the significance level was $P<0.05$.

7.3 Results

7.3.1 Worm infection

Rams were generally very effective at resisting the larval challenge. Only a few worms were observed in the gut contents and abomasal digest washings. Mainly larval stages were observed, as was to be expected given the rams were killed just after three weeks since the larval challenge began. Control rams, as well as rams challenged with only *T. colubriformis* or *T. circumcincta,* were completely free of all stages of *T. colubriformis* at post-mortem examination. Of the five control rams, despite being drenched and removed from pasture, three still harboured small numbers of immature *T. circumcincta,* as did one ram that was challenged only with *T. colubriformis* (Table 7.2). Rams challenged with *T. circumcincta* had slightly higher immature numbers of that species, but were mainly free of adult worms. WEC taken just before necropsy were all negative apart from two rams, both in the group that received the mixed challenge, which had a positive count of 50 eggs per gram of faeces.

7.3.2 Faecal dry matter

There were no differences (ca 30%) in faecal dry matter between treatment groups before larval dosing began (baseline faecal dry matter, average of three values, see materials and methods). After dosing commenced, the FDM of the control group stayed relatively constant at around 32%. After dosing commenced, there was a trend for faecal dry matter in the challenged groups to decrease, reflected in an interaction between time and treatment group ($P<0.001$; Figure 7.1). At each sampling, faecal dry matter was lowest in those rams receiving only the *T. colubriformis* dose. Seven days after dosing commenced, faecal dry matter in this group was lower ($P<0.001$) than in both the control group and the group receiving only *T. circumcincta*. The significant differences between the *T. colubriformis* group and the control group persisted throughout the experiment; however, after seven days there were no further differences between the three challenged groups. In rams dosed with both species, faecal dry matter was lower ($P<0.05$) than the control rams from seven days after challenge until the end of the experiment. Faecal dry matter was also lower ($P<0.05$) than the controls in the group receiving only T. *circumcincta* at 15 and 23 days after challenge began.

Table 7.2. Arithmetic worm burdens of challenged and control rams.

		Tric. Colubriformis			*Tela. Circumcincta*		
Group	Sheep ID	EL_4	DL_4	Adult	EL_4	DL_4	Adult
Control	667	0	0	0	0	0	0
	675	0	0	0	0	0	0
	681	0	0	0	75	0	0
	690	0	0	0	0	75	0
	872	0	0	0	0	200	0
	Group Mean	**0**	**0**	**0**	**15**	**55**	**0**
Tela	661	0	0	0	350	0	0
	710	0	0	0	150	0	0
	750	0	0	0	450	225	75
	771	0	0	0	75	275	0
	783	0	0	0	500	75	75
	Group Mean	**0**	**0**	**0**	**350**	**115**	**30**
Tric	674	0	0	0	0	0	0
	775	0	0	0	0	0	0
	813	0	0	0	0	0	0
	845	0	0	0	75	0	0
	851	0	0	0	0	0	0
	Group Mean	**0**	**0**	**0**	**15**	**0**	**0**
Tela + *Tric*	634	0	50	0	75	0	0
	649	0	0	100	75	75	0
	760	0	0	0	350	275	0
	856	0	0	0	275	475	200
	859	0	0	0	200	75	0
	Group Mean	**0**	**10**	**20**	**195**	**180**	**40**

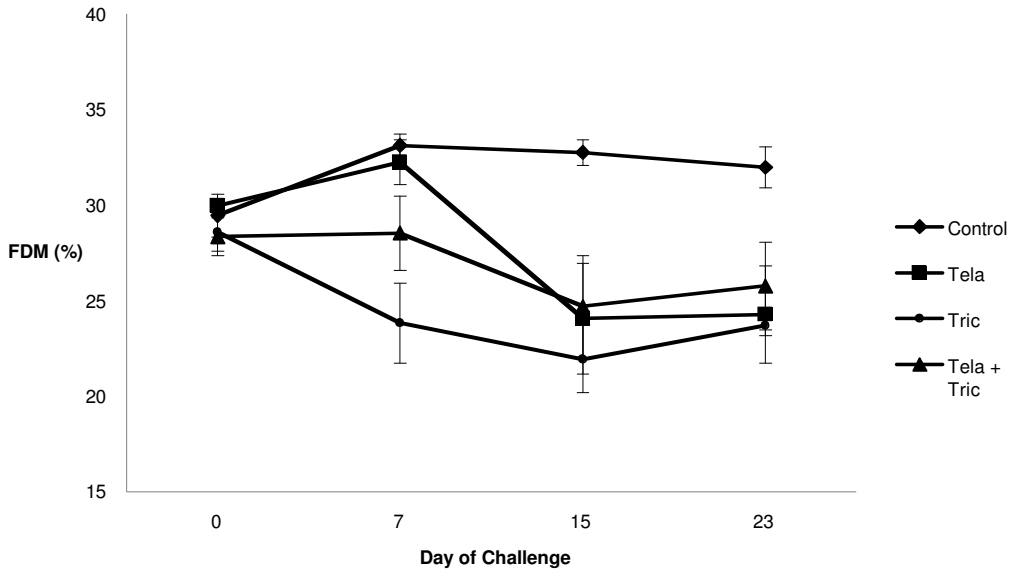

Figure 7.1. FDM (means ± s.e.m) in rams challenged with *T. circumcincta* (*Tela*), *T. colubriformis* (*Tric*), both species (*Tela* + *Tric*) or not challenged (control). For each group n=5. Larval challenge began on day one. FDM values at day 0 are the average of three estimations of FDM taken during a two-week acclimatisation period before dosing commenced.

7.3.3 Histology

Within the abomasum, there were no differences in the numbers of mast cells, globule leukocytes or eosinophils between control rams and those rams that were challenged with only *T. colubriformis* (Table 7.2). In contrast, those rams that were challenged with only *T. circumcincta* had higher numbers of all three cell types than control rams ($P<0.05$). Rams challenged with both species had higher numbers of cells than control rams, but only significantly so for eosinophils. Within the three challenged groups, the trend was for increased numbers of cells in those rams that were challenged with *T. circumcincta.* Rams challenged with only *T. circumcincta* had higher numbers of mast cells ($P<0.05$) than rams challenged with either *T. colubriformis* alone or with both species. Rams that were challenged with both species had higher numbers of eosinophils ($P<0.05$) compared to those that were only challenged with *T. colubriformis.*

Table 7.2. Numbers of inflammatory cells (cells/mm^2 of tissue) in the abomasum of rams challenged with either *Teladorsagia circumcincta* (*Tela*), *Trichostrongylus* (*Tric*), both species (*Tela* + *Tric*) or not challenged (control). Within each cell type, numbers followed by different letters are different (P<0.05).

	Group (each n=5)			
Cell Type	Control	*Tela*	*Tric*	*Tela* + *Tric*
Mast Cells	15 ± 2.5^{A}	87 ± 25.4^{B}	15 ± 4.2^{A}	31 ± 7.7^{A}
Globule leukocytes	20 ± 6^{A}	97 ± 29.9^{B}	30 ± 5.2^{AB}	58 ± 19.6^{AB}
Eosinophils	12 ± 1.8^{A}	59 ± 5.9^{BC}	22 ± 5.5^{AB}	86 ± 16.3^{C}

Within the small intestine, there were no significant differences in the numbers of eosinophils, although numbers were higher in challenged rams (Table 7.3). For mast cells and globule leukocytes, there was a consistent trend whereby rams that were challenged with both species had the highest numbers of cells, followed by those that were challenged with only *T. colubriformis,* then those challenged with only *T. circumcincta* and finally control rams had the lowest numbers of cells. However, there were no significant differences between the three challenged groups. Compared to control rams, numbers of mast cells and globule leukocytes were higher (P<0.05) in rams that were challenged with both species, and numbers of globule leukocytes were also higher (P<0.05) in rams that were challenged with only *T. colubriformis.*

Table 7.3. Numbers of inflammatory cells (cells/mm^2 of tissue) in the small intestine of rams challenged with either *Teladorsagia circumcincta* (*Tela*), *Trichostrongylus* (*Tric*), both species (*Tela* + *Tric*) or not challenged (control). Within each cell type, numbers followed by different letters are different (P<0.05).

	Group (each n=5)			
Cell Type	Control	*Tela*	*Tric*	*Tela* + *Tric*
Mast Cells	20 ± 4.7^{A}	47 ± 20^{AB}	69 ± 10^{AB}	99 ± 16.7^{B}
Globule leukocytes	26 ± 5.3^{A}	52 ± 10.9^{AB}	64 ± 6^{B}	77 ± 12.7^{B}
Eosinophils	31 ± 3	53 ± 13.5	46 ± 7.9	60 ± 6.5

Within the colon, numbers of all cell types were low (ca 15 cells/mm^2 tissue) and there were no differences between challenged and unchallenged sheep.

7.3.4 Serum antibodies

Significant interactions were observed between time and treatment group for IgA specific for both species and IgE specific for *T. colubriformis.* The IgA interactions represented a rapid increase in antibody in the challenged groups 10 days after sampling (Figures 7.2 and 7.3). This was in contrast to the control group where no increase was observed. No further increase was observed in the challenged groups between days 10 and 20. *T. colubriformis*-specific IgA levels in rams challenged only with *T. colubriformis* decreased sharply to baseline levels by day 20 (Figure 7.2). IgE increased sharply at day 10 in rams challenged with either *T. colubriformis* or *T. circumcincta* alone, however returned to baseline levels by day 20. There was a slight linear increase in IgE over the course of the experiment in rams challenged with both species. There was no change in IgE in the control group. There were no significant main effects or interactions for total light chain antibody, IgG_1 or IgM.

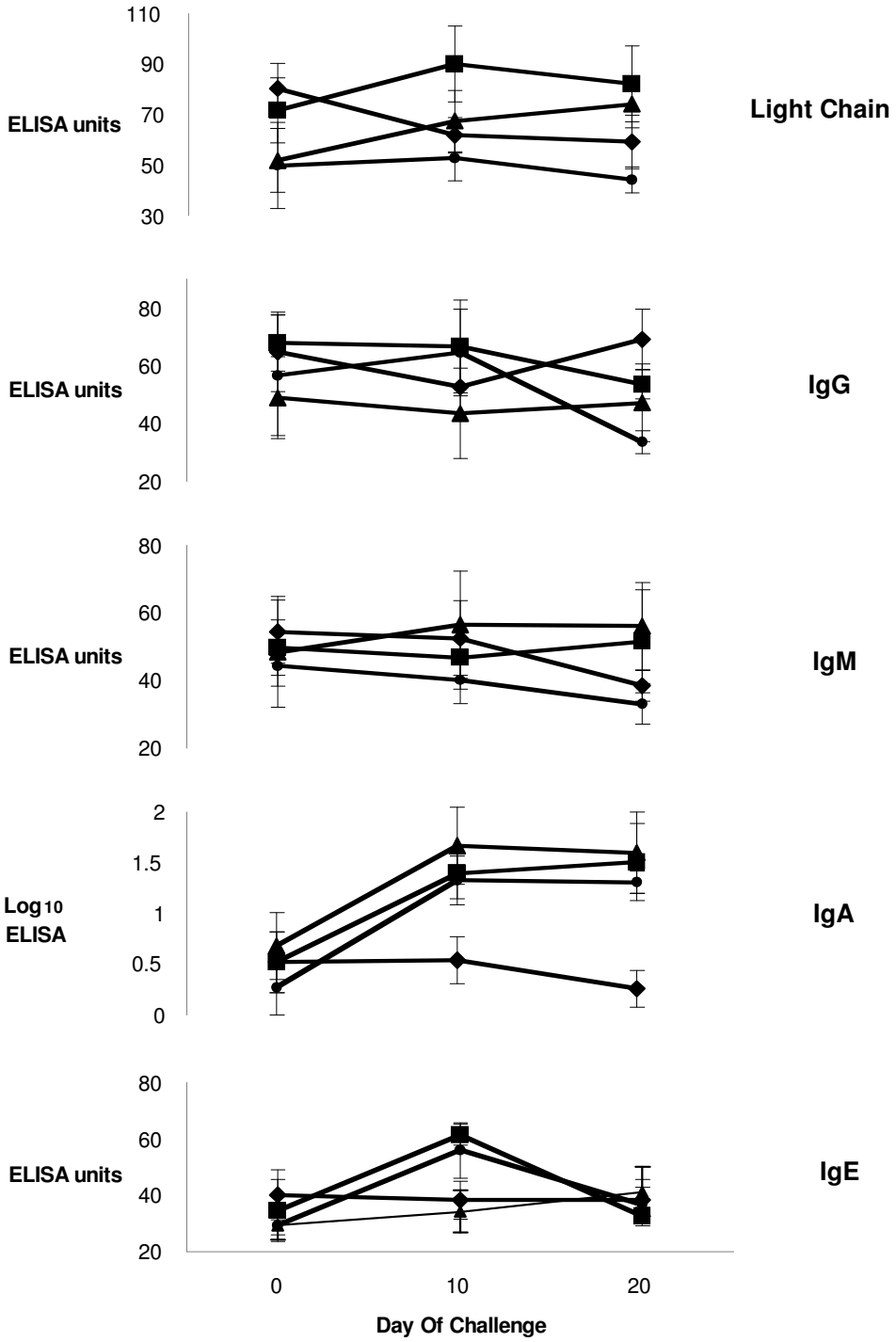

Figure 7.2. Levels of *T. colubriformis*-specific antibodies in serum from sheep challenged with *T. colubriformis* (●), *T. circumcincta* (■), both species (▲) or not challenged (◆). Means of each group (n=5) and s.e.m. are shown.

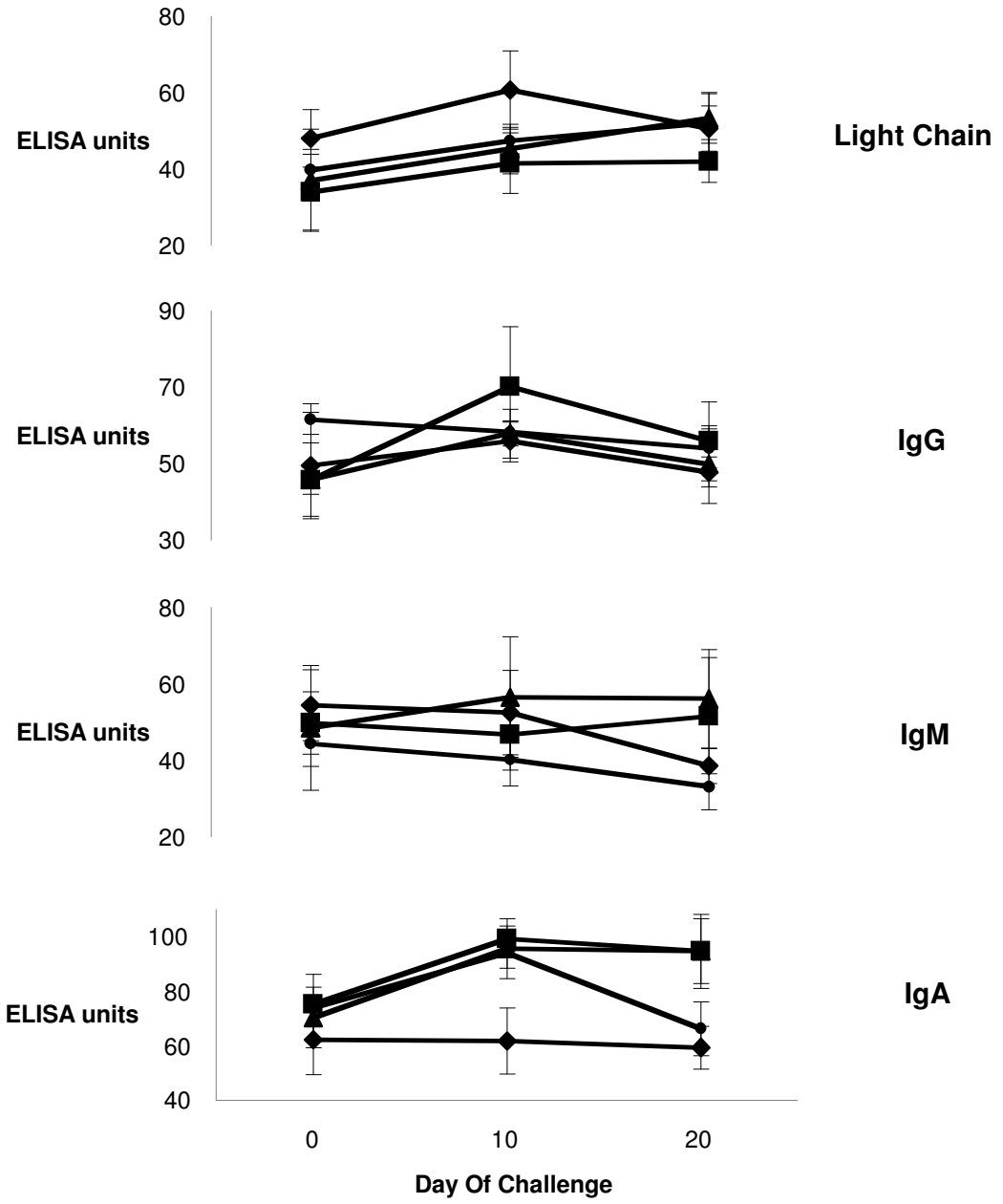

Figure 7.3. Levels of *T. circumcincta*-specific antibodies in serum from sheep challenged with *T. colubriformis* (●), *T. circumcincta* (■), both species (▲) or not challenged (◆). Means of each group (n=5) and s.e.m. are shown.

7.3.5 Interluekin-5

There was an interaction between time and treatment group (P<0.0001) in the analysis of the IL-5 concentrations (Figure 7.4). There was an increase from baseline levels at day 10 in all three challenged groups, whereas the control group slightly declined and then remained constant. From day 10-20, the IL-5 concentrations in the groups challenged with only *T. colubriformis* or *T. circumcincta* tended to level off, whereas the concentration in the group challenged with both species rose sharply to nearly twice baseline levels.

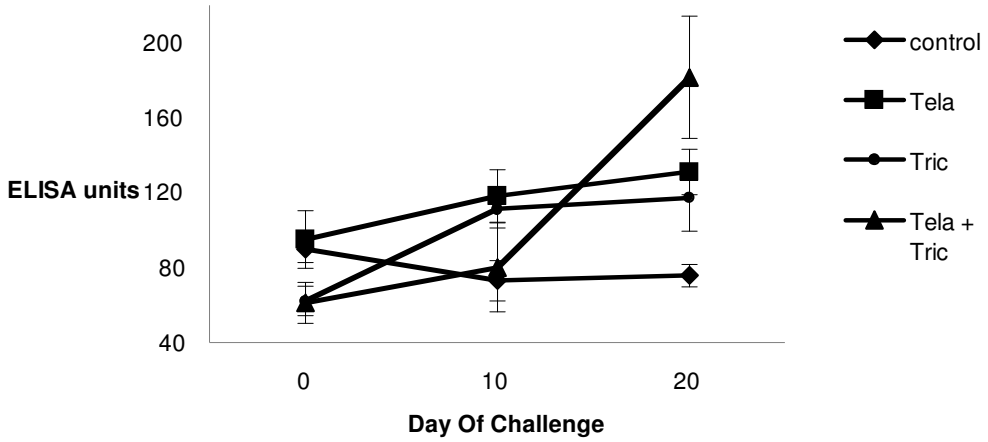

Figure 7.4. Concentration of interleukin-5 in serum from sheep challenged with *T. circumcincta* (*Tela*), *T. colubriformis* (*Tric*), both species (*Tela* + *Tric*) or unchallenged controls (means and standard errors are shown, each group n=5). Day zero represents pre-challenge values.

7.4 Discussion

I found that faecal dry matter was lower in challenged rams than in unchallenged controls, but there were no differences in faecal dry matter between the three challenged groups. Therefore, the first part of my hypothesis was not supported. I also hypothesised that there would be higher numbers of inflammatory cells and serum antibodies in sheep that received the mixed larval challenge. There was a trend for inflammatory cells in the small intestine, and also concentrations of IL-5 in serum, to be higher in rams that were challenged with both species. However, serum antibodies and

inflammatory cells in the abomasum were not higher in the group that received the mixed challenge. Hence, there is only partial support for this hypothesis.

I found that faecal dry matter was lower in challenged sheep compared to controls, but from seven days after the challenge began there was no difference between the three challenged groups. Faecal dry matter in the rams challenged with *T. colubriformis* decreased faster than in rams challenged only with *T. circumcincta*. However, at both 15 and 23 days after challenge commenced, there were no differences between any of the challenged groups. Therefore, it appears there are no multiplicative effects of concurrent challenge, at least in terms of faecal dry matter. Both *T. circumcincta* and *T. colubriformis* are capable of causing immune-mediated scouring, and concurrent infection with both species will not necessarily lead to worse diarrhoea.

Within the abomasum, I found that only those sheep that were challenged with *T. circumcincta* larvae had increases in inflammatory cells compared to control sheep. There were no differences between those sheep that were challenged with *T. circumcincta* only, and those sheep that received the mixed challenge. This suggests that challenge with *T. colubriformis* does not stimulate any immunopathology in the abomasum. Stewart (1955) and Barnes and Dobson (1993) have previously noted that sheep immunised with *T. colubriformis* have no protection when given a challenge infection of an abomasal parasite such as *H. contortus* or *T. circumcincta*. This is consistent with my results, which suggest that no immunity is generated in the abomasum by the presence of *T. colubriformis* larvae. Even though *T. colubriformis* pass through the abomasum, and probably exsheath there en route to the duodenum, there does not seem to be any antigenic challenge there that results in an immune response. Therefore, challenge with both *T. circumcincta* and *T. colubriformis* has no additional effects on mucosal pathology in the abomasum, as none of the immune response towards *T. colubriformis* acts non-specifically towards *T. circumcincta.* Thus, it seems that concurrent challenge with *T. colubriformis* has little or no impact on the immune response towards *T. circumcincta*.

Within the small intestine the response was different because there was a trend for those sheep that received the mixed challenge to have the highest numbers of inflammatory cells. While this was not significant, it can be explained by the relatively low numbers of sheep in each treatment group. In addition, there seemed to be an increase in inflammatory cells in the small intestine in those sheep that were only challenged with *T. circumcincta*, compared to controls. This suggests that challenge with

T. circumcincta can also lead to an infiltration of inflammatory cells into the small intestine. Stewart (1955) observed that sheep that were immunised with *H. contortus* and then challenged with both *H. contortus* and *T. colubriformis* were able to expel both species. However, sheep that were immunised with *T. colubriformis* and then challenged with both species were only able to expel *T. colubriformis.* Similar results were obtained by Dobson *et al.* (1992) who found that immunising sheep with *T. circumcincta* conferred some protection against *T. colubriformis* but the reverse was not true. Therefore, it appears that non-specific immune mechanisms only operate downstream of the site of larval challenge. This is consistent with my results that showed no effect of *T. colubriformis* challenge on inflammation in the abomasum, but a slight effect of *T. circumcincta* challenge on inflammation in the small intestine. This may also explain the trend for the mixed challenge to lead to higher numbers of inflammatory cells in the small intestine, as the inflammation is a result of both the local response to *T. colubriformis* and also a 'trickle-down' effect of the response to *T. circumcincta* higher up in the gut. However, the low numbers of inflammatory cells in the colon shows that this inflammation does not extend all the way to the large intestine. These findings could also partially explain the results in Chapters 4 and 5, where I found lower establishment of *T. colubriformis* compared to *T. circumcincta* when rams were dosed with equal numbers of both species. It appears that the immune response towards *T. colubriformis* can be enhanced when there is a concurrent challenge with *T. circumcincta*.

I hypothesised that sheep that were challenged with larvae from both nematode species would have the highest levels of antibodies and IL-5 in serum, but this was only true for IL-5. There was a clear trend for IgA and IgE levels to increase as a result of larval challenge, but concentrations in rams that received the mixed larval challenge were not noticeably higher compared to rams that were challenged with only one species. Interestingly, rams challenged only with *T. circumcincta* had increases in *T. colubriformis*-specific IgA and IgE, and rams challenged only with *T. colubriformis* showed an increase in *T. circumcincta* specific IgA. Similarly, *T. circumcincta*-specific total antibody increased linearly throughout the larval challenge period in rams challenged only with *T. colubriformis.* This clearly shows that the two worm species produce common antigens that are recognised by sheep immunoglobulin. Therefore, it would be expected that rams that were dosed with both species of larvae would have higher serum antibody levels, if only through a simple additive effect of being challenged with more larvae. It is possible that the relatively low numbers of sheep in

each treatment group, combined with high between-animal variation, did not allow me to detect a significant trend. This is probably the case with the IgG_1 and IgM results, where no trends where observed even between challenged and unchallenged sheep. This is in contrast to the results in Chapter 6, where I found that larval challenge increased IgG_1 and IgM levels in sheep challenged with a mixture of *T. colubriformis* and *T. circumcincta*. IL-5 did tend to show to a greater rise in concentrations in sheep challenged with both species. IL-5 is the major cytokine responsible for eosinophil proliferation, and eosinophils tended to be higher in both the abomasum and small intestine of sheep that were challenged with both species. Therefore, it is possible that the concurrent challenge does lead to increased eosinophil production than either species alone. However, the results in this chapter suggest that serum antibody levels, like faecal dry matter, are similar in sheep given a mixed larval challenge or either species by itself.

Chapter 8 - General Discussion

The work presented in this thesis supports the theory that in parasite-resistant adult Merino sheep, scouring is mainly due to an inflammatory immune response to ingested larvae rather than a large worm burden. Challenging parasite-resistant sheep with nematode larvae softened faeces, and faeces were softer in those sheep that had high-dag scores in the field compared to sheep with low-dag scores. Numbers of inflammatory granulocytes in tissue, concentrations of inflammatory mediators in mucus and levels of parasite-specific antibodies and IL-5 in serum were all generally increased by larval challenge. Overall, there was a consistent trend for both granulocytes and inflammatory mediators to be negatively correlated with both WEC and total worm burdens. Only tissue eosinophils in the small intestine were significantly correlated with faecal softening, although there were some weak trends for inflammatory mediators to also be associated with low faecal dry matter. Therefore, the general hypothesis tested in this thesis was partially supported. It is likely that the inflammatory response associated with larval challenge was responsible for rejection of larvae, but may also have led to more fluid faeces, scouring and dags in grazing sheep.

I have demonstrated that faecal dry matter in parasite-resistant sheep is significantly reduced by larval challenge indoors in an animal house. Challenging the sheep indoors showed that the larvae are definitely responsible for the changes in faecal dry matter, as both challenged and unchallenged sheep were consuming the same daily ration of chaff and lupins. Therefore, the changes in faecal dry matter can only be attributed to the larval challenge and not components of lush green pasture or differing levels of food intake. This reduction in faecal dry matter is correlated with the level of scouring and, consequently, dag scores that are observed in sheep while they are grazing in the field. This reduction in faecal dry matter occurred even though sheep were very effective at resisting the larval challenge and did not develop clinical worm burdens. This suggests that it is the larval challenge itself that is responsible for parasite-related scouring. Therefore, scouring in grazing sheep can be mostly attributed to larval challenge, rather than non-parasitic sources such as lush green pasture or species such as capeweed. It was interesting to note that in all three animal house experiments, faecal dry matter tended to increase in the unchallenged control sheep. This may indicate that the sheep were still adjusting to the animal house diet. Ideally, control sheep should have experienced a constant faecal dry matter throughout the experiments. It was not

possible to allow the sheep any more time than two weeks to acclimatise to the animal house due to time and budgetary constraints. However, all sheep were grazed under the same conditions before entering the animal house and there were no differences in faecal dry matter between treatment groups before larval challenge began. Therefore, changes in faecal dry matter between challenged and unchallenged groups can still be ascribed to the larval challenge rather than any dietary factors. Even though faecal dry matter was reduced, the majority of the sheep did not suffer clinical diarrhoea during the experimental period. Most dag formation occurs when the dry matter percentage of faeces is <15% (L.J.E. Karlsson pers. comm.) and only a few sheep had this level of moisture. Therefore, severe scouring observed in the field is probably caused by larval challenge that may be exacerbated by a complex set of factors including pasture composition and level of food intake (Jacobson, 2006). However, my results show clearly that in parasite-resistant sheep larval challenge is a major cause of scouring.

The results presented here are the first characterisation of the immune response of the parasite-resistant Rylington Merino flock. Challenge with *T. circumcincta* and *T. colubriformis* larvae increased numbers of eosinophils, mast cells and globule leukocytes in tissue sections from both the abomasum and small intestine. In addition, larval challenge increased concentrations of cysteinyl leukotrienes, prostaglandin E_2 and bradykinin in mucus from the abomasum and small intestine. Concentrations of parasite-specific IgE, parasite-specific IgG_1 and IL-5 in serum were also increased by larval challenge. The Rylington Merino flock is a unique resource as sheep have been selected for low WEC for over twenty years in an environment with highly seasonal rainfall, which results in a larval challenge for only half the year. The results in this thesis suggest that this selection for low WEC has selected for sheep that have an immune response strongly skewed towards a Th2 or hypersensitive immune response. The environmental conditions probably contribute to the development of this skewed response. Modulation of the immune system, whereby it is programmed to respond to antigens by either a Th1 or Th2 response, occurs as animals are first exposed to antigenic challenge (Khan and Collins, 2004). The absence of a larval challenge for six months, shortly after weaning, probably results in the immune system mounting a strong Th2 or allergic response after the reappearance of infective larvae on winter pastures. This response is extremely effective at rejecting incoming worm larvae, leading to the Rylington Merino flock being considered by sheep breeders to be the most worm-resistant Merino flock in the world (L.J.E. Karlsson, pers comm.). The effectiveness of this response is clearly demonstrated by the negative correlations

between WEC/ total worm numbers and numbers of globule leukocytes and eosinophils, and also concentrations of leukotrienes and bradykinin. Even when correlations were significant, they were generally weak (less than 0.5). This could perhaps be explained by the fact that all sheep in the experiments were from a flock bred for parasite-resistance. As a result, there was not as much variation in immunological responses as would be seen in a group of random, outbred sheep. Comparing the responses of these highly resistant sheep with parasite-susceptible sheep could add further weight to the data obtained here. This inflammatory immune response results in mucous hypersecretion, leakage of plasma proteins (e.g. immunoglobulins) into the intestinal lumen and increased peristalsis due to contraction of non-vascular smooth muscle. This response prevents larvae from establishing and/or expels them from the mucosa by creating a microenvironment that is unsuitable for their survival.

An important finding was that eosinophils in the small intestine were negatively correlated with faecal dry matter. This confirms the findings of Larsen *et al.* (1994), who found higher eosinophils numbers were the main difference in Merino ewes with high-dag scores, compared to unaffected sheep. Shaw *et al.* (1998) also noted a positive, but non-significant correlation between eosinophils and faecal consistency score in sheep challenged with *T. colubriformis.* None of the inflammatory mediators measured were significantly correlated with faecal dry matter, but there was a general trend for sheep with lower faecal dry matter to have higher concentrations of mediators in mucus. Other researchers have demonstrated that sheep bred to be naturally resistant to parasites have higher numbers of eosinophils and also higher concentrations of leukotrienes following larval challenge than unselected sheep (Buddle *et al.*, 1992, Gray *et al.*, 1992, Bisset *et al.*, 1996). Therefore, my results could explain the unfavourable correlations observed between WEC and scouring in parasite-resistant sheep in Western Australia (Karlsson *et al.*, 2004) and in New Zealand (Bisset *et al.*, 1997). They suggest that the mechanisms that expel larvae from the gut of parasite-resistant sheep are also responsible for scouring.

The strong Th2 immune response raises questions about the 'balance' between resistance to parasites and immunopathology. In the winter-rainfall regions of Western Australia, sheep producers are now advised to select for both low WEC and low-dag scores (Karlsson and Greeff, 2006). The obvious drawback of this approach is that it will slow progress in the rate of genetic gain for low WEC, as less selection pressure is being applied for this trait. However, it then must be considered whether having sheep

that are slightly less resistant to worms will have benefits in terms of fewer side effects of the Th2 immune response, such as scouring. Depending on environmental and other management factors, sheep breeders and producers must decide on the relative importance of these factors in a selection index. Similarly, sheep producers in Mediterranean environments should be aware of the consequences of having a significant part of the year with no worm challenge followed by a sudden increase of infective larvae on pastures. In previous years, farmers in these environments have been advised to treat sheep with anthelmintics during the dry summer months to ensure they are worm-free and to try and prevent the degree of pasture contamination following the start of winter-rainfall. In adult sheep, this approach is now advised against due to the need to maintain a worm population in refugia to slow anthelmintic resistance (Besier, 2004). An additional benefit of allowing some worms to persist over summer may be to modulate the immune system away from a strong hypersensitive immune response, by allowing young sheep to become 'accustomed' to a small larval challenge.

It has now been established that in a winter-rainfall environment scouring is a consequence of a strong immunity to worms. One of the major unanswered questions is why some sheep are highly resistant to worms (low WEC) but are not prone to scouring. One possible explanation is that these sheep have a low WEC due to suppression of the egg-laying potential of female worms or arrested development of the fourth larval stage as their primary immune response. The fact that rams in the unchallenged control groups had immature *T. circumcincta* present at post-mortem examination, despite being removed from pastures for over eight weeks, confirms that arrested development is occurring in this species. This would lower WEC but, as worms are not actually being rejected from the gut, scouring may not be a consequence. In addition, while the percentage of faecal dry matter is an important determinant of dag formation, other factors such as the level of wool coverage around the breech are also crucial factors (Waghorn *et al.*, 1999, Scobie *et al.*, 2008). Therefore, it could also be postulated that sheep with low WEC and low-dag scores do have reduced faecal dry matter as a result of larval rejection, but this does not lead to dags because the conformation of the breech is not suitable for faecal accumulation. However, both of these hypotheses are not supported by my results. Sheep selected as having low WEC EBV and also low-dag scores in the field maintained these characteristics during the experimental larval challenge. That is, they had high faecal dry matter but also had low WEC and low worm numbers at post-mortem examination. Therefore, there seems to be a strong correlation between dag formation and the percentage of faecal dry matter. In addition, within the

low WEC EBV category, total worm numbers (especially *T. colubriformis*) were similarly low in sheep with both high and low-dag scores, and there was no difference in the number of fourth-stage larvae. Therefore, sheep with low-dag scores are definitely able to reject incoming larvae, and their low WEC is not due to suppression of female fecundity or arrested development of larvae without actually reducing their total worm burden. This means that scouring is not an inevitable consequence of larval rejection and sheep can be selected that have both low WEC and low-dag scores. This is an encouraging result for sheep breeders and producers.

The mechanisms by which these sheep reject the larval challenge without an associated decrease in faecal dry matter are not clear. Eosinophils in the small intestine were negatively correlated with faecal dry matter, but not worm numbers. Therefore, it could be postulated that sheep susceptible to immune-mediated scouring have an allergic response in the small intestine characterised by an infiltration of eosinophils that do not play a role in larval rejection. This was the hypothesis of Larsen *et al.* (1994) when they noted a correlation between eosinophil infiltration and scouring that was unrelated to worm burdens. Conversely, sheep that are not susceptible to immune-mediated scouring could reject larvae using mechanisms that were not correlated with faecal dry matter, e.g. globule leukocyte production. It is likely that globule leukocytes and eosinophils play slightly different roles in worm rejection. Globule leukocytes probably mediate rapid expulsion of incoming L_3, while eosinophils are recruited once larvae penetrate the mucosa (Balic *et al.*, 2000). The precise role that eosinophils play in parasite infection has not yet been elucidated and remains controversial (Bruschi *et al.*, 2008). While some authors have reported that eosinophils play no role in worm rejection in sheep (Gruner *et al.*, 2004, Sykes *et al.*, 2007), there are many other reports of negative correlations between eosinophil numbers and worm burdens and/or WEC (Douch *et al.*, 1986, Stevenson *et al.*, 1994, Dobson *et al.*, 1992, Doligalska *et al.*, 1999, Woolaston *et al.*, 1996). Therefore, it seems more probable that eosinophils are directly involved in worm rejection from the gut mucosa.

I found a significant negative correlation between eosinophils in the abomasum and WEC. Even though this correlation was not evident in the small intestine, it is unlikely that eosinophils would have a distinctly different role in response to challenge with *T. colubriformis,* compared to challenge with *T. circumcincta,* as they are both mucosa-dwelling nematodes that feed directly on soluble nutrients in host tissue. Eosinophil numbers are consistently higher in sheep that are bred for low WEC (Dawkins *et al.*,

1989, Rothwell *et al.*, 1993, Thamsborg *et al.*, 1999). It is also interesting to note that numbers of eosinophils are positively correlated with numbers of mast cells and globule leukocytes in blackface sheep in the UK (Stear *et al.*, 1995), and also positively correlated with concentrations of parasite-specific antibodies in genetically resistant Romney sheep in New Zealand (Bisset *et al.*, 1996). In addition, I found a positive correlation between eosinophils and globule leukocytes in the abomasum. This suggests that eosinophils, mast cells and antibodies play complementary roles in immunity to worms, and breeding sheep on the basis of low WEC is selecting for the same general immune response. Therefore, all parasite-resistant sheep have the same inflammatory mechanism to reject larvae and variation in WEC stems from the magnitude of this response. The reason(s) why some sheep have the inflammatory response without suffering diarrhoea is probably to do with underlying physiological differences. Larsen (1997) noted that sheep with high-dag scores had significantly lower faecal dry matter under field conditions even when there was no larval challenge. Therefore, it seems that sheep with low dag-scores have a better capacity to absorb fluid in the hindgut than do sheep with high-dag scores. During times of larval challenge, this capacity might allow them to reject larvae with an inflammatory immune response and then nullify the effects of this response through efficient absorption of fluid in the hindgut. This might also explain the rather weak correlations between inflammatory mediators and faecal dry matter as inflammation does not always lead to diarrhoea in some sheep. However, the overall negative trends and the clear relationship between eosinophils and faecal dry matter suggest that it is a minority of sheep that are able to expel larvae with an inflammatory response that does not lead to scouring. For example, in the Rylington parasite-resistant Merino flock, only a small percentage of sheep have consistently low WEC and low-dag scores (L.J.E. Karlsson pers. comm.). Therefore, sheep breeders need to be aware that breeding sheep for low WEC in a winter-rainfall environment without also concurrently breeding for low-dag scores will almost certainly bring about a moderate increase in scouring.

I found that even though levels of parasite-specific antibodies and IL-5 were increased in serum during larval challenge, there were no correlations between any serum parameter that was measured and faecal dry matter. Serum samples are routinely used in human medicine, where individuals are screened for a range of allergies by measuring antibody and cytokine responses in their blood against certain allergens (Oppenheimer and Nelson, 2006). However, in the case of gastrointestinal nematodes, it appears that the correlation between measurable parameters in serum and what

happens at the mucosal interface between the nematode and the host is too far removed. This is especially the case in the mucosa-dwelling nematodes, whereas there may be a better correlation for the blood-sucking nematode *H. contortus*. Single serum measurements on an individual animal may have had value as a diagnostic test, for example to differentiate immune-mediated scouring from functional intestinal disorders such as diarrhoea due to nutritional factors. My results suggest that this is unlikely to be a viable option. However, other researchers have found significant, positive correlations between parasite-specific IgG_1 and IgE in serum and dag score (Douch *et al.*, 1995a, Shaw *et al.*, 1999). These studies were carried out in large flocks of animals and involved several repeated measurements. Therefore, it could be possible, on a whole-flock scale, to reduce immune-mediated scouring by breeding animals with lower parasite-specific IgG_1 and IgE titres in serum. The problem with this approach is that the levels of these antibodies are also negatively correlated with WEC (Shaw *et al.*, 1999) so this will drastically slow progress in breeding for resistance to parasites. In the continued absence of genomic markers for susceptibility to immune-mediated scouring, breeding on the basis of phenotypic dag scores and including these in a selection index with low WEC still appears to be the most appropriate strategy for controlling immune-mediated scouring.

Challenging parasite-resistant sheep with either *T. circumcincta* or *T. colubriformis* larvae reduced faecal dry matter compared to unchallenged sheep. A mixed challenge of both species did not result in reduced faecal dry matter compared to either species by itself. This was a surprising result as it was expected that the immunopathology associated with larval intake would be greater with the concurrent challenge. In addition, serum antibodies were no higher in sheep receiving the mixed challenge, even though it was apparent that the two nematode species shared many L_3 antigenic epitopes between. Inflammation in the small intestine seems to be increased with a mixed challenge, as challenge with *T. circumcincta* seems to have the potential to cause an infiltration of granulocytes distal to the site of larval establishment. However, challenge with *T. colubriformis* caused no pathology in the abomasum so the mixed challenge has no extra effects in the abomasum compared to challenge with only *T. circumcincta*. This result is consistent with those of Emery *et al.* (1993b) who observed that non-specific immune mechanisms can operate downstream in the gut, but not the reverse. However, I would then expect that, if there were increased inflammation in the small intestine as a result of the concurrent larval challenge, this would translate to a decrease in faecal dry matter. The numbers of sheep in each treatment group (n=5) relatively low

but should have been sufficient at least to see a trend if there were going to be differences in faecal dry matter, as standard errors were low. The results then suggest that both *T. circumcincta* and *T. colubriformis* are capable of causing immune-mediated scouring in parasite-resistant sheep and a concurrent challenge will not necessarily exacerbate the condition.

The exact mechanisms by which the inflammatory response leads to diarrhoea have not been investigated in this thesis, but there is evidence that the increased peristalsis caused by the release of inflammatory mediators is responsible. The influx of granulocytes and mediators into the abomasum and small intestine causes fluid secretion and leakage of protein into the lumen. Ruminants have a high capacity for re-absorption of water and electrolytes in the distal parts of the small intestine and the colon (Sykes and Greer, 2003), so it would be expected that any secretions and endogenous protein would be reabsorbed and would not lead to diarrhoea. However, this process may be compromised when the flow rate of digesta is too fast to allow an effective osmotic gradient to be established and/or there is local inflammation in the colon that affects its absorptive capacity. I showed that there was no increased infiltration of inflammatory cells in the colon of challenged sheep compared to controls. Therefore, the inflammation that is caused by challenge with larvae in the abomasum and/or small intestine does not extend all the way to the colon. The most likely explanation is that inflammatory mediators released in the upper part of the tract lead to increased contraction of non-vascular smooth muscle and, therefore, increased peristalsis. This probably increases the flow of digesta along the intestinal tract. Whilst this is an effective immune response, it also probably causes diarrhoea.

With anthelmintic resistance continuing to increase, breeding sheep that are naturally resistant to parasites is a sustainable option for control. At the same time, with increasing pressure on the Australian sheep industry to phase out the practice of mulesing, reducing scouring and the incidence of dags are of paramount importance. Therefore, future research should focus on sheep that are highly resistant to parasites, but also not predisposed to scouring. There is a need to increase our understanding of the mechanisms by which these sheep reject nematodes without suffering diarrhoea.

Some sheep are able to expel incoming larvae without suffering diarrhoea. I speculated that this is due to an underlying physiological difference in these sheep, which allows them to reabsorb efficiently large amounts of water and soluble nutrients in the colon. This is a difficult hypothesis to test. It requires a carefully designed experiment

involving pair-fed controls and a quantification of the flow rate of digesta throughout different segments of the gut, using labelled markers, as well as a measurement of the inflammatory response to larval challenge.

Another approach is to examine the underlying physiological differences between scouring and non-scouring resistant sheep, through the use of genomic and proteomic microarrays. This would not only increase our understanding of the mechanism of immune-mediated scouring, but would also provide an opportunity for marker-assisted selection to breed parasite-resistant sheep that are also not prone to diarrhoea. This would be a major step forward as using dag scores can be an unreliable indicator trait because they are subjective and susceptible to influence from environmental factors. Other approaches to identify the underlying mechanisms of scouring could be immunosuppressing challenged sheep, to see if this negates the decrease in FDM, and also attempting to elicit scouring in worm-free animals through injection of specific mediator compounds such as bradykinin and histamine.

Researchers are continuing efforts to produce vaccines against gastrointestinal nematodes (Smith and Zarlenga, 2006, McClure, 2009). The results in this thesis indicate that undesirable immunopathology may be invoked by stimulating a Th2 immune response – this needs to be taken into account when designing possible candidate vaccines and should include the study of their modes of action.

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