# Mitigation of Production Losses Associated with Ruminant Gastrointestinal Nematode Infections by Induction of Mucosal Tolerance

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# Abstract

## Mitigation of Production Losses Associated with Ruminant Gastrointestinal Nematode Infections by Induction of Mucosal Tolerance

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This thesis describes a series of studies designed to further develop an antigen treatment regime involving mucosal immunisation with somatic nematode antigens to modify the immune response and reduce production losses associated with *Trichostrongylus colubriformis* infections in sheep.

The aim of the first study (Chapter 3) was to determine the larval stage specifity of *T. colubriformis* antigen to induce successful tolerance. Thirty-six five-month-old Poll Dorset x East Friesian lambs were born and raised indoors and allocated hierarchically by live weight (mean 30.50 ± 0.67 kg) into one of six treatment groups (n=6). The antigens investigated for successful immunisation were evaluated using single somatic and purified antigens that were prepared from each larval stage of *T. colubriformis* (L3, L4 and L5) and one combination antigen consisting of all three larval stages. Antigen doses containing either 7.5 µg L3, 5.5 µg L4 and 10.5 µg L5 or a combination of all three were contained in 200 µl PBS and injected into the rectal submucosa once per week for three weeks. The ability to induce mucosal tolerance was determined by measuring liveweight gains of lambs trickle infected with 2,000 *T. colubriformis* day$^{-1}$ and fed *ad libitum* of a pelleted complete ruminant diet containing 12.15 MJME kgDM$^{-1}$ and 173 g CP kgDM$^{-1}$. Apart from liveweight gains antibodies and cytokines in serum, faecal egg counts (FEC), worm burden, worm length, carcass composition and serum levels of phosphorous, urea, total protein and albumin were measured. The antigen treatment reduced worm establishment and worm length in all groups with the greatest effect in the lambs injected with the antigen combination. Furthermore, it boosted a Th2 immune response reflected as elevated IL-4 cytokines and induced immunity rather than tolerance indicated by elevated antibodies and reduced FEC.

The objective of the second study (Chapter 4) was to identify the optimal delivery route of *T. colubriformis* antigen to stimulate mucosal tolerance by investigating the intra-rectal and subcutaneous routes in four-month-old lambs. Thirty-five Coopworth lambs were born on pasture, allocated hierarchically by sex and live weight (mean 35.73 ± 1.32 kg) into one of five treatment

groups (n=7) and housed indoors for the trial. The most efficient antigen from Chapter 3, i.e. the larval combination antigen, was used. Two groups were injected with antigen into the rectal submucosa or subcutaneously in the neck area using the treatment protocol from Chapter 3. One group was immunosuppressed with weekly intramuscular injections of methylprednisolone acetate 40 mg $ml^{-1}$ at a dose 1 ml 30 kg live $weight^{-1}$ for six weeks. The lambs were trickle infected with 3,000 *T. colubriformis* $day^{-1}$ and fed *ad libitum* lucerne pellets containing 10.26 MJME $kgDM^{-1}$ and 214 g CP $kgDM^{-1}$. The ability to induce mucosal tolerance or immunity was determined by measuring the same parameters as in Chapter 3. Treatment reduced the worm establishment and female worm length in both antigen treatment groups with the greatest effect in the lambs injected intra-rectally. The antigen treatment induced a Th2 immune response with increased serum antibody responses and elevated IL-4 cytokine concentrations. Reductions in serum protein and serum albumin concentrations were alleviated in the antigen treated groups. Moreover, the intra-rectally injected group had greater performance during the last 21 days of the study compared with the untreated infected group.

The final study (Chapter 5) was conducted as a field study with 72 sets of crossbred mixed-sex twin lambs that were born on low contaminated pasture and sequentially allocated into one of six treatment groups (n=24) based on birth order, and balanced for sex. The lambs were injected with a somatic antigen either at birth or at weaning (three months of age) to explore the importance of age and previous nematode exposure on the ability to induce mucosal tolerance or immunity to gastrointestinal nematodes. Two groups were injected with the *T. colubriformis* larval combination used in Chapters 3 and 4 at the intra-rectal submucosa three times a week apart. One group was injected subcutaneously with 1 mg kg live $weight^{-1}$ of the long-acting anthelmintic Cydectin and used for positive growth control. One of the twins from each set was used as a control for the respective treatment and three of the groups were injected with PBS and acted as control for the level of pathogenicity of infection. The lambs were trickle infected from four months of age with 2,000 *T. colubriformis* $day^{-1}$. The results provided no evidence that the antigen treatment induced mucosal tolerance; however it induced immunity indicated by increased antibody production and reductions in FEC. Treatment did not mitigate the costs of immunity on growth.

In summary, these studies provided evidence that injection of lambs with a somatic *T. colubriformis* antigen is associated with a Th2 immune response and that the combination larval antigen was more efficient than individual larval antigens. The intra-rectal route was the most sufficient delivery route for inducing immunity and reducing the establishment of parasites. Extended protection through tolerance was not able to be achieved by administration of larval antigen to neonates.

Keywords: sheep, nematodes, *Trichostrongylus colubriformis,* larvae, antigen, desensitising, tolerance, immune response

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# Chapter 1
# General Introduction

Gastrointestinal parasitism is considered a major animal health problem to livestock production throughout the world, causing significant production losses (Jackson & Coop, 2000; Waller, 2006). The economic importance of gastrointestinal nematode infections in sheep farming in New Zealand has been recognised since the late 19$^{th}$ century (Gilruth, 1895) and following more than a century of research, gastrointestinal nematodes are still a key factor limiting sheep production (Leathwick, Pomroy, & Heath, 2001). Parasite control in sheep farming has strongly relied on anthelmintic treatment and the frequent use of anthelmintics has seen an increased resistance to the drugs (Vlassoff & McKenna, 1994). There is an ever increasing demand from consumers for products that originate from farming systems that utilise lower levels of chemicals and are more sympathetic towards the sustainability of the environment. As a result of the escalating anthelmintic resistance and the increasing consumer demand, alternative control methods are required.

Considerable research has been directed towards alternative methods for parasite control through enhancing the immune system of the host, including vaccines and selective breeding, however sheep selected for immunity have been observed to have a lower performance than sheep bred for productivity (Bisset et al., 1996; Bisset, Vlassoff, West, & Morrison, 1997; Morris et al., 1997). It has been established that production losses related to parasitism are attributed to a strong immune response against the nematodes and vaccination has so far, with the exception of Barbervax®, been largely unsuccessful at mitigating the effects of the nematodes (Greer, 2008; Greer et al., 2008). Therefore, it may be anticipated that methods to reduce or modify the immune responses can abrogate production losses associated with nematode parasitism in sheep.

This study focused on developing a desensitising regime involving mucosal immunisation with nematode antigens to modify the immune response and reduce production losses associated with *Trichostrongulus* infections in sheep. The study comprised of three major trials where the optimal antigen and route(s) of delivery were investigated. Additionally, the importance of the age of the animal and previous nematode exposure was examined for its ability to induce mucosal tolerance.

# Chapter 2
# Review of literature

## 2.1 Introduction

The control of gastrointestinal nematode infections in ruminants relies heavily on anthelmintic treatment in order to prevent production losses in farming systems. A major problem in livestock production worldwide is rapidly growing anthelmintic resistance (Vlassoff & McKenna, 1994; Waller, 2006) and there is a demand for alternative control methods. Considerable research has been directed towards this with the primary focus being on methods that enhance the immune response of the host animal (Waller, 1999). However, it has been established that production losses associated with parasitism in temperate parasite species are caused by a strong immune response of the host to the parasite rather than direct effects of the parasites (Greer, 2008; Williams, Palmer, et al., 2010). Alternative methods, specifically those that aim to reduce the immune responses, are of interest and hold potential for further research.

## 2.2 Host-pathogen interaction

Sheep in New Zealand farming systems are frequently exposed to gastrointestinal nematodes (Vlassoff, Leathwick, & Heath, 2001). The most prominent parasites of small ruminants are *Trichostrongylus colubriformis, Teladorsagia circumcincta* and *Haemonchus contortus* which cause major production losses and clinical disease in sheep production in New Zealand. The relative abundance of nematode species can vary between regions (Vlassoff & McKenna, 1994), with *H.contortus* being a problem mainly in the North Island of New Zealand (Bruere & West, 1993; Herve, McAnulty, Logan, & Sykes, 2003) and *Teladorsagia* followed by *Trichostrongylus* spp. being the dominating genera in the South Island (Herve et al., 2003).

### 2.2.1 Lifecycle

The life cycle of most gastrointestinal nematodes is direct, i.e. there is no intermediate host. Adult female worms in the host lay eggs which are passed out in faeces. The eggs hatch and each egg releases one first-stage larva, L1. The L1 develops and moult into a second stage larva, L2. First and second stage larvae are active feeders feeding off bacteria in faecal material and are vulnerable to desiccation. Development to the third larval stage occurs through a second moult from L2 to the infective stage, L3 (Familton & McAnulty, 1997). At this moult the cuticle of the L2 remains as a sheath, protecting L3 until they enter the host, but also preventing them from feeding. Usually, it takes two to three weeks for eggs to hatch and develop into the infective larval stage, L3 (Bruere &

West, 1993). Optimal development is temperature and moisture dependent and occurs in temperatures between 15 ˚C and 30 ˚C under adequate moisture conditions (Vlassoff et al., 2001). In moist environments a significant number of eggs develop to L3, although during dry periods the surface on the faecal mass can form a protecting crust allowing the eggs inside to hatch and develop into infective larvae. To become accessible to the animals, the L3 migrate up the grass blades where they are ingested by the sheep. In the digestive tract L3 larvae exsheath and become L4 larvae. Small intestinal parasites normally exsheath in the abomasum, whereas abomasal parasites exsheath in the rumen. Over a period of 8-10 days, L4 larvae moult to form immature adults (L5), which then develop to mature adult worms. Female and male worms mate inside the host and females produce eggs that are passed out in the faeces (Familton & McAnulty, 1997). The pre-patent period (the time from ingestion of larvae to egg production) is typically 21-28 days (Bruere & West, 1993). Under unfavourable conditions, some gastrointestinal nematodes can inhibit their development at the L4 (or L3) stage and persist in hypobiosis until more favourable conditions when they can continue their development. This arrested stage of development is generally seen in larvae ingested during late autumn and winter (Urquhart, 1987). Resumption of larval development can be induced by environmental factors, e.g. temperature and humidity suitable to the free-living development, or be triggered by host factors such as depressed immunity or changes in hormone levels around parturition (Kassai, 1999; Urquhart, 1987).

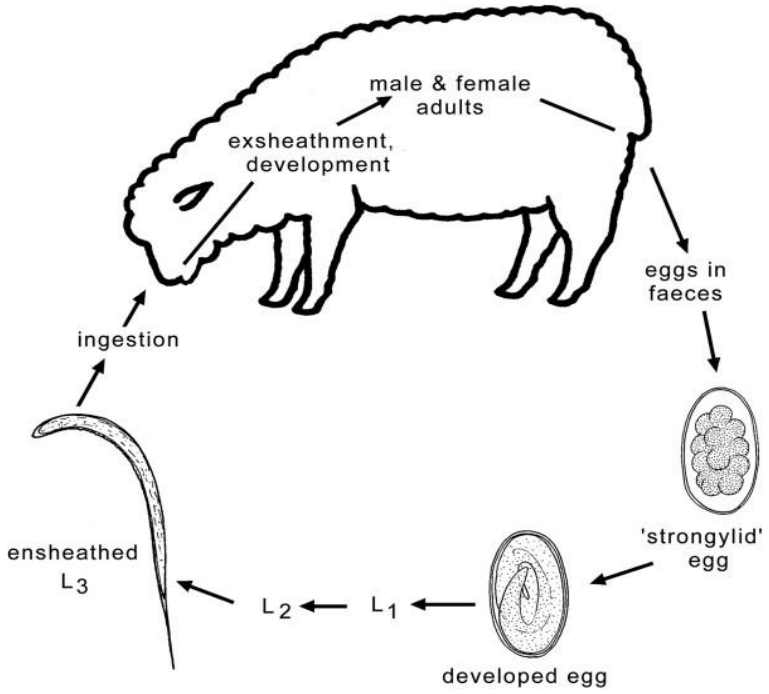


Figure 2.1: Direct life cycle of gastrointestinal nematodes (Roeber, Jex, and Gasser (2013)).

### 2.2.2 Larvae on pasture

There is a variation in numbers of infective larvae on pasture throughout the seasons with a minor peak in larval numbers seen in the spring and a major peak in the autumn. The spring peak is caused by the maturation of over-wintered larvae as well as larvae derived from the peri-parturient rise in faecal egg counts (FEC) explained by a temporary drop in immunity of ewes in late pregnancy and early lactation. Worm numbers in the lambs build up slowly during spring and early summer to the major peak seen in the autumn. The high infectivity seen on pastures in the autumn is caused by eggs deposited by lambs in spring and early summer (Taylor, 1999). A rapid decline in worm numbers is seen in the winter when the lambs are 10-12 months of age and is associated with development of immunity (Brunsdon, 1970).

### 2.2.3 Impacts on the host

Infections with gastrointestinal nematodes can have various effects on the animal depending on factors such as nematode species and infection rate. Infections with low numbers of *Trichostrongylus* spp. are often asymptomatic but when present in large numbers, between 10,000-100,000 worms, the parasites cause clinical disease (Georgi, 1985). Severe clinical parasitism may lead to sick moribund animals of which some will die despite anthelmintic treatment with the rest of the mob usually suffering from chronic, subclinical parasitism (Sutherland & Scott, 2010). Although losses from clinical parasitism are still commonly seen, subclinical losses are of greater economic importance (Bruere & West, 1993; Vlassoff et al., 2001).

Gastrointestinal nematodes generally limit the host productivity through reduction in voluntary feed intake and impaired nutrient absorption. Depression of appetite is a common feature in *Teladorsagia*-infected sheep while infections with *Trichostrongylus* spp. typically are associated with reduced nutrient utilisation for maintenance and growth (Coop & Sykes, 2002). Studies on infected sheep and uninfected control groups offered the same amount of feed have shown that gastrointestinal parasitism leads to reduced nutrient utilisation of the feed (Coop & Holmes, 1996; Houtert & Sykes, 1996). A decrease in energy utilisation by 40-50% and reductions in liveweight gain by up to 60% have been reported in sheep subclinically infected with either *T. colubriformis* or *T. circumcincta* (Coop, Sykes, & Angus, 1982; Sykes & Coop, 1976, 1977). A decrease in feed intake around 10-30% is commonly seen in subclinical intestinal parasitism (Poppi, Sykes, & Dynes, 1990) and has been seen to be responsible for 60-90% of performance loss during nematode infections (Coop & Holmes, 1996; Houtert & Sykes, 1996). The level of reduction in feed intake and the impaired nutrient utilisation are influenced by the numbers of larvae ingested and the nematode species (Coop et al., 1982; Houtert &

Sykes, 1996). Steel, Symons, and Jones (1980) reported that lambs that received 30,000 *T. colubriformis* week$^{-1}$ suffered a greater reduction in feed intake than lambs receiving 3,000 or 9,500 larvae week$^{-1}$ which had a greater reduction than lambs infected with 300 or 950 larvae week$^{-1}$.

#### 2.2.3.1 Voluntary feed intake

The causes of the anorexia are not fully established but it appears that the presence of parasites play an important role as it has been proved that the feed intake returns to normal following anthelmintic treatment or the acquisition of immunity (Kyriazakis, Anderson, Oldham, Coop, & Jackson, 1996). It is suggested that the immune system can interact with the central nervous system and modify feed intake by stimulating leukocytes to produce pro-inflammatory cytokines such as IL-1 and TNF-α. The cytokines stimulate the hypothalamus to cause fever and inappetence (Johnson, 1998). A hypothesis is that increased leptin levels are a contributing factor to anorexia in parasitised sheep in that it stimulates the production of IL-1, TNF-α, IL-6 (Zaralis, Tolkamp, Houdijk, Wylie, & Kyriazakis, 2008). However, a number of studies have rejected this hypothesis concluding that circulating leptin is not associated with the feed intake in infected sheep (Greer, Boisclair, et al., 2009; Zaralis, Tolkamp, Houdijk, Wylie, & Kyriazakis, 2009; Zaralis et al., 2011). The gastrointestinal hormone cholecystokinin (CCK) has been proposed as having an influence on the regulation of feed intake. Dynes, Poppi, Barrell, and Sykes (1998) studied the effect of CCK in sheep infected with *T. colubriformis* that had a 10-60% reduced feed intake. Whereas no effect was seen on voluntary feed intake when a CCK antagonist was given systemically before feeding an increase in short-term feed intake and duration of feeding was noted when the CCK antagonist was administered to the central brain. In this study, a 188% rise in feed consumption was reported during the second hour of feeding in infected animals and it was concluded that central satiety signals might be involved in the reduction in voluntary feed intake in parasitised sheep.

#### 2.2.3.2 Nutrient utilisation

Increased loss of endogenous protein into the gastrointestinal tract due to damage to the abomasum and small intestine is a feature typical of many nematode infections. *T. colubriformis* reside in the small intestine and, when ingested, larvae penetrate the epithelial glands causing loss of absorption and leakage of plasma proteins into the lumen of the intestines. These losses can increase four-fivefold in parasitised animals and Poppi, MacRae, Brewer, and Coop (1986) reported an increase of protein loss from 20 g to 125 g day$^{-1}$ in sheep infected with *T. colubriformis.* Although some of the protein is reabsorbed, up to 30 g of non-reabsorbable nitrogen (N) has been found to leave the terminal ileum of parasitised sheep day$^{-1}$ (Kimambo, MacRae, Walker, Watt, & Coop, 1988; Poppi et al., 1986). It has been concluded that the increase in endogenous loss of N is the main cause of an impaired N balance, possibly due to increased secretion of plasma proteins towards the

gastrointestinal tract and sloughing of epithelial cells (Bown, Poppi, & Sykes, 1991; Poppi et al., 1986). The loss of plasma protein has been estimated to only make up 10-30% of the total endogenous N losses in infected sheep (Poppi et al., 1990). A redirection of protein synthesis in production tissues into the liver and the gastrointestinal tract for repair of the gastrointestinal epithelium and maintenance of the immune system is a major consequence of nematode infection (Jones & Symons, 1982) and leads to reduced production, such as muscle deposition and wool growth (Holmes, 1993; Poppi et al., 1990; Sykes, 1994). Once animals have developed immunity their growth rates have been seen to return to levels comparable with their uninfected counterparts (Kimambo et al., 1988), presumably reflecting a lack of damage in immune animals due to failure of larval establishment (Jackson et al., 2004).

### 2.2.4 Resistance and resilience

Improving resistance and resilience to reduce the impact of nematode infection in young lambs is desirable in sheep production systems and has been an area of interest for many years (Hunt, McEwan, & Miller, 2008). Resistance is the ability of an animal to prevent or limit the establishment or development of infection (Albers et al., 1987) and is typically assessed by the host's ability to limit faecal egg counts (FEC), worm burden, worm length and female fecundity (Coop & Sykes, 2002). Increased levels of circulating immunoglobulins are also used to measure resistance (Iposu, Greer, McAnulty, Stankiewicz, & Sykes, 2010; Shaw, Morris, Wheeler, Tate, & Sutherland, 2012). Resilience is defined by the ability of an animal to maintain a reasonable level of productivity, e.g. weight gain or milk and wool production, under parasite challenge (Albers et al., 1987). Different phenotypic traits have traditionally been used in the selection for resistance and resilience although the identification of genetic markers associated with resistance has made progress in breeding for nematode resistance (Hunt et al., 2008). Improved nutrition has in several studies been reported to increase resistance and resilience and reduce production losses and parasite burdens as a consequence of an enhanced immune response and a better capacity to replace endogenous losses (Coop & Kyriazakis, 2001; Houtert & Sykes, 1996; Waller, 1999), potentially through increased protein rather than energy intake (Bown et al., 1991; Houtert & Sykes, 1996; Kyriazakis et al., 1996). In sheep infected with *T. colubriformis* and given the option of high (21% CP) or low (9% CP) protein feed with the same ME concentration, 10.4 MJ $kg^{-1}$, the animals changed to the higher protein diet when challenged with nematodes (Kyriazakis et al., 1996). In this study, despite a 10% reduction in feed intake in infected animals, their total CP intake was similar to their uninfected counterparts indicating that infected sheep may display some nutritional wisdom and alter their diet to mitigate the effects of subclinical infection (Kyriazakis et al., 1996; Kyriazakis, Oldham, Coop, & Jackson, 1994a).

## 2.3 Immunity to gastrointestinal nematodes

The immune response of the animal against pathogens is complex and not yet fully understood. Protective immunity against most gastrointestinal nematodes does not occur after primary parasite infection but takes several weeks or months after recurrent infection to develop (Dineen & Wagland, 1966; Smith, 1988). The degree of clinically observable immunity and the rate with which it develops appear to be influenced by the parasite species, number of parasites and the age, sex, and breed of the host (Emery, McClure, & Wagland, 1993). Lambs rapidly develop resistance to small intestinal parasites, such as *T. colubriformis* but take longer to gain the ability to control abomasal parasites (McClure, Emery, Bendixsen, & Davey, 1998; Smith, Jackson, Jackson, & Williams, 1985). Greer and Hamie (2016) suggested that relative maturity of the lambs may also be of importance in the timing of development of immunity and that physiological age, rather than chronological age, may explain differences in responses to gastrointestinal nematodes within and between breeds. Very young ruminants are believed to have an increased susceptibility to infectious diseases considered to be caused by an immunological hyporesponsiveness (Colditz, Watson, Gray, & Eady, 1996) and a lacking ability to acquire immunity to parasites (Dineen, Gregg, & Lascelles, 1978; Dineen & Wagland, 1966; Iposu et al., 2010; Kambara, McFarlane, Abell, McAnulty, & Sykes, 1993; Smith et al., 1985). Kambara et al. (1993) showed that young lambs did not respond immunologically to nematode infection at 8-20 weeks of age challenged with *T. colubriformis* L3 larvae but when challenged at 33 weeks of age there was a difference in lymphocyte responsiveness between infected and uninfected lambs.

Typical indicators of acquired immunity are: reduced numbers of female worms and female fecundity, a decreased size of adult worms and increased numbers of inhibited larvae (Stear, Strain, & Bishop, 1999a, 1999b). Other factors associated with acquired immunity include reduced FEC, increased worm expulsion, increased numbers of cytokines and inflammatory cells, such as eosinophils, mast cells and lymphocytes, as well as production of parasite-specific antibodies (Lee et al., 2011). Increased levels of Immunoglobulin A (IgA), IgG1, and IgE antibodies are commonly seen in immune sheep (Stear et al., 1997; Williams, Palmer, et al., 2010). Local IgA secretion has been reported to reduce female worm length and female fecundity (Stear et al., 1995; Stear et al., 1999a; Strain et al., 2002) and elevated IgG1 and IgE antibody levels have been negatively correlated with FEC (Bendixsen et al., 2004; Bisset et al., 1996; Douch, Green, & Risdon, 1994; Shaw et al., 1999). However, Shaw et al. (1999) reported unfavourable effects on liveweight gain associated with elevated serum IgE responses to *T. colubriformis* antigen. IgE is suggested to play a significant role in parasite rejection (Huntley et al., 2001; Miller, 1996; Shaw, Gatehouse, & McNeill, 1998) which can occur at different times following infection. Rapid rejection of infective larvae may occur within hours of infection and

delayed rejection normally takes place several days post-infection (Emery et al., 1993) and continues for a number of days. The rapid expulsion is associated with mucosal mast cell hyperplasia and globule leucocytes in the gastrointestinal tissue (Miller, 1996).

### 2.3.1 Mechanisms of the immune response

Immunity may be mediated by a number of different protective mechanisms acting together and can be divided into innate and acquired immunity (Emery, 1996). Innate immunity aids in initiating the acquired immunity which consists of humoral immunity (B cells) and cell-mediated immunity where T cells play a crucial role. T cells can be separated into cytotoxic T cells (Tc) and T helper cells (Th). Th cells can be further classified as Th1, Th2 or Th17 cells depending on the profile of cytokines they produce. Th1 cells are involved in cellular immunity against intracellular bacteria and viruses and Th2 cells are involved in the humoral immunity to parasitic infection. It is considered that Th17 cells link innate and adaptive immunity to produce a strong antimicrobial inflammatory response (Bettelli et al., 2006; Peck & Mellins, 2010; Veldhoen, Hocking, Atkins, Locksley, & Stockinger, 2006) and the primary role appears to be clearance of pathogens that are not sufficiently controlled by Th1 or Th2 cells such as extracellular bacteria and fungi (Korn, Bettelli, Oukka, & Kuchroo, 2009; Weaver, 2009). Another subset of Th cells is regulatory T cells (Tregs). Tregs suppress the activity of other lymphocytes and play an important role in the induction and maintenance of tolerance (Sakaguchi, Yamaguchi, Nomura, & Ono, 2008).

The immune response to gastrointestinal nematodes is typically characterised by a Th2 response (Williams, Palmer, et al., 2010) and normally includes enhanced levels of IgG and IgE as well as local mast cell and eosinophil responses in the tissues (Bendixsen et al., 2004; Mosmann & Coffman, 1989; Williams, Karlsson, et al., 2010). Mucosal mast cells express a high-affinity receptor (FcεRI) for the Fc region of IgE, and mast cells are normally coated in parasite-specific IgE. The combination of the parasitic antigens and mast cell-bound IgE triggers mast cell degranulation and the release of inflammatory mediators, such as histamine, leukotrienes and proteases, that stimulate smooth muscle contraction and mucus secretion, and flush larvae from the gut (Meeusen, Balic, & Bowles, 2005; Williams, Palmer, et al., 2010). Eosinophils are also suggested to be directly involved in the rejection of parasites although their exact role is not known (Meeusen et al., 2005).

Th1 and Th17 responses are typically associated with susceptibility to nematode infections rather than immunity (Meeusen et al., 2005). The differentiation of naïve CD4+ T cells into effector Th cells is initiated by the connection of cells bearing T cell receptors, TCRs, and co-stimulatory molecules in the presence of cytokines produced by the innate immune system, in response to certain pathogens.

IL-12 from dendritic cells and IFN-γ trigger the differentiation of Th1 cells which is characterised by high production of IFN-γ and is effective in clearing intracellular pathogens (Korn et al., 2009). Th1 cells also release IL-2 and TNF-α that are responsible for activation of macrophages and induction of IgG antibodies that mediate phagocytosis (Jankovic, Liu, & Gause, 2001). Macrophages release pro-inflammatory cytokines including IL-1, IL-6 and TNF-α that cause an inflammatory response and activate the hypothalamus to induce fever and cause inappetence and sickness behaviour in the host (Johnson, 1998). The differentiation of Th2 cells is induced by the cytokine IL-4. Th2 cells are crucial for the host defence against extracellular pathogens and in helping B cells to produce antibodies (Kimura & Kishimoto, 2010; Korn et al., 2009) and release cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 which promote growth and differentiation of mast cells and eosinophils. TGF-β is a suppressor of Th1 and Th2 differentiation and together with IL-2 plays an important role in the differentiation of naïve T cells into Tregs expressing the transcription factor Foxp3 (Korn et al., 2009). Although, in combination with IL-6, it inhibits the differentiation of T cells into Foxp3+ Tregs and initiates the differentiation of Th17 cells (Bettelli et al., 2006). Th17 cells have been found to be important for the host defence against many microorganisms. However, they can also contribute to immunopathology detrimental to the host during infection, as Th17 responses have been associated with chronic inflammation as seen in autoimmune diseases (Bettelli et al., 2006; Korn et al., 2009). Whether or not Th17 cells are harmful depends on the disease and target tissue (Lohr, Knoechel, Caretto, & Abbas, 2009). Generally, it is considered that a balance between Th17 cells and Tregs are crucial for immune homeostasis and IL-6 is a crucial factor for regulating this balance (Kimura & Kishimoto, 2010). Further, cytokines produced by the different subsets of T helper cells can suppress the production of other subsets (Mosmann & Coffman, 1989). A number of factors such as the type and dose rate of antigen, type of antigen presenting cells (APC), and surrounding cytokines at antigen presentation influence which subset of Th cells will be predominant in an immune response following a nematode infection (Constant & Bottomly, 1997).

### 2.3.2 Mucosal immunity

The mucosal surfaces are the main entry sites of many pathogens with the majority of infectious processes taking place here. They comprise an important component of the immune system of both humans and animals and have three main functions: (i) to protect the mucosa against invasion and colonisation by microbes, (ii) to prevent uptake of antigens and (iii) to prevent development of potentially harmful immune responses to the antigen (Holmgren & Czerkinsky, 2005; McNeilly, McClure, & Huntley, 2008). Mucosal surfaces of the gastrointestinal, respiratory and reproductive tracts have a barrier consisting of epithelia which differ slightly from one tract to another. This barrier is part of the innate immune system and, in addition to being a physical barrier, also consists of

proteins and antimicrobial peptides along with a number of cell types such as mucosal mast cells, dendritic cells and natural killer (NK) cells. Another cell type found on mucosal surfaces are γδ-T cells which make up around 50% of the peripheral T lymphocytes in ruminants compared with only 5% in humans and rodents (Hein & Dudler, 1993; Schreurs, Pernthaner, Hein, & Barry, 2010). The exact role of γδ-T cells in the immune response has not yet been established but it is believed that they are part of the innate defence acting through the release of immunoregulatory cytokines (Casetti & Martino, 2008; Jutila, Holderness, Graff, & Hedges, 2008). Infective organisms are characterised by pathogen associated molecular patterns (PAMPs). They are detected through pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) found on many cells including epithelial cells, macrophages, mast cells and dendritic cells and respond by sending cytokine and chemokine signals to underlying mucosal cells which trigger the innate immune system and promote adaptive responses (Basset, Holton, O'Mahony, & Roitt, 2003). A key feature of the adaptive mucosal immune system is the local secretion of IgA antibodies. IgA in secretions are synthesised by local plasma cells as a dimer and released into the interstitial fluid where they are taken up by specific polymeric immunoglobulin receptors, pIgR, a secretory component expressed on the interstitial epithelial cells (Woof & Kerr, 2006). The secretory component bound to the dimeric IgA is secreted into the mucous layer on the epithelium as secretory IgA (S-IgA) where its main role is to bind to antigens and prevent direct contact between pathogens and the mucosal surface and attachment to the epithelium (Husband, 1987). The mucosal immune system is composed of different lymphoid tissues that are associated with the mucosal surfaces, MALT (mucosa-associated lymphoid tissue) and can be divided into two parts; induction sites and effector sites. Induction sites consist of organised MALT and are responsible for the induction of the immune response. They are found in areas where pathogens are most likely to enter the body, e.g. the tonsils, bronchi, Peyer's patches and mesenteric lymph nodes (Mowat, 2003). Induction sites involve mucin-producing glandular cells, lymphocytes, plasma cells, dendritic cells, macrophages and the specialised epithelial cells, M cells. The M cells play a crucial role in luminal uptake and transport of antigens presented on the mucosal surfaces (Lamm, 1997). Effector sites also referred to as diffuse MALT, consist of leukocytes scattered throughout the epithelium and lamina propria (Mowat, 2003). MALT can be further divided into GALT (gut-associated lymphoid tissue), BALT (bronchus-associated lymphoid tissue), NALT (nasopharynx-associated lymphoid tissue) and mammary and salivary glands, depending on their location.

During primary infections with *T. circumcincta* and *T. colubriformis* in sheep, larvae penetrate the mucosa of the abomasum and small intestine respectively, where they reside and excrete antigens. The nematode antigens are taken up into the Peyer's patches by M cells located on top of the Peyer's

patches and passed on to APCs such as dendritic cells which present the antigen to CD4+ Th cells via their MHCII receptors within the GALT (Gossner, Venturina, Shaw, Pemberton, & Hopkins, 2012; Pernthaner, Cole, Morrison, & Hein, 2005). While IgA is the predominating antibody class secreted on mucosal surfaces (Lamm, 1997; Lascelles, Beh, Mukkur, & Watson, 1986), IgG1 also appears to play an important role in mucosal immunity in ruminants (Lascelles et al., 1986).

### 2.3.3 Common Mucosal System

Cytokines associated with a Th2 response, such as IL4, IL-5, IL-6 and IL-10, are produced in MALT and influence B cells to proliferate and differentiate into IgA plasma cells (Lamm, 1997; Meeusen et al., 2005). The activated B cells leave the mucosa via the lymph and are guided by 'homing receptors' through the draining mesenteric lymph nodes and the bloodstream, before returning to the mucous membranes. Some of these receptors are broadly expressed and B cells can be attracted to several different mucosal sites. Mucosal immunisation at one site can, therefore, lead to IgA secretion in other mucosal tissues. This phenomenon is known as the 'common mucosal immune system' (Mestecky, 1987). IL-5 also stimulates proliferation of eosinophils and mast cells (from the bone marrow into lamina propria in the gut) which typically appear in increased numbers during nematode infection (Miller, 1990). Mucosal immunity is most effectively induced by delivery of vaccines directly onto mucosal surfaces, however, the understanding of the mucosal immune system is incomplete and the development of mucosal vaccines is complicated. Consequently, there are no mucosal vaccines specific to gastrointestinal nematodes in sheep for use in veterinary medicine as yet (Pavot, Rochereau, Genin, Verrier, & Paul, 2012).

## 2.4 Cost of immunity

A strong immune response against parasites has benefits for the host, such as a reduced parasite burden and decreased susceptibility to reinfection, although it may also have a nutritional cost to the host animal (Colditz, 2002). It has been calculated that the immune response is responsible for 39% and 75% of the entire cost of nematode infection in grazing lambs exposed to *Trichostrongylus vitrinis* and *T. colubriformis,* respectively (Blackburn, Carmichael, & Walkden-Brown, 2015; Dever, Kahn, Doyle, & Walkden-Brown, 2016) and the total cost in lambs housed indoors and infected with *T. colubriformis* or *T. circumcincta* (Greer et al., 2008; Greer, Stankiewicz, Jay, McAnulty, & Sykes, 2005). The ability of the parasitised animal to maintain production is therefore greatly affected by host nutrition (Coop & Kyriazakis, 1999). Sykes (1994) estimated that a production cost of 15% is required in sheep to maintain immunity to parasites, a figure supported by the immunosuppression studies of Greer et al. (2005).

FEC have been utilised extensively in genetic selection for resistance to gastrointestinal nematodes. Selection for low FEC leads to significantly reduced worm burdens of most of the sheep nematodes of economic importance (Bisset et al., 1996), however low FEC does not always mean increased productivity and resistant animals are not necessarily unaffected by the effects of nematodes during grazing (Bisset & Morris, 1996). Sheep breeds selected more intensively for production traits such as growth and fleece weight have been found to be more susceptible to gastrointestinal nematode infections than breeds that have been selected less intensively (Bisset, Morris, McEwan, & Vlassoff, 2001). It has been established that sheep selected for low FEC have no better growth than sheep selected for high FEC when grazed together on contaminated pastures (Morris et al., 1997; Morris, Watson, Bisset, Vlassoff, & Douch, 1995; Morris, Wheeler, Watson, Hosking, & Leathwick, 2005). Morris et al. (2005) reported a 6-12% lower live weight and 24-26% lower fleece weight in lines bred for low FEC when grazed together with lines bred for high FEC. Similarly, Morris et al. (1997) reported a lower fleece weight in low FEC lines compared with high FEC lines when grazed on shared pastures. Ewes bred for low FEC had on average a 10.9% lower fleece weight than the high FEC lines and yearling low lines had an average 9.1% lower fleece weight than the yearling high lines. No significant difference in fleece weight was seen when the groups were grazed apart. This indicates that when the sheep were grazed together the pasture contamination was greater, which is unfavourable for production, and it has been suggested that real advantages from selecting for low FEC only are seen when sheep are grazed separately (Bisset & Morris, 1996). Increased dag scores and negative effects on liveweight gain in lambs have also been reported in lines selected for low FEC (Bisset & Morris, 1996; Bisset et al., 1997). The increased dag scores have been attributed to a stronger immune response in the low FEC lines, i.e. resistant animals, than in their more susceptible counterparts resulting in increased hypersensitivity to nematode infection.

The nutritional cost of immunity can also be observed in supplementation studies. Supplementary feeding with dietary protein to infected lambs has been proven to reduce the severity of infection, however, it is considered to have little impact on the ability to prevent the early establishment of nematode infection in young growing animals due to a lacking immune response (Sykes, 2008). Rather, it seems to have a greater effect on the extent to which the animal can gain immunity (Bown et al., 1991; Houtert & Sykes, 1996; Kambara et al., 1993). Bown et al. (1991) also showed that protein supplementation of lambs with a post-ruminal infusion of casein can have an effect on immune development. No effect was seen on worm burdens and FEC after six weeks of infection, but after 12 weeks a threefold reduction was noted in lambs that received a casein infusion of 50 g of metabolisable protein $day^{-1}$. Supplementary protein has also been shown to influence the expulsion

rate of parasites from the host. The reduction in FEC and the rate of expulsion was seen to be related to the level of protein given in a study where sheep infected with *T. colubriformis* were fed a supplementary diet of either 50 g or 100 g of fishmeal $day^{-1}$ (Vanhoutert, Barger, Steel, Windon, & Emery, 1995). As such, it appears supplementation has the primary advantage of allowing sufficient nutrient resources to assist with meeting the cost of pathological disturbances and development of immunity.

A strong immune response against the parasite is acquired throughout the acquisition period of immunity, about 4-14 weeks after infection. This is thought to have a higher priority than growth in young naïve lambs, otherwise, the animal may suffer severe costs of the parasitic burden before it reaches reproductive maturity (Coop & Kyriazakis, 1999). Iposu et al. (2010) reported that nematode infection in the very young lamb, up to 12 weeks of age, does not impact on production suggesting young lambs avoid the nutritional costs associated with immune development due to lacking an ability of to acquire immunity to parasites. McClure and Emery (2007) found that the body weight at the start of infection was important for lambs' ability to develop immunity against parasites. Merino lambs with a body weight of less than 23 kg at the time of their first challenge with *T. colubriformis* had a reduced ability to develop protective immunity upon a subsequent challenge. Infection with *T. colubriformis* has a significant impact on production during the acquisition period, but once immunity developed after about 14 weeks, performance returns to normal (Sykes & Greer, 2003). It has also been demonstrated that naïve lambs around three to four months of age had a 15-30% reduction in feed intake during the first two to four weeks of infection which was associated with an increased FEC. The intake was restored after 8-10 weeks following a decrease in FEC and a return of immunological indicators, such as eosinophils and IgA, to pre-infection levels, i.e. an established immunity (Houtert & Sykes, 1996; Sykes, 2008).

During the immune response, there is a redirection of nutrients away from production tissues towards the liver and gastrointestinal tract for the production of acute phase proteins (Colditz, 2002). Acute phase proteins are synthesised in the liver by pro-inflammatory cytokines which activate the acute phase response (APR). The APR may reduce protein synthesis and alters nutrient availability, nutrient uptake and nutrient utilisation in production tissues (Colditz, 2003). Jones and Symons (1982) noted an increase in protein synthesis in the liver from 29.4 g to 47.7 g $day^{-1}$ and a reduction in amino acid incorporation in muscle from 0.345 g to 0.242 g $day^{-1}$ in lambs infected with *T. colubriformis*. Wool growth can be impaired in nematode-challenged sheep due to a redirection of protein synthesis towards the liver and the gastrointestinal tract. The requirement for glutamine and cysteine for

activation of the acute phase response may be increased during nematode infection, and with these amino acids also being important for wool growth, especially cysteine, which is a limiting amino acid for wool synthesis, the immune response may limit the availability of cysteine for wool growth (Colditz, 2002). Barger, Southcott, and Williams (1973) and Lobley, Hoskin, and McNeil (2001) have reported supplements of 5 g glutamine plus 1 g cysteine $day^{-1}$ during infection with *T. colubriformis* had no effect on wool growth. In contrast, supplements of 2 g of cysteine $day^{-1}$ were found to increase wool growth by 33% in both infected and non-infected sheep, although it did not completely reverse the negative impact of infection on wool growth (Barger et al., 1973).

Decreased efficiency of nutrient utilisation for growth is a factor influencing the costs of immunity with reduced weight gain being a major consequence of gastrointestinal nematode infection (Colditz, 2003). It is generally believed to be caused by inappetence and reduced feed intake as an effect of the immune response, rather than malabsorption of nutrients in the intestine (Coop & Kyriazakis, 1999; Sykes, 2008; Williams, Palmer, et al., 2010). Many components of the immune system, including immunoglobulins, mast cells, globule leukocytes and lymphokines, are proteinaceous in nature and it can, therefore, be anticipated that they drain the protein resources during an immune response (Coop & Holmes, 1996; Lewis & Austen, 1981). Depression of the host's immune response by corticosteroid treatment has been reported to reverse the inappetence in lambs during infection with *T. circumcincta* (Greer et al., 2008) and *T. colubriformis* (Greer et al., 2005). Greer et al. (2008) also found that the immunosuppressed lambs had a greater feed intake and grew faster than their immunologically normal counterparts, despite having a distinctly larger worm burden, which supports this theory.

### 2.4.1 Immunopathology

Pathology associated with nematodes includes direct damage by the parasite, e.g. from penetration and feeding on the host or the host's immune response to the parasite (Garside, Kennedy, Wakelin, & Lawrence, 2000). Pathology linked to immune responses can be due to direct effects of immune mechanisms on tissue or associated with metabolic factors and loss of body weight as a result of competition for nutrients between growth and the immune system (Williams, Palmer, et al., 2010). A strong immune response to nematodes may have unfavourable side effects for the host, and many of the clinical signs of nematode infection may in fact be effects of to the developing immunity rather than direct effects of the parasite (Greer, 2008; Williams, Palmer, et al., 2010). Characteristics for pathology associated with nematodes are villus atrophy, hyperplasia of the intestinal crypts and goblet cells, as well as infiltration by inflammatory cells in the mucosa, e.g. eosinophils and mast cells (Garside et al., 2000). Infection with *T. colubriformis* is also characterised by hypophosphataemia due

to decreased absorption of P (Bown, Poppi, & Sykes, 1989; Poppi, MacRae, Brewer, Dewey, & Walker, 1985; Wilson & Field, 1983), and reduced serum albumin concentrations as a result of loss of plasma proteins into the gastrointestinal tract and decreased albumin synthesis in the liver (Coop, Sykes, & Angus, 1976; Steel et al., 1980; Sykes & Coop, 1976). Lack of reduction in serum albumin has been reported in immunosuppressed lambs (Greer et al., 2005) although leakage of plasma albumin still exists (Greer et al., 2005; Vaughan, Greer, McAnulty, & Sykes, 2006) indicating that the hypoalbuminaemia in immune animals is a result of physical damage caused directly from the nematodes rather than immunopathology. Vaughan et al. (2006) showed that losses of albumin in corticosteroid treated animals still occurred suggesting that the lack of reduction in albumin in immunosuppressed animals arose from a greater capacity to replace endogenous losses.

During gastrointestinal nematode infections, there is an increased maintenance demand from the gastrointestinal tract. Amino acids are redirected from muscles to replace endogenous mucus and plasma secretions in the gut and to repair the mucosal epithelium, leading to protein deficiency commonly seen in infected animals (Sykes & Greer, 2003). Typical for a developing immune response is the release of pro-inflammatory cytokines such as IL-1, TNF-α, IL-6 and increased acute phase protein synthesis in the liver (Basset et al., 2003; Colditz, 2002; Johnson, 1998). Acute phase proteins facilitate tissue repair and many of the amino acids used in the acute phase protein synthesis are derived from muscle degradation mediated by the pro-inflammatory cytokines (Johnson, 1997). Diarrhoea and breech faecal soiling are often seen in infections with *T. circumcincta* and *T. colubriformis*. It is typically connected with heavy worm burdens but has been demonstrated to be linked to immunity to nematodes, triggered by Th2 cytokines and associated with high numbers of inflammatory cells such as eosinophils and mucosal mast cells (Meeusen, 1999), as such can be considered to contribute to the cost of immunity.

## 2.5 Vaccines

There are very few commercially available vaccines against nematode parasites, although with the increasing demand for alternative non-chemical parasite control methods, this is an area that has received considerable attention. Vaccines have some advantages over chemical anti-parasitic treatments as they leave no chemical residues nor do they damage the environment (Dalton & Mulcahy, 2001). As yet there are some major obstacles to the production of vaccines in a commercially viable way, e.g. the number of vaccine components must be reduced and appropriate antigens capable of delivering cross-protection yet have to be discovered (Knox, 2000; Matthews, Geldhof, Tzelos, & Claerebout, 2016). The efficacy of vaccines has been estimated through computer

models. It is suggested that vaccines do not require 100% efficacy, unlike anthelmintics, but are required only to be efficient enough to prevent clinical disease in animals and thereby prevent production losses with an efficacy level of around 80% considered enough to provide sufficient control (Barnes, Dobson, & Barger, 1995). The effect of vaccination can be influenced by the antigen dose and the number of doses, adjuvants, delivery systems and administration site. Anthelmintics are inexpensive and relatively easy to administer (Dalton & Mulcahy, 2001) and for a vaccine to substitute for drugs it has to be cost-effective, deliver protection against several nematode species with satisfactory duration, and be practical to administer under farm conditions (Dalton & Mulcahy, 2001; Knox, 2000). Factors impacting on the immunological effect of vaccines include the age of the animal (raised corticosteroid levels due to stress at weaning can interfere with induction of immunity) (Emery et al., 1993), farm conditions and the genetic make-up of individuals (Knox, 2006).

The first commercially available vaccine against nematode infections in ruminants was an irradiated vaccine against the bovine lungworm, *Dictyocaulus viviparous* (Matthews et al., 2016; Meeusen, Walker, Peters, Pastoret, & Jungersen, 2007; Smith, 1999). Attempts were also made to develop irradiated vaccines against the gastrointestinal nematodes *H. contortus* and *T. colubriformis* but these only provided protection in adult sheep and not in young lambs and were never commercialised (Smith, 1999). In the case of *H. contortus*, this problem has now been overcome through the use of vaccines based on 'hidden' antigens expressed on the surface of the parasite's intestine and there is now a commercial vaccine available in Australia for the control of haemonchosis (Matthews et al., 2016). When these antigens are administered to the animal, antibodies are produced (Knox, 2006). An advantage with hidden antigens is that they induce immunity where natural immune responses are weak and therefore work well in both lambs and older animals (Smith, 1999). Targeting intestinal antigens in non-blood feeding nematodes has been less effective suggesting that they do not produce enough antibodies (Knox, 2006). A drawback with this kind of vaccine is that since the antigens are not seen during natural infection, antibody levels are not boosted by infection with the parasite so repeated immunisation is required (Miller & Horohov, 2006).

In the 1980's the development of recombinant DNA technology and the opportunity to produce recombinant parasite proteins were major steps forward in the development of antigen vaccines against parasites (Emery, 1996) and DNA vaccines have been studied widely over the last two decades. The concept of DNA vaccines is based on plasmid vectors carrying an inserted gene or cDNA of interest. The DNA plasmid is delivered to the target cell where it is taken up and expressed by the host cells. The host immune system recognises it as foreign and an immune response is induced

(Knox, 2000; Sedgmen, Meeusen, & Lofthouse, 2004). DNA vaccination has been successful in rodents but has been less so in large animals (Eriksson & Holmgren, 2002). Much research has focused on the use of 'conventional' antigens found in worm somatic tissue, excretory/secretory (ES) products, including enzymes and a number of proteins capable of altering the host immune response (Knox, 2006; Miller & Horohov, 2006). Several studies on *H. contortus* and *T. colubriformis* have shown significant potential in the use of antigens purified from L3 and adult ES products from the nematodes (Emery, 1996). Recent research on lambs where a recombinant subunit vaccine mix of eight parasite antigens was used showed successful immunisation against *T. circumcincta.* Two trials were carried out with the lambs being of different ages; 204-206 days and 172-178 days respectively. 400 µg of the antigen mix with 10 mg of Quil A as adjuvant was administered subcutaneously on three occasions three weeks apart to both groups. Control groups were injected with urea/PBS/Quil A. The mean FECs were reduced by 92% and 73% respectively and on average the abomasal worm burden decreased to 55% and 57% respectively compared with the control groups (Nisbet et al., 2013). When twin-bearing ewes were immunised with the same recombinant subunit vaccine the authors noted a 45% reduction in FEC and elevated antigen-specific IgA and IgG in serum and colostrum following immunisation (Nisbet et al., 2016).

Most vaccines are given parenterally and induce systemic immunisation, but are poor inducers of mucosal immunity (Mestecky, 1987). Since the majority of infections occur at the mucosal surfaces vaccination through a mucosal route, rather than systemically, would be preferable. With the awareness of the importance of mucosal immunity the interest in vaccines capable of inducing strong mucosal immunity is growing. Mucosal vaccines would ideally induce both a humoral and cell-mediated immunity locally, at the site of administration, and throughout the body, to give optimal protection (Dalton & Mulcahy, 2001). Stimulation of strong mucosal responses through administration via the mucosal route has proved difficult in practice and to date, there has been little progress in the development of mucosal vaccines for ruminants. There is, therefore, potential scope for the development of vaccines that can promote a strong mucosal immunity. DNA vaccines for mucosal immunisation have been studied in cattle with promising results against bovine herpesvirus 1 (Loehr et al., 2001), but appropriate strategies for mucosal immunisation of sheep still remain unexplored. Anticipated obstacles to be overcome in regards to mucosal immunisation are the practical difficulties of accessing mucosal tissue for repeated immunisation, and delivery of sufficient antigen over the mucosal barrier with the use of adequate and harmless adjuvants (Sedgmen et al., 2004).

### 2.5.1 Parasite antigens

Antigens associated with nematodes can be divided into two groups; natural/conventional and hidden/covert. Natural or conventional antigens induce an immune response in the host during infection and are surface antigens or ES antigens. Natural antigens are effective against both blood feeding and non-blood feeding nematodes (Smith & Smith, 1993). Hidden or covert antigens are normally derived from the gut of the parasite and not generally recognised by the host during infection. They become accessible to sensitised host cells or antibodies once immunity is generated. Hidden antigens are efficient against blood feeding nematodes such as *H. contortus* because elevated levels of specific antigen antibodies are taken up through feeding of the blood meal inducing a protective response, but less effective against non-blood feeding nematodes. It is commonly acknowledged that proteins expressed on the intestinal surface of gastrointestinal nematodes can be used as protective antigens and attempts have been made to develop recombinant vaccines based on these antigens (Knox, Redmond, Skuce, & Newlands, 2001). Considerable research has focused on *H. contortus* and developing vaccines based on hidden antigens. Several protective gut-expressed proteins have been identified from the parasite, such as Contorin, H11 and H-gal-GP, however no recombinants have yet been developed into commercial vaccines. H11 has been shown to be the most effective antigen isolated from *H. contortus* and the most promising candidate for a vaccine (Knox, 2000; Newton & Munn, 1999) as results have shown an average reduction in FEC greater than 90%, and an average reduction in worm burden greater than 75% (Newton & Munn, 1999). Since 2014, a vaccine containing H11 and H-gal-GP, Barbervax®, is available against *H. contortus* in Australia. It has been reported to reduce FEC by up to 80% in vaccinated lambs compared with non-vaccinated controls (Bassetto et al., 2018; Bassetto et al., 2014) and is the first effective vaccine developed against gastrointestinal nematodes in ruminants (Matthews et al., 2016; Pomroy, 2017). Attempts have also been made to vaccinate sheep against *T. circumcincta* using homologues of H11 and H-gal-GP but these have produced highly variable results, indicating that the level of antibodies ingested is unsatisfacility for effective control (Knox, 2000).

The process of developing vaccines for gastrointestinal nematodes based on natural antigens is complex. The mechanism of natural immunity to sheep parasites is not fully understood, causing difficulties in inducing appropriate immune responses and in selecting suitable adjuvants for a vaccine. ES products of different larval stages of *T. circumcincta* are currently being investigated. The surface of L3 larvae is the main site for host-parasite interaction and is, therefore, a target for nematode vaccine development (Newton & Meeusen, 2003). Maass, Harrison, Grant, and Shoemaker (2007) studied mucosal antibody responses in sheep naturally infected with *T. circumcincta,*

*T. colubriformis* and *H. contortus* and reported that three L3 surface antigens are the dominating target antigens of the immune response. Wedrychowicz, Bairden, Dunlop, Holmes, and Tait (1995) also demonstrated potential for the use of *T. circumcincta* L3 surface antigens to induce protective immunity. Subcutaneous vaccination with two low doses (25 µg antigen kg body weight$^{-1}$) and beryllium hydroxide (1 mg kg body weight$^{-1}$) as adjuvant given two weeks apart, to animals challenged with parasites three weeks after the final vaccination, showed a reduction in worm burdens of 72% and elevated serum IgA levels, compared with the control group. A reduction in post-challenge live weight was also noted in all immunised groups. Nisbet et al. (2009) investigated the L3 surface antigen *Tc*-SAA-1 isolated from *T. circumcincta* as a potential vaccine in five-month-old lambs, using an antigen dose of 5 µg. No significant reduction in worm burden and limited binding of anti-*Tc*-SAA-1 antibody on the nematode surface was reported. Studies on ES antigens from L4 have shown varying levels of IgA response in primary infected lambs and stronger IgA responses to the antigen in immunised ewes (Nisbet et al., 2010; Nisbet et al., 2011; Smith et al., 2009). It is likely that immune responses against a number of antigens are required for protective immunity and that several antigens may have to be included in future vaccines. This could explain the less successful immunisation results when only one antigen was tested. Recent research on the immunisation of sheep against *T. circumcincta* with a recombinant subunit vaccine with Quil A adjuvant showed successful immunisation with a mean FEC reduction of 73-92% and a 55-57% decrease in worm burden. This represents a greater level of protection than has previously been observed with recombinant vaccines against nematodes in ruminants, suggesting that vaccination against nematodes using a recombinant subunit vaccine may be an alternative to anthelmintic treatment (Nisbet et al., 2013).

### 2.5.2 Mucosal/oral tolerance

Mucosal or oral tolerance is a specific type of peripheral tolerance being the induction of local and systemic immune unresponsiveness after mucosal administration of an antigen (Czerkinsky et al., 1999; Pabst & Mowat, 2012; Weiner, 1997). It is characterised by suppressed T cell responses and reduced serum antibodies, in particular, IgE and IgG, with or without variations in mucosal IgA responses (Ogra, 1999; Ogra, Faden, & Welliver, 2001; Pabst & Mowat, 2012) while the induction of tolerance has been proposed to be associated with a Th2 immune response and production of Th2 cytokines such as IL-4, IL-5 and IL-10 (Strobel, 2001; Weiner, 1997). Mucosal tolerance is the oldest model of peripheral tolerance and was explained in the literature for the first time by Wells and Osborne (1911). It has since been described in many animal species, including humans and is applied to areas such as autoimmune diseases e.g. experimental autoimmune encephalitis (EAE), experimental arthritis, organ transplantation and hypersensitivity (Mestecky, Moldoveanu, & Elson,

2005; Mowat, Parker, Beacock-Sharp, Millington, & Chirdo, 2004). Theories of mucosal tolerance suggest that the optimum timing for desensitising animals occurs in the neonate, particularly during the first 24 h of life when the immune response is naïve to a number of potential pathogens and in the process of developing an ability to determine self and non-self. During this time colonisation of the gut fauna occurs and, in a majority of cases, develops life-long commensal partnerships with their host (Torow & Hornef, 2017).

#### 2.5.2.1 Mechanisms of mucosal tolerance

The induction of immunity and mucosal tolerance depends on many factors including age and sex of the animal, type and dose of the antigen, route of delivery, frequency of administration and the use of adjuvants (Mestecky et al., 2005; Mestecky, Russell, & Elson, 2007). In general, intermediate doses of an antigen induce immunity and very high or low doses induce tolerance (Zouali, 2001). Most studies on tolerance have been focused on oral administration of antigens in rodents (Mestecky et al., 2005) and little is known about mucosal tolerance in sheep. The mechanism of mucosal tolerance is dependent on the dose of antigen given. Administration of several low doses favour induction of regulatory T cells (Tregs) suppressing specific immune responses in the target organ which then migrate to the systemic immune system, whereas single high doses lead to clonal deletion of antigen-specific T cells in the peripheral immune system (Czerkinsky et al., 1999; Weiner, da Cunha, Quintana, & Wu, 2011). Buchanan, Mertins, and Wilson (2013) reported that a single high dose (2.27 g) of ovalbumin, administered orally to neonatal lambs led to lower levels of serum IgG and IgG in lung washes compared with groups immunised with several low antigen doses (0.023 g and 0.2 g) indicating that one single high dose of antigen can promote tolerance. T cells with γδ-receptors appear to be important for induction and maintenance of mucosal tolerance and mucosal IgA and IgE responses (Fujihashi et al., 1999; Ke, Pearce, Lake, Ziegler, & Kapp, 1997; Mengel et al., 1995). Fujihashi et al. (1999) reported decreased IgG levels and reduced T cell responsiveness in mice with γδ-TCRs given a low dose (2.5 mg) of oral ovalbumin and immunised parentally with 100 µg ovalbumin/CFA or 100 µg HEL/CFA seven days later, indicating that mucosal tolerance was induced. Ke et al. (1997) experienced difficulties in inducing tolerance in γδ-TCR-deficient mice with a low antigen dose (2.5 mg ovalbumin) and concluded that γδ-T cells were essential for tolerance. In contrast, Fujihashi et al. (1999) reported hyporesponsiveness in γδ-TCR-deficient mice given 25 mg ovalbumin orally and suggested that γδ-T cells are not crucial for induction of systemic tolerance with high antigen doses.

Interactions between competing cytokines determine the nature of the immune response and influence whether immunity or tolerance is induced (Ogra, 1999). An important factor for the

function of Tregs is IL-2 which facilitates the differentiation of naïve CD4+CD25+ T cells into Foxp3 expressing Tregs in combination with TGF-β as well as inhibiting the induction of Th17 cells (Bettelli et al., 2006; Mucida et al., 2007; Sakaguchi et al., 2008). The transcription factor Foxp3 is responsible for the regulation of development and function of this subset of Tregs (Sakaguchi et al., 2008). Low doses of nasally and orally administered antigens appear to promote the activation of Treg cells producing IL-10 and TGF-β, both having a suppressive effect on the immune response (Cools, Ponsaerts, Van Tendeloo, & Berneman, 2007; Holmgren & Czerkinsky, 2005; Sun, Raghavan, Sjöling, Lundin, & Holmgren, 2006). Fujihashi et al. (1999) found that γδ-T cells can up-regulate IL-10 synthesis in mice given a low dose (2.5 mg) of ovalbumin antigen orally, followed by systemic immunisation. It has been established by Bettelli et al. (2006) that TGF-β strongly facilitates the development of Treg cells in the absence of IL-6. Sun et al. (2006) demonstrated an increase in TGF-β and enhanced numbers of Foxp3+ Treg cells associated with mucosal tolerance in mice induced by three doses of 200 µg ovalbumin antigen/CTB adjuvant, or 20 mg ovalbumin administered orally, suggesting that the increase in TGF-β and concomitant decrease in IL-6 may lie behind the efficient induction of mucosal tolerance by an antigen-CTB complex.

It is generally believed that APCs serve as an important component in the induction of tolerance or immunity following antigen challenge. The APCs responsible for peripheral tolerance are dendritic cells which are essential for the induction and expression of CD4+ Tregs (Sakaguchi et al., 2008). The physiological induction of tolerance occurs in the GALT (Weiner et al., 2011). The dendritic cells in the GALT secrete retinoic acid which, under influence of TGF-β, facilitates the differentiation of naïve CD4+ T cells into Foxp3+ Tregs and inhibits the induction of Th17 cells (Mucida et al., 2007). Antigens administered orally and presented by retinoic acid-producing dendritic cells may, therefore, induce Foxp3+ Tregs which is also a suggested mechanism of tolerance (Sakaguchi et al., 2008).

#### 2.5.2.2 Routes of delivery

Most vaccines available on the market are administered via the systemic route, i.e. intramuscular or subcutaneous injection, and induce strong systemic immune responses with production of serum antibodies, but often weak mucosal immunity. Mucosal vaccination, on the other hand, can induce both systemic and mucosal immune responses and the vaccines may be easier to administer and often less invasive (Loehr et al., 2001). The common mucosal immune system, where induction of an immune response at one mucosal site spreads to other mucosal effector sites, plays an important role in mucosal immunisation. However, it has been suggested that this system does not cover all mucosal sites but that only some mucosal parts may be linked, favouring the migration of effector cells from some inductive sites to certain effector sites (Vujanic, Sutton, Snibson, Yen, & Scheerlinck, 2012). This

is facilitated by interactions between mucosal chemokines and receptors which are essential for linking induction sites with certain effector sites and results in some routes being more favourable for inducing immunity than others (Gerdts, Mutwiri, Tikoo, & Babiuk, 2006). This information is important to take into consideration when designing mucosal vaccines and delivery routes.

Mucosal vaccines require sufficient adjuvants and antigen delivery systems, however research to compare adjuvants and delivery routes has been limited in ruminants (Matthews et al., 2016). Antigens are vulnerable to degrading enzymes so delivery systems would ideally protect the vaccine from physical degradation, target induction sites in the mucosa and enhance antigen uptake and, depending on the desired response, induce either protective immunity or mucosal tolerance (Czerkinsky et al., 1999; Holmgren & Czerkinsky, 2005). Adjuvants can be carrier systems, immunostimulatory substances, or a combination of the two. The challenge in adjuvant research is to find a safe preparation that helps induce an immune response (Guy, 2007). Rose et al. (2002) compared intramuscular, subcutaneous and epidermal delivery of DNA vaccines with microcarriers in sheep challenged with *Corynebacterium pseudotuberculosis*. The sheep were injected twice, four weeks apart with 1 mg (500 µg plasmid and 500 µg vector) DNA vaccine via the intramuscular and subcutaneous routes, and 5 µg (2.5 µg plasmid and 2.5 µg vector) vaccine via the epidermal route. Intramuscular immunisation resulted in a threefold increase in serum IgG2 antibodies two weeks after the challenge compared with subcutaneous or epidermal immunisation, and intramuscular vaccination was the only delivery route that induced protective immunity. None of the three routes induced mucosal immunity. This supports the understanding that systemic administration of vaccines is unsuccessful at inducing a mucosal immune response, although it has been demonstrated that a combination of mucosal and systemic immunisation can lead to protective mucosal immunity (Carmichael, Pal, Tifrea, & Maza, 2011).

Oral administration of antigens has gained popularity because it is easy to carry out and known to induce immune responses at the site of antigen contact, as well as at other mucosal sites. A challenge with oral vaccination is that it requires significantly higher (up to 100-fold) doses of antigen to induce an immune response compared with immunisation through injection, as many vaccines are degraded by enzymes in the gastrointestinal tract (Pavot et al., 2012). Administration of an antigen via the intra-nasal route requires lower doses of antigen and induces a stronger immune response than oral administration (Çuburu et al., 2007). Intra-nasal immunisation has been found to induce immune responses in the female reproductive tract as well as in the airways (Holmgren & Czerkinsky, 2005; Voyich, Ansotegui, Swenson, Bailey, & Burgess, 2001). Cattle intra-nasally immunised with 100 µg

Tf190 antigen of *Trichomonas foetus* three times had significantly elevated IgA levels in nasal and cervical mucus compared with control animals (Voyich et al., 2001). Results from research in ruminants suggest that nasal and rectal immunisation may be suitable options to the traditional oral administration of antigens for induction of mucosal immunity (McClure, 2009). However, a disadvantage with intra-nasal delivery is the risk of inhaled antigens passing into the brain via the olfactory nerve, which can cause severe side effects (Pavot et al., 2012). Jacobs, Wiltshire, Ashman, and Meeusen (1999) showed in a study on sheep infected with *H. contortus* that intra-rectal immunisation with a formulation containing 20 µg Hc-sL3 antigen and either 10 µg cholera toxin (CT) or 250 µg aluminium hydroxide adjuvant when placed on the mucosal surface of rectum provoked a protective immune response against the parasite, with reductions in FEC and worm burden of 52-55% and 67-69% respectively. However, no serum antibodies were detected. Another study by the same group showed similar levels of protection by intra-rectal injection of the antigen, and detectable levels of serum antibody with aluminium hydroxide as an adjuvant. McClure (2009) also demonstrated protective mucosal immunity in sheep in several experiments after delivery of *Trichostrongylus* antigen via the rectal route. Both native and recombinant antigens induced immunity.

Sublingual administration of antigens can induce mucosal and systemic immune responses, including local and systemic antibody secretion and cytotoxic T cell responses (Çuburu et al., 2007; Song et al., 2008). Studies in mice have shown protective immune responses with significantly greater numbers of MHC II+ cells in the sublingual mucosa 2 h after administration of ovalbumin antigen and CT adjuvant (Pavot et al., 2012), as well as in the respiratory tract and peripheral lymph organs with the same antigen and adjuvant (Çuburu et al., 2007). Sublingual administration of ovalbumin antigen together with CT adjuvant induced a mucosal immune response in the female genital tract similar to that induced by intra-nasal immunisation (Amuguni et al., 2011; Çuburu et al., 2007; Cuburu et al., 2009). Çuburu et al. (2007) reported comparable humoral and cytotoxic T cell responses after sublingual and intra-nasal immunisation of mice using the same adjuvant (CT) and the same antigen doses (50 µg and 200 µg) for the two routes indicating that the sublingual route is effective for inducing immunity. Likewise, Amuguni et al. (2011) reported similar or greater IgA levels in saliva, vaginal wash and faeces in mice immunised three to four times at fortnightly intervals with 10 µg *Bacillus subtilus* cells via the sublingual route compared with intra-nasally immunised animals. The sublingual route is considered a safer delivery route than the intra-nasal as there is no risk for antigen redirection to the CNS (Çuburu et al., 2007; Song et al., 2008).

Most studies on intra-vaginal vaccination have been carried out on mice with varying results. Intra-vaginal immunisation of mice with CT administered into vagina with a micropipette on day 0 (20 µg) and days 10, 17 and 24 (10 µg) resulted in a weak vaginal IgA response. However, in the same study oral, gastric and rectal administration induced strong IgA responses at the local site and in the vagina (Haneberg et al., 1994). Similarly, no mucosal response was induced when mice were intra-vaginally immunised with a recombinant adenovirus vector expressing herpes simplex virus-1, although intra-nasal immunisation resulted in significant vaginal IgA levels (Scott Gallichan & Rosenthal, 1995). Hunter, Tumban, Dziduszko, and Chackerian (2011) studied the effect of intra-vaginal immunisation of mice with a virus-like particle and concluded that it induced significant levels of IgA and IgG antibodies in the female genital tract as well as elevated serum IgG. Less research has been performed on the intra-vaginal administration in ruminants, although Loehr et al. (2001) and Babiuk, Pontarollo, Babiuk, Loehr, and Drunen Littel-van den Hurk (2003) demonstrated a strong mucosal immune response after DNA vaccination with bovine herpesvirus-1 in cattle. Ocular antigen delivery has not been widely studied but in rabbits vaccinated three times with 15 µg HSV-2 gB2/gD2 antigen and MF59 adjuvant or twice with 2 x $10^5$ PFU KOS it has been reported to induce strong ocular immune responses only following infection with herpes simplex virus (HSV). It provided better protection than systemic immunisation against eye disease (Nesburn et al., 1998).

#### 2.5.2.3 Adjuvants

There is less restriction on the use of adjuvants in veterinary medicine than in human medicine and a variety of adjuvants are licensed for use in animal vaccines, including microparticles, immunostimulatory complexes (ISCOMs), liposomes and live attenuated bacterial or viral vectors (Gerdts et al., 2006; Holmgren & Czerkinsky, 2005; Holmgren, Czerkinsky, Eriksson, & Mharandi, 2003; Pashine, Valiante, & Ulmer, 2005). Alginate microparticles have been shown to be effective for delivery of mucosal vaccines in both small and large animals (Gerdts et al., 2006). 5 mg orally administered *P. multocida* antigen encapsulated in alginate microspheres administered to rabbits on three occasions at weekly intervals induced nasal IgA responses after intra-nasal challenge with virulent *P. multocida*. Cattle that were given two oral doses of ovalbumin and alginate microspheres, or subcutaneously primed with 5 mg ovalbumin in Freund's adjuvant and thereafter orally immunised with 5 mg ovalbumin and alginate microspheres, developed enhanced IgA production in the respiratory tract (Bowersock et al., 1999). This indicates that alginate microspheres administered orally can induce lymphocyte migration from the intestine to the lung and stimulate the common mucosal immune system in large animals (Bowersock et al., 1999; Bowersock et al., 1998). In pigs, oral immunisation with microsphere encapsulated *E. coli* provided no protective mucosal response. A possible explanation could be that the composition of the formulation and antigen dose administered

was not sufficient to induce mucosal immunity (Felder, Vorlaender, Gander, Merkle, & Bertschinger, 2000). This suggests that oral immunisation of livestock with microsphere adjuvants is dependent on optimal antigen dosage and the composition of the particles delivered (Sedgmen et al., 2004). One of the most successful ways of delivering a mucosal vaccine is by the use of bacterial or viral immunogens, such as subunit B of cholera toxin (CTB) and *E. coli* heat-labile enterotoxin (LTB), that actually infect the mucosal surfaces (Gerdts et al., 2006). CTB is the most effective and investigated mucosal adjuvant and a promising antigen vector for induction of mucosal tolerance (Holmgren et al., 2003). Live vectors can deliver recombinant proteins incorporated in the vector or plasmid DNA and can be used to induce protective immunity to several diseases at once in an animal (Gerdts et al., 2006). Oral administration of 100 µg CTB and PRRS antigen in pigs induced an IgA secretion in the saliva, the intestines and the reproductive tract but the distal IgA secretion was 10-20 times lower in relation to the local IgA production (Hyland, Foss, Johnson, & Murtaugh, 2004). LTB and CTB promoted mucosal IgA responses and systemic IgG production in animals when administered nasally, but had poor adjuvant properties when given orally (Eriksson & Holmgren, 2002).

Another successful adjuvant and delivery system for inducing mucosal immunity is based on immunostimulatory CpG motif-containing DNA (CpG ODN) (Holmgren et al., 2003; McCluskie, Weeratna, & Davis, 2001). CpG ODN has been found to be a potent adjuvant in several studies on mucosally immunised mice. Oral delivery of 100 µg HBsAg or 100 µg tetanus toxoid (TT) together with 50 µg, 100 µg or 500 µg CpG ODN led to elevated serum IgG levels and significant IgA levels in vaginal, lung and intestinal washes, with no obvious dose-response for the three doses used (McCluskie, Weeratna, Krieg, & Davis, 2000). In two other studies, mice were immunised intra-nasally with 1 µg or 10 µg HBsAg antigen together with 1 µg or 10 µg of either CT or CpG or a mixture of 1 µg of each adjuvant. All combinations induced significant serum IgG levels with the best effect seen when the two adjuvants were used together. Mice treated with HBsAg and CT (high and low doses) had IgA in lung washes in contrast to when CpG was used as an adjuvant. The greatest local IgA response was detected when the high antigen dose and the combination of adjuvants were used. Only the combination of 10 µg HBsAg and 10 µg CpG induced significant IgA levels in faeces (McCluskie & Davis, 1998; McCluskie & Davis, 1999). CpG ODN binds to a Toll-like receptor and has been found to stimulate immune responses in the mucosa after oral, nasal or vaginal administration and the stimulatory properties have been connected to activation of dendritic cells and secretion of cytokines and chemokines (Harandi & Holmgren, 2004; Jakob, Walker, Krieg, Udey, & Vogel, 1998; McCluskie, Weeratna, Payette, & Davis, 2001). CpG motifs vary with different species and therefore it is important that plasmids contain the sequence appropriate for each species (Rankin et al., 2001).

Over the last few years, much research has aimed at utilising nanoparticles as adjuvants for oral and intra-nasal vaccination (Chadwick, Kriegel, & Amiji, 2010; Pavot et al., 2012; Sun et al., 2018). Nanoparticles are solid particles that enclose the active substance and are thought to be taken up by M cells in MALT and transported to the lymphatic system and bloodstream. It is one of few adjuvant systems known to increase the amount of antigen to reach the immune system (Csaba, Garcia-Fuentes, & Alonso, 2009). Nanoparticles include liposomes and ISCOMs and can have delivery and immunostimulatory effects (Pavot et al., 2012). ISCOMs can stimulate both MHC I and MHC II immune responses and are potentially good delivery systems for administering antigens to the mucosa (Gerdts et al., 2006). ISCOMs have proven to induce a protective IgA antibody response in the lung and nasal washes and the large intestine in mice and sheep after intra-nasal administration with an influenza antigen (Coulter et al., 2003). Most studies on liposomes as adjuvants have been conducted on mice with variable immune responses described after oral administration. One reason for the variable results can be an inefficient uptake of the liposomes by the GALT because liposomes get trapped in the mucous layer on mucosal surfaces, hence do not reach the mucosal epithelium and underlying induction sites (Harokopakis, Hajishengallis, & Michalek, 1998). This problem seemed to be overcome by Harokopakis et al. (1998) who used CTB conjugated to liposomes including *Streptococcus mutans* to immunise mice orally and reported greater serum IgG and mucosal IgA levels than when liposomes alone were used as an adjuvant. Significantly higher mucosal immune responses were also seen in the respiratory tracts of mice intra-nasally immunised with an inactivated *Yersinia pestis* vaccine together with liposomes compared with a group that received the vaccine alone. In the same study, some mice were given the antigen-liposome combination subcutaneously. They responded with elevated antibodies in serum, but very low levels of mucosal antibodies indicating that induction of a mucosal response in the respiratory tract depends on mucosal delivery (Baca-Estrada et al., 2000).

Adjuvants were long thought to be effective only in the induction of immunity and to prevent induction of tolerance, although studies in mice have indicated that antigens given with CTB adjuvant can efficiently induce mucosal tolerance (Sun et al., 2006; Sun, Czerkinsky, & Holmgren, 2010; Sun, Holmgren, & Czerkinsky, 1994; Sun, Rask, Olsson, Holmgren, & Czerkinsky, 1996; Weiner, 1997). *E. coli* heat-labile enterotoxin has also proven effective in delivering tolerogenic proteins to the mucosa (Sun et al., 2006; Sun et al., 2010). CTB in conjugation with an antigen can enhance mucosal tolerance via oral, nasal and sublingual administration (Jacobs et al., 1999; Sun et al., 2010; Weiner et al., 2011) and oral administration of antigen-CTB complex has been seen to decrease the amount of antigen needed for induction of tolerance by many hundred-fold, as well as decrease the number of doses required (Sun et al., 1996).

Recent research on mucosal tolerance in sheep, based on a novel method of desensitising lambs to nematode antigens through repeated immunisations, has shown promising results on animal performance (Greer et al., 2014). An antigen combination of 15 µg L3, 11 µg L4 and 21 µg L5 *T. colubriformis* larvae was administered without an adjuvant via the intra-rectal route once a week for three weeks. The desensitising regime improved performance in sheep challenged with *T. colubriformis* and reduced breech faecal soiling. Mean carcass weight increased by 2.03 kg per desensitised lamb compared with lambs in the non-desensitised control group. This appears to be a promising approach to enhance animal production under nematode challenges, however, the mechanism involved in the desensitisation of sheep is not known and the optimal route of delivery is yet to be determined.

Table 2.1: Antigen delivery systems.

| Animal species | Antigen | Dose | Dose rate | Delivery routes | Adjuvant | Immune response | Author |
|---|---|---|---|---|---|---|---|
| Mice | Cholera toxin for all four routes | 20 µg day 0<br>10 µg day 10, 17, 24 | 4 x days 0, 10, 17 and 24 | oral<br>intra-gastric<br>intra-rectal<br>intra-vaginal | | ↑ IgA at each local site; saliva, intestines, rectum and vagina, highest in colonic-rectal secretions after intra-rectal administration<br>↑ serum IgA + IgG after gastric + rectal<br>serum IgA reflected local IgA responses | (Haneberg et al., 1994) |
| Mice | Adenovirus vector containing Herpes simplex virus type 1 (HSV-1), AdgB8 | $10^8$ PFU | 2 x 2 weeks apart | intra-nasal | | significant vaginal IgA<br>IgG in vaginal wash | (Gallichan & Rosenthal, 1995) |
| Mice | AdgB8 | $10^8$ PFU | 2 x 2 weeks apart | intra-peritoneal | | undetectable levels of IgA in vaginal wash | |
| Mice | AdgB8 | $10^8$ PFU | 4 x 2 weeks apart | intra-vaginal | | IgG in vaginal wash<br>little detectable IgA | |
| Sheep | L3 surface proteins (*Ostertagia circumcincta*) | 25 µg $kgBW^{-1}$ | 2 x 2 weeks apart | subcutaneous | 4 mg $kgBW^{-1}$ beryllium hydroxide subcutaneous | 72% ↓ worm burden<br>↑IgA | (Wedrychowicz et al., 1995) |
| Sheep | Ovalbumin | 25 µg $kgBW^{-1}$ | 2 x 2 weeks apart | subcutaneous | 4 mg $kgBW^{-1}$ beryllium hydroxide | 54% ↓worm burden<br>↑ serum IgM, lower IgA | |
| Mice | MBP (killed *Mycobacterium tuberculosis*) | 25 µg<br>50 µg | 1 x or 3 x at 2 days interval | oral<br>subcutaneous after oral adm. | cholera toxin B unit | protection against EAE<br>↓ IL-2 | (Sun et al., 1996) |
| Cattle | Ovalbumin | 5 mg | 2 x 2 weeks apart | subcutaneous /oral | Freund's | ↑ serum IgA, IgG<br>greater IgA, IgG1 and IgG2 in BAL than oral/oral | (Bowersock et al., 1998) |
| | Ovalbumin | 5 mg | | oral/oral | alginate microspheres | | |
| Rabbit | HSV-2 gB2/gD2 | 15 µg | 3 x 3 weeks apart | ocular | MF59 | protection against eye disease | (Nesburn et al., 1998) |
| | KOS | 2 x $10^5$ PFU | 2 x 3 weeks apart | | | | |

| Animal species | Antigen | Dose | Dose rate | Delivery routes | Adjuvant | Immune response | Author |
|---|---|---|---|---|---|---|---|
| Cattle | Ovalbumin | 5 mg | up to 5 x at different times | subcutaneous + 5 x oral | alginate microspheres | ↑ IgA + IgG ASCs in BAL subcutaneous<br>comparable levels of IgG ASCs for subcutaneous and oral boost | (Bowersock et al., 1999) |
| Cattle | Ovalbumin | 5 mg | up to 5 x at different times | oral + 3 x oral | alginate microspheres | comparable levels of IgG ASCs for subcutaneous and oral boost | |
| Cattle | Ovalbumin | 5 mg | up to 5 x at different times | 3 x oral | alginate microspheres | no increase in serum Ab | |
| Rabbits | *P. multocida* KSCN | 5 mg | 3 x days 0, 7 and 14 | oral<br>intra-nasal | alginate microspheres | ↑ IgA in the respiratory tract<br>intra-nasal as effective as oral | |
| Mice | Ovalbumin | 2.5 mg | 3 x days 0, 2 and 5 | oral + 100 µg ovalbumin subcutaneous | | ↓ IgG in serum and spleen | (Fujihashi et al., 1999) |
| Mice | Ovalbumin | 25 mg | 1 x | oral + 100 µg ovalbumin intra-peritoneal | Freund's | ↓ serum IgG, 2-4 x ↓ IgE<br>3-6 x↓IgG in the spleen<br>↓ IgG in lamina propria | |
| Sheep | Hc-sL3 (*H. contortus)* | 20 µg | 3 x week 0, 6, 11 | intradermal in the thigh area | 3 mg dextran sulphate | ↓ FEC<br>no effect of adjuvant | (Jacobs et al., 1999) |
| Sheep | Hc-sL3 (*H. contortus)* | 20 µg | 3 x week 0, 6, 11 | intradermal in the thigh area | 1 µg pertussis toxin | 64% ↓ FEC<br>↑ protective effect | |
| Sheep | Hc-sL3 (*H. contortus)* | 20 µg | 3 x week 0, 6, 11 | intra-rectal | 10 µg cholera toxin<br>or<br>250 µg aluminium hydroxide | 69% ↓ FEC<br>55% ↓ worm burden<br>67% ↓ FEC<br>55% ↓ worm burden | |
| Mice | Formalin-inactivated tetanus toxoid | 10 µg | days 0, 1 ,2 | oral | CpG 10 µg | ↑ serum IgG | (McCluskie, Weeratna, & Davis, 2001) |
| Mice | Recombinant HBsAg | 1 µg | days 0, 1, 2 | intramuscular | CpG 10 µg | ↑ serum IgG<br>levels comparable to oral immunisation | |
| Cattle | *Limulus* haemocyanin | 1 mg | 2 x 16 days apart | subcutaneous | Freund's | weak serum IgA<br>no serum IgG1, IgG2, IgM | (Rebelatto, Siger, & Hogenesch, 2001) |
| Cattle | *Limulus* haemocyanin | 1 mg | 2 x 16 days apart | intra-nasal | cholera toxin 100 µg | no serum IgA<br>5 x↑ serum IgG1, IgG2, IgM | |

| Animal species | Antigen | Dose | Dose rate | Delivery routes | Adjuvant | Immune response | Author |
|---|---|---|---|---|---|---|---|
| Cattle | Tf190 | 100 µg | 3 x days 0, 21 and 58 | 1 x subcutaneous | aluminium hydroxide, AH, 250 µg | ↑ serum IgG | (Voyich et al., 2001) |
| Cattle | Tf190 | 100 µg | 3 x days 0, 21 and 58 | 1 x subcutaneous + 2 x intra-nasal | AH, 250 µg subcut. cholera toxin 20 µg intra-nasal | ↑ IgA in cervical mucus<br>no serum IgG response 50% reduction in infection rate | |
| Mice | Influenza | 1 µg (6 µL nostril$^{-1}$) | 2 x 3 weeks apart | intra-nasal | ISCOM 100 µg | ↑ IgA nasal | (Coulter et al., 2003) |
| | | | | | IMX 10 µg | ↑ IgA nasal, lung, large intestine | |
| Sheep | Influenza | 15 µg (0.25 ml nostril$^{-1}$) | 2 x 3 weeks apart | intra-nasal | ISCOM 1 mg | ↑ IgA nasal | |
| | | | | | IMX 1 mg | ↑ IgA nasal, lung, large intestine | |
| Pigs | CT-B-N-myc or CT-B-N-myc$_9$ | 1 mg CT-B<br>20 µg N<br>175 µg myc | 1 x | oral | CT 100 µg | 50% ↑ IgA intestine<br>↑ IgA reproductive tract | (Hyland et al., 2004) |
| Mice | *Yersinia pestis,* KWC | 5 µg | 2 x | intra-nasal | liposomes | 5 x ↑ serum IgG<br>1 5x ↑ IgG, 8 x↑ IgA in nasal wash<br>1,000 x ↑ IgG, 600x↑ IgA in lung wash | |
| Mice | *Yersinia pestis,* KWC | 12.5 µg or 5 µg | 2 x | subcutaneous + intra-nasal boost | | 3 x↑ IgA in nasal wash<br>80x ↑ IgG in lung wash | |
| Mice | *Yersinia pestis,* KWC | 12.5 µg or 5 µg | 2 x | subcutaneous + subcutaneous | | low IgA and IgG in lung and nasal wash | |
| Mice | Ovalbumin | 200 µg | 1 x or 3 x 2 days apart | oral | CTB 4 µg | ↑ TGF-β<br>↑ Foxp3 Tregs | (Sun et al., 2006) |
| Mice | Ovalbumin | 20 mg | 1 x or 3 x 2 days apart | oral | | ↑ TGF-β<br>↑ Foxp3 Tregs | |
| Mice | Ovalbumin | 10, 50, 200 µg | 3 x days 0, 7 and 21 | intra-nasal | CT 2 µg | greatest mucosal IgG, IgA with 50 µg -200 µg | (Çuburu et al., 2007) |
| Mice | Ovalbumin | 10, 50, 200 µg | 3 x days 0, 7 and 21 | sublingual | CT 2 µg | same as intra-nasal<br>more effective at 10 µg | |

| Animal species | Antigen | Dose | Dose rate | Delivery routes | Adjuvant | Immune response | Author |
|---|---|---|---|---|---|---|---|
| Mice | *Bacillus subtilis* expressing tetanus toxin TTFC | 10 µg | 3-4 x 2 weeks apart | intra-nasal<br><br>sublingual | mutant heat labile toxin from *E. coli* (mLT) 5 µg | ↑ IgA in saliva, vaginal wash, faeces<br>similar or greater IgA than intra-nasal<br>mLT → 40% protection<br>no mLT → 100% protection | **(Amuguni et al., 2011)** |
| Mice | Virus-like particle (16L2-PP7 VLP) | 10 µg | days 0 and 14 boost days 42 and 200 | intramuscular | Freund's incomplete adjuvant | high serum IgG<br>high vaginal IgG<br>low vaginal IgA | **(Hunter et al., 2011)** |
| Mice | Virus-like particle (16L2-PP7 VLP) | 25 µg | | intra-vaginal | | high vaginal IgG<br>high vaginal IgA | |
| Sheep | Ovalbumin | 2.27 g | 1 x | oral + 10 mg intra-peritoneal | Freund's | ↓ IgG in serum and lung wash (tolerance) | **(Buchanan et al., 2013)** |
| Sheep | Ovalbumin | 0.23 g | 3 x days 1, 2, 3 after birth | oral + 10 mg intra-peritoneal | Freund's | ↑ IgG in serum and lung wash | |
| Sheep | Ovalbumin | 0.023 g | 6 x days 1, 2, 3, 5, 7 and 9 after birth | oral + 10 mg ovalbumin intra-peritoneal | Freund's | ↑ IgG in serum and lung wash | |
| Sheep 204-206 days old<br><br>172-178 days old | Antigen mix with eight antigens (*Teladorsagia circumcincta*) | 400 µg (50 µg of each antigen) | 3 x 3 weeks apart | subcutaneous at two sites of neck | Quil A<br><br>Quil A | 55% fewer worms than control<br>↓ FEC 92%<br><br>57% fewer worms than control<br>↓ FEC 73% | **(Nisbet et al., 2013)** |

## 2.6 Conclusion

Gastrointestinal nematodes cause significant production losses in livestock including sheep, mainly through a reduced feed intake and a redirection of protein from the production tissues to the gastrointestinal tract and the immune response. Alternative methods for nematode control are required due to a rapidly growing anthelmintic resistance. With the majority of infections occurring at mucosal surfaces, immunisation via the mucosal route would be desirable since, unlike systemic administration, mucosal immunisation can induce both systemic and mucosal immunity and is less invasive. Many attempts have been made to develop conventional and mucosal vaccines against gastrointestinal nematodes but stimulating a strong mucosal immune response through the mucosal route has proved difficult and most vaccines have been unsuccessful to date and few have become commercially available. It is anticipated that methods to reduce the strength of the immune responses specifically against gastrointestinal nematodes can mitigate production losses and increase the performance, i.e. reduced growth and feed intake, of the animals. Mucosal tolerance is a local and systemic immune unresponsiveness specific to antigens characterised by reduced T cell responses and serum antibodies induced by mucosal administration of antigens without adjuvants. Previous research has shown promising results in inducing mucosal tolerance in sheep and potentially this could be a new route to parasite control. However, little is known about mucosal tolerance in sheep infected with *T. colubriformis* and further studies are required to establish the mechanism behind tolerance and its importance to mitigate the effects of gastrointestinal nematode infections.

# Chapter 3
# Determination of larval stage specificity of *Trichostrongylus colubriformis* antigen

## 3.1 Introduction

Infections with gastrointestinal nematodes can have detrimental nutritional costs for the infected animal. Production losses associated with temperate parasite species, such as *T. colubriformis,* generally limit the host productivity through reduced voluntary feed intake and impaired nutrient utilisation for maintenance and growth (Coop & Sykes, 2002). They have been attributed to components of the animal's immune response against the parasites and associated with the acquisition phase of immunity (Greer, 2008; Williams, Palmer, et al., 2010). Substantial research has been directed towards control methods for gastrointestinal nematodes through enhancing the immune system of the host, including vaccines and selective breeding (Waller, 1999). However, little is yet known about tolerance to parasites. The surface of L3 larvae is the main site for host parasite interaction and is believed to be the site towards which natural immunity is directed. Hence, it has been the target for nematode vaccine development (Newton & Meeusen, 2003) and vaccine development has focused on L3 surface antigens of *T. circumcincta, T. colubriformis* and *H. contortus* with various outcomes (Maass et al., 2007; McClure, 2009; Newton & Meeusen, 2003; Wedrychowicz et al., 1995). Similarly, Nisbet et al. (2009), Nisbet et al. (2011) and Smith et al. (2009) analysed the effect of L4 *T. circumcincta* excretory/secretory products on IgA antibody responses in sheep with varying results. It is likely that immune responses towards a number of antigens are required for protective immunity and that several antigens may have to be included in a future vaccine, which could explain less successful immunisation results when only one antigen was tested.

It is considered that sheep that are resilient to nematodes may have a greater productivity than resistant animals. Approaches to induce resilience through reducing the immune responses, such as inducing tolerance through desensitising sheep to nematode antigens, may therefore hold potential for mitigation of production losses associated with parasitism. Greer et al. (2014) desensitised sheep to *T. colubriformis* with a somatic antigen comprising *T. colubriformis* L3, L4 and L5 antigen (*Tcol*-L3/L4/L5) and noted increased performance and feed intake in desensitised animals compared with their non-desensitised counterparts. However, this was with a combination of antigens and the exact larval stage which is important is not yet known. This study compared the immunostimulatory effect of the three individual larval stages of *T. colubriformis*; L3, L4 and L5 in comparison to an antigen combination *Tcol*-L3/L4/L5, previously used by Greer et al. (2014) to determine and refine the optimal parasite antigen for desensitisation.

## 3.2 Methods and materials

The study was carried out at Johnstone Memorial Laboratory (JML), Lincoln University, with approval from and in accordance with Lincoln University Animal Ethics Committee (LUAEC# 2016-21). Thirty-six five-month-old parasite naïve Poll Dorset x East Friesian lambs were born and raised indoors and injected with a nematode antigen consisting of one of three different *T. colubriformis* larval stages or a combination of all three to investigate the tolerising effect of each larval stage. The lambs were fitted with electronic ear tags (Allflex, New Zealand), allocated hierarchically by live weight (mean 30.50 ± 0.67 kg) into one of six treatment groups (n=6), including one infected and one uninfected group, and housed indoors in individual pens on day -28. They were delivered from a sheep milking facility in a poor condition, below the requested minimum weight, with several lambs suffering from and treated for pneumonia and contagious ecthyma upon arrival.

### 3.2.1 Treatment

The treatment group structure is shown in table 3.1. Lambs from four groups; TL3, TL4, TL5 and COMB were injected with a somatic *T. colubriformis* antigen consisting of one of the larval stages L3, L4, L5 (immature adults) or a combination of the three (COMB), prepared as described below. The amount of protein in each antigen dose was based on previous indoor studies (A.W. Greer, unpublished data) where a positive response had been observed for each fraction*, viz,* 7.5 µg of L3 somatic antigen; 5.5 µg of L4 somatic antigen; 10.5 µg of L5 somatic antigen. The antigen combination contained 7.5 µg of L3, 5.5 µg of L4 and 10.5 µg of L5 antigen. Doses were contained in 200 µl PBS and injected three times into the rectal submucosa with the use of a rectal speculum with injections one week apart. INF and CON were sham injected with 200 µl PBS. Care was taken to ensure a blister was visually observed at the site of injection reflecting administration of the antigen cocktail correctly into the rectal submucosa. Doses were administered on days -21, -14, and -7.

All animals were raised worm free, but in addition were treated with 1 ml 5 kg live weight$^{-1}$ ComboSheep combination drench (37.5 g l$^{-1}$ levamisole hydrochloride, 24.0 g l$^{-1}$ albendazole, Ravensdown New Zealand Ltd, Christchurch, New Zealand) at housing. Five groups; TL3, TL4, TL5, COMB and INF were challenged with the equivalent of 14,000 *T. colubriformis* L3 larvae week$^{-1}$, administered three times week$^{-1}$ from day 0 until the end of the trial while the remaining group (CON) was uninfected. The INF group was control for the level of infection and CON the positive growth rate control being the only non-infected group. Larvae were distributed onto filter paper which was rolled and administered orally by the use of a balling gun.

Table 3.1: Experimental design.

| Group (n=6) | Immunisation | Challenge from day 0 |
|---|---|---|
| TL3 | *Tcol*-L3 | 14,000 *T. colubriformis* L3 week$^{-1}$* |
| TL4 | *Tcol*-L4 | 14,000 *T. colubriformis* L3 week$^{-1}$* |
| TL5 | *Tcol*-L5 | 14,000 *T. colubriformis* L3 week$^{-1}$* |
| COMB | *Tcol*-L3/L4/L5 | 14,000 *T. colubriformis* L3 week$^{-1}$* |
| INF | NA | 14,000 *T. colubriformis* L3 week$^{-1}$* |
| CON | NA | No challenge |

*administered in split doses three times week$^{-1}$

### 3.2.2 Feed

Animals had *ad libitum* access to fresh water and were offered Homestead Rumatain Multi-Nut (Seales Winslow, New Zealand) *ad libitum* daily consisting of a pelleted complete ruminant diet containing 12.15 MJME kgDM$^{-1}$ and 173 g CP kgDM$^{-1}$ and *ad libitum* access to a salt lick. Symptoms of copper (Cu) toxicity, i.e. jaundice, haematuria and lethargy, were observed on day 36 and the lambs were supplemented with 50 mg day$^{-1}$ ammonium molybdate and 500 mg day$^{-1}$ sodium sulphate. Individual feed refusals were collected and weighed weekly. Subsamples of the refusals were collected and dried at 90 °C for seven days for determination of dry matter (DM) percentage.

### 3.2.3 Live weight

Live weights were recorded weekly using a Prattley weigh crate fitted with Tru-Test load bars. Animal identification was recorded using an Edit Display Wand and Tru-Test XR3000 (Tru-Test Ltd, New Zealand) with a sensitivity of 0.2 kg.

### 3.2.4 Antigen preparation

Soluble somatic parasite antigen was prepared prior to the trial by a similar method to that described by Knox and Jones (1990). Six sheep were infected with 50,000 *T. colubriformis* L3 larvae each and two were sacrificed on each of 10 and 21 days post infection for retrieval of L4 larvae and immature adults (L5 larvae), respectively, from the small intestine. Intestinal washings with L4 and L5 larvae separate were mixed with agar and set to coagulate and placed on a mesh in 8% saline solution overnight at 28 ˚C. The small intestines were dissected and also placed in 8% saline overnight. Larvae were collected from the saline solutions and stored at 4 ˚C until further use. For the remaining sheep, faecal collection was undertaken from day 21 and cultured for L3 larvae. The faecal material was placed on a

mesh in lukewarm water and cultured in 28 ˚C for 10 days. The culture solution was collected and siphoned down to 20 ml, thereafter placed on a Baermann apparatus for 24 h before larvae were collected and stored at 4 ˚C until use. L3, L4 and L5 *T. colubriformis* larvae were homogenised using a Biospec Mini Bead Beater ™. The antigen was clarified by centrifugation (Eppendorf 5415D, Eppendorf AG, Hamburg, Germany) at 12,000 x g for two minutes at 4 ˚C and the protein concentration estimated using a Pierce ™ BCA Protein Assay kit according to the instructions (Thermo Fisher Scientific, New Zealand) and stored at -20 ˚C until use.

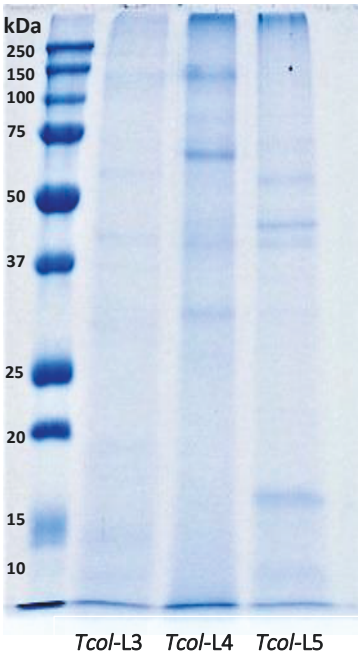


Figure 3.1: Gel electrophoresis of *T. colubriformis* somatic antigens; *Tcol*-L3, *Tcol*-L4, and *Tcol*-L5 antigen and expression of associated proteins.

## 3.2.5 Parasitology

### 3.2.5.1 Faecal egg count

Faecal samples were collected weekly directly from the rectum of the lambs. Following collection, the samples were stored at 4 °C and faecal egg counts were determined using a modified McMaster technique as described by MAFF (1986). Subsamples of 1.7 g faeces from each animal were placed in a glass jar and 10 ml water added and left overnight. The following day 40 ml of saturated saline was added and each sample was homogenised to ensure that the eggs were uniformly distributed throughout the sample prior to determination. Subsamples were then taken and used to fill each chamber of a McMaster counting slide. The slide was allowed to stand for a few minutes to allow the eggs to float to the surface before microscopic counting of the eggs. Each egg counted was equivalent to 100 eggs gram$^{-1}$ of faeces (epg).

#### 3.2.5.2 Total worm burden

Post-mortem worm recovery from the small intestine was performed in accordance with the method described by Donaldson, Houtert, and Sykes (2001). At slaughter, the animals were stunned with a captive bolt gun followed by severance of the carotid arteries and jugular veins. The small intestine was collected from all animals and ligated at both ends to contain the content using the first five metres distal to the pylorus. It was gently flushed with tap water and the washings collected in a beaker and made up to a volume of two litres. Four 50 ml aliquots (a total of 200 ml) were thoroughly mixed before being taken out and fixed in 10% formalin. 20 ml were transferred to a petri dish for microscopic counting of the larvae. For recovery of worms residing in the tissue of the small intestine, the tissues were digested in an acidified pepsin solution containing 1% pepsin and 3% HCl at 37 °C for 12-16 h and passed through a mesh sieve with 45 µm pore size to retain the worms as described by Herlich (1956). The recovered worms were fixed in 10% formalin and stored until microscopic examination and counting using a compound binocular microscope.

For worm length measurements, slides with worms were mounted onto a microscope connected to a camera and images of 20 female and 20 male adult worms from four animals in each group were taken. The length of the individual worms was recorded using Image J 2009 that was calibrated with a one-millimetre microtome.

### 3.2.6 Blood samples

Blood samples were collected weekly from all animals by jugular venepuncture using 2 x 10 ml lithium heparin and non-heparinised vacutubes (Becton Dickinson Vacutainer®, Belliver Industrial Estate, UK) starting on day -21. The non-heparinised tubes were set to coagulate overnight and then centrifuged at 1,200 x g for 10 minutes at 4 °C. Serum was collected to 1.2 ml tubes and stored at -20 °C until examined for *T. colubriformis* specific IgA, IgG and IgE antibodies by enzyme-linked immunosorbant assay (ELISA). The heparinised blood samples were prepared immediately for leukocyte stimulation assays.

Serum albumin, total protein, urea and P concentrations were analysed by colourimetric assays on a Randox Rx Daytona analyser (Randox Headquarters Co. Antrim, UK) with all concentrations determined photometrically. Serum albumin concentration was measured at 578 nm UV light utilising Bromocresol green (Albumin kit #AB3800, Randox Headquarters Co. Antrim, UK). Serum total protein was analysed with a Biuret reagent (Total protein kit #TP3869, Randox Headquarters Co. Antrim, UK). Serum urea concentration was determined with an enzymatic kinetic method (Urea kit #UR3825, Randox

Headquarters Co. Antrim, UK). Serum P concentration was measured at 340 nm UV light utilising the Inorganic P UV method (Inorganic phophorous kit #PH3820, Randox Headquarters Co. Antrim, UK).

#### 3.2.6.1 Antibodies

IgA, IgG and IgE antibody responses to *T. colubriformis* antigen in serum were detected using an ELISA similar to that described by Douch et al. (1994). 96-well high-binding ELISA plates (Jetbiofil ®, China) were coated with 50 µl well$^{-1}$ of a coating buffer containing distilled $H_2O$ and 1.59 g l$^{-1}$ $Na_2CO_3$, 2.93 g l$^{-1}$ $NaHCO_3$ and 2 µg of L3, L4 or L5 *T. colubriformis* antigen ml well$^{-1}$ for detection of antibody production against the different larval stages. The plates were incubated at 4 °C overnight then washed with PBS + 0.05% Tween 20 (PBST20) five times. 200 µl blocking buffer containing PBS with 5% bovine skim milk powder was added to each well and the plates incubated at room temperature for 1 h, thereafter washed with PBST20 five times. For IgA and IgG serum was added in duplicates, 50 µl well$^{-1}$ diluted to 1:10 and 1:400, respectively. Plates were incubated at room temperature for 2 h before being washed in PBST20 five times. The following procedures were all carried out at room temperature. 100 µl of horseradish rabbit anti-sheep IgA (A130-108P, Bethyl Laboratories Inc., USA) or peroxidase (HRP) conjugated polyclonal rabbit anti-sheep IgG antibodies (Pierce Immunopure Antibodies, USA cat#31480, lot#GI959969) diluted 1:2,000 was added to each well before incubation of the plates for 1 h. The plates were washed five times in PBST20. To develop colour, 100 µl well$^{-1}$ of TMB substrate containing 0.05 M P-citrate buffer (0.2 M $Na_2CO_3$ + 0.1 M citrate), 2 µl 30% $H_2O_2$+ tetramethylbenzidine (TMB; Sigma Aldrich, USA) tablet 10 ml buffer$^{-1}$, was added and the plates incubated in darkness for 40 minutes. The reaction was stopped by adding 100 µl well$^{-1}$ 1.25M $H_2SO_4$ STOP solution, and relative absorbance was read on a microplate reader (Multiskan Go, 1510-01462C, Thermofisher Scientific, Finland) at 450 nm.

For IgE detection, 50 µl of serum diluted 1:10, was added in duplicates and incubated for 2 h, then washed in PBST20 five times. 50 µl of 1:100 mouse anti-sheep IgE 2F1 (Moredun Research Institute, Edinburgh, Scotland) was added and plates incubated for 1 h at room temperature. Thereafter 50 µl 1:1,000 HRP conjugated goat anti-mouse IgG (cat#A90-131P, Bethyl Laboratories INC., USA) was added and the plates incubated for 1 h. Colour was developed as described for IgG and IgA and the absorbance read at 450 nm. Results of the ELISAs were corrected for background absorbance and expressed as mean optical density (OD) values of the duplicates and adjusted to a positive control standard value on each plate. Cross-reactivity between *T. colubriformis* L3, L4 and L5 antigen was not assessed.

### 3.2.6.2 Leukocyte stimulation assay

For the leukocyte stimulation assays L3, L4 or L5 *T. colubriformis* antigen was added to a mix of 250 µl RPMI medium (RPMI 1640, Gibco®, USA, 10% heat activated calf serum, 2 mM L-glutamine, 100 U $ml^{-1}$ penicillin, 100 µg $ml^{-1}$ streptomycin and 50 µM 2-mercaptoethanol) to give a final antigen concentration of 5 µl $ml^{-1}$. 96-well ELISA plates were coated with 250 µl of the RPMI medium/antigen mixture and 250 µl blood $well^{-1}$. The plates were incubated in a $CO_2$ incubator at 37 ˚C in an atmosphere containing 5% $CO_2$ for 48 h and then centrifuged at 1,500 x g for 5 minutes at 4 ˚C. The supernatant was collected to 1.2 ml tubes and stored at -80 ˚C until analysed. IL-4 and IFN-γ cytokine levels were measured using commercial ELISA kits (3118-1H-6 and 3119-1H-6, MabTech AB, Nacka Strand, Sweden) following the manufacturer's instructions and expressed as pg $ml^{-1}$. 96-well high protein binding ELISA plates were coated with 50 µl $well^{-1}$ of either mAb bIL-4-I or mAb MT17.1, diluted to 2 µg $ml^{-1}$ in PBS and incubated overnight at 4 °C. The plates were washed twice with 200 µl PBS $well^{-1}$ and blocked with 200 µl $well^{-1}$ of PBST20 containing 0.1% bovine serum albumin (BSA) (Sigma, Aldrich, USA) before being incubated at room temperature for 1 h and thereafter washed five times with PBST20. The following procedures were carried out at room temperature and plates were washed five times in PBST20 following each incubation. Supernatant samples or standard samples diluted in incubation buffer containing PBST20 and 0.1% BSA were added in duplicates, 100 µl $well^{-1}$, and the plates incubated for 2 h. 50 µl $well^{-1}$ of mAb bIL4-II biotin at 0.5 µg $ml^{-1}$ incubation buffer or mAb MT307-biotin at 0.25 µg $ml^{-1}$ incubation buffer was added and the plates incubated for 1 h. 50 µl $well^{-1}$ of Streptavidin-HRP diluted 1:1,000 in incubation buffer was added before incubating for 1 h. To develop colour, 100 µl TMB substrate containing 0.05 M P-citrate buffer (0.2 M $Na_2CO_3$ + 0.1 M citrate), 2 µl 30% $H_2O_2$+ tetramethylbenzidine (TMB) tablet 10 ml $buffer^{-1}$, was added to each well and the plates incubated in darkness for 40 minutes. The reaction was stopped by adding 100 µl 1.25 M $H_2SO_4$ STOP solution and absorbance was read on an ELISA plate reader at 450 nm.

## 3.2.7 Body composition

To determine changes in the tissue deposition that may have resulted from treatment, body composition at day -2 and day 46 of infection was assessed using computed tomography (CT). Animals were fasted for 24 h, mildly sedated with 1.0 ml 50 kg live $weight^{-1}$ of 13.5 mg $ml^{-1}$ acepromazine maleate intramuscularly (Acezine 10, Delvet Pty Ltd, NSW, Australia) 30 minutes prior to scanning. They were immobilised on their backs in a cradle to minimise movements during scanning. Seven cross-sectional slices were taken at each scanning, as described by Jay, Ven, and Hopkins (2014), at the 1st thoracic, 5th thoracic, 1st lumbar, 6th lumbar, 3rd sacral, 2nd caudal vertebrae, and ischium. The CT images were segmented into fat, bone and lean tissues based on their density value measured in

Hounsfield units (HU) ranging from -174 to -16, 106 to 254, and -14 to 104 pixels for fat, bone and lean tissue, respectively. Tissue areas were converted to volume using the equation:
*volume ($cm^3$) = total area of carcass tissue ($cm^2$) x section distance (cm)* (Jay et al., 2014).
Tissue weights were converted from tissue volumes using estimated tissue density CT image HU values (True density =HU x 0.00106 + 1.0062 (Campbell et al., 2003) using the equation:
*mass (g) = tissue volume ($cm^3$) x tissue density (g $cm^{3-1}$).*
The energy deposited in the carcass gain was calculated using energy values of 38.9 MJ $kg^{-1}$, 10 MJ $kg^{-1}$ and 4.44 MJ $kg^{-1}$ for fat, bone and lean tissue, respectively (Blaxter & Rook, 2007).

### 3.2.8 Statistical analysis

Statistical analysis were carried out using the GENSTAT statistical package (GENSTAT 2018. GenStat Eighteenth edition, VSN International Limited, UK). Blood analyses, live weight and FEC were subjected to sequential comparison for ante-dependent structure prior to analysis as repeated measures using a Mixed Models Restricted Maximum Likelihood (REML) with treatment groups and time included as factors and missing values estimated. Worm burdens, worm length, liveweight gain, feed intake, feed conversion efficiency and carcass composition were analysed by analysis of variance (ANOVA) using Minitab ®17 Statistical Software (Minitab Inc. Seventeenth edition, USA). FEC and worm burden were log transformed (log10 (count+10)) before analyses and uninfected control animals were excluded from the analysis. $P \leq 0.05$ was regarded as significant and all values are group means and expressed as mean ± SEM unless otherwise specified.

## 3.3 Results

### 3.3.1 Clinical observations

Four animals from the groups TL4, TL5, INF and CON died from Cu poisoning on days 36, 37 (two animals), and 44. Diagnosis was confirmed by autopsy. Data from the dead animals were included in the statistical analyses using missing value estimates from sampling days 42 and 49. There were no clinical signs of parasitism observed in any animals throughout the study.

### 3.3.2 Live weight

Mean live weights (kg) are given in Figure 3.2. Overall, there was a tendency for treatment by time interaction (P=0.065) reflecting similar initial live weights in all groups which increased with time to peak on day 49 *viz*. 43.08 ± 1.49, 39.90 ± 2.16, 43.80 ± 2.13, 45.00 ± 2.79, 44.60 ± 1.80, 46.63 ± 4.47 kg for TL3, TL4, TL5, COMB, INF and CON respectively.

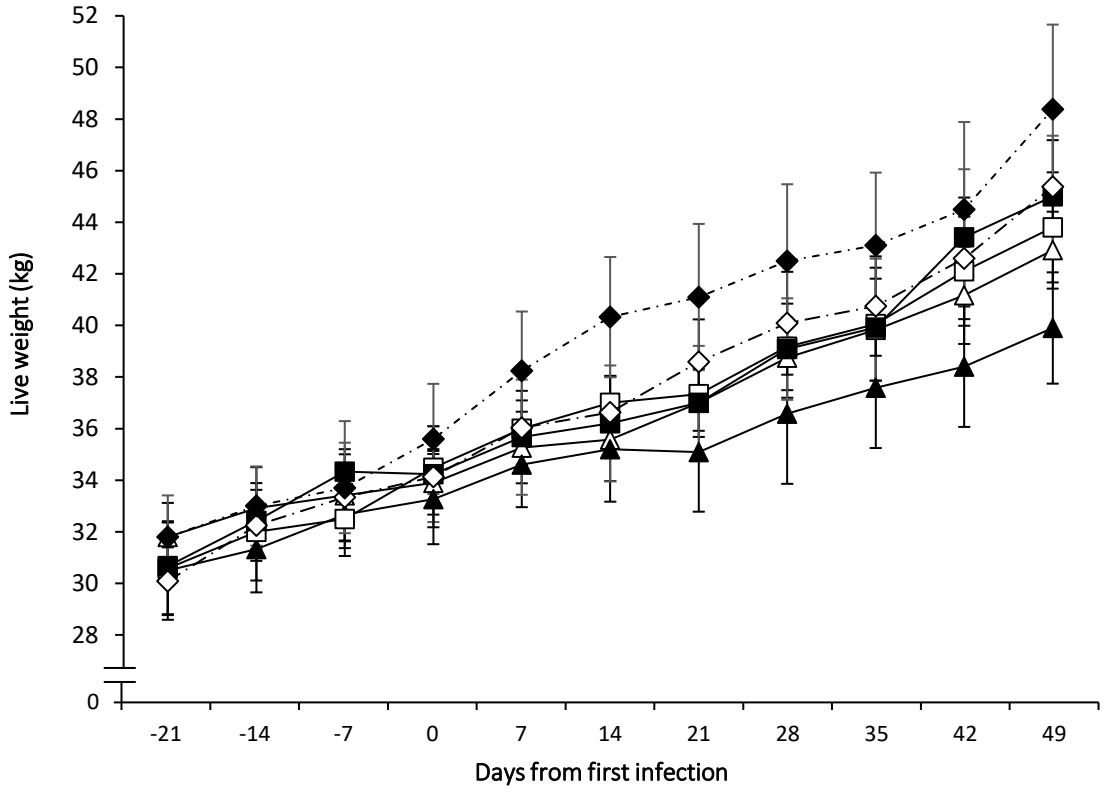


Figure 3.2: Mean live weight (± SEM) of lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

### 3.3.3 Liveweight gain

Mean liveweight gain (g day$^{-1}$) during the different periods of infection is given in Figure 3.3. There was no difference in liveweight gain between treatment groups pre-infection (day -21 to day 0, P=0.945), during the first 21 days of infection (day 0 to 21, P=0.521), nor the last 28 days of infection (day 21 to 49, P=0.131).

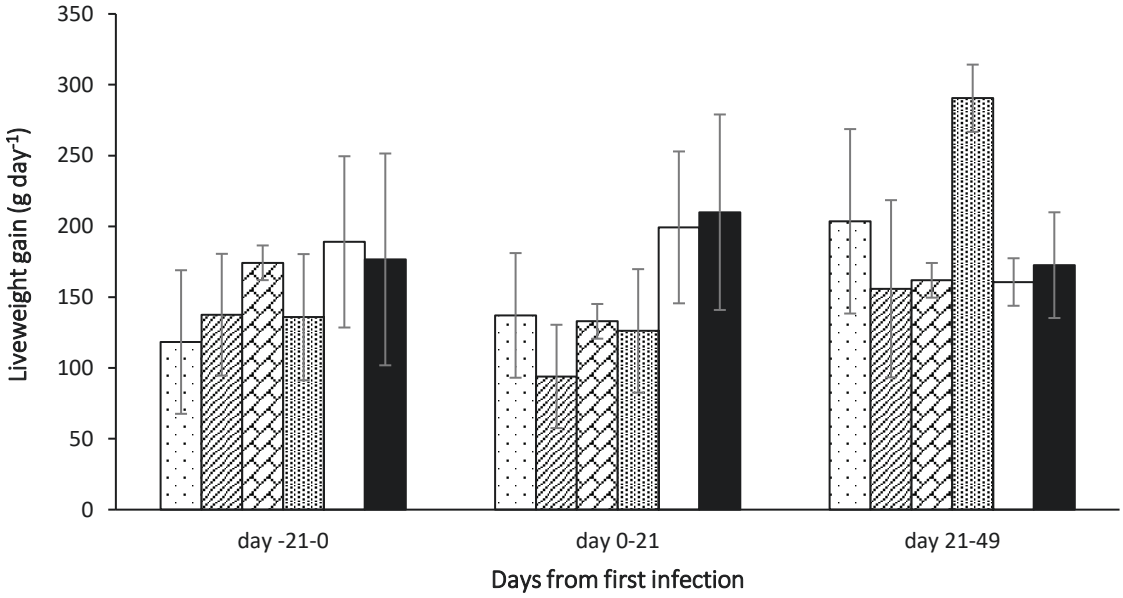


Figure 3.3: Mean liveweight gain (± SEM) of lambs infected with 14 000 *T. colubriformis* week$^{-1}$ (INF □) infected and injected with *Tcol*-L3 (TL3 ⁙), *Tcol*-L4 (TL4 ▨), *Tcol*-L5 (TL5 ▩), or *Tcol*-L3/L4/L5 antigen (COMB ▦), and non-infected controls (CON ■).

### 3.3.4 Feed intake

Mean voluntary feed intake (kgDM day$^{-1}$) during the different periods of infection is given in Figure 3.4. Feed intake was not different between the treatment groups pre-infection (day -21 to day 0, P=0.154), during the first 21 days of infection (day 0 to 21, P=0.472), nor during the last 28 days of infection (day 21 to 49, P=0.660).

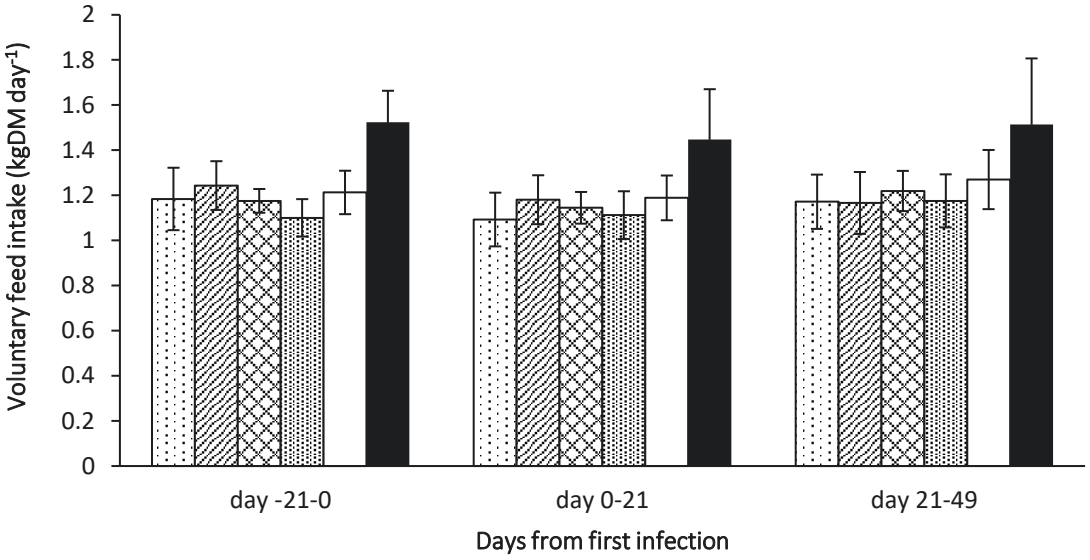


Figure 3.4: Mean voluntary feed intake (± SEM) of lambs infected with 14 000 *T. colubriformis* week$^{-1}$ (INF ☐) infected and injected with *Tcol*-L3 (TL3 ⁘), *Tcol*-L4 (TL4 ▨), *Tcol*-L5 (TL5 ▩), or *Tcol*-L3/L4/L5 antigen (COMB ▦), and non-infected controls (CON ■).

### 3.3.5 Feed conversion efficiency

The mean feed conversion efficiency (FCE, g live weight gained kgDM$^{-1}$ of feed consumed) is given in Figure 3.5. Overall, there was no difference in FCE between treatment groups during pre-infection (P=0.747) nor during the first 21 days of infection (P=0.624). However, there was a tendency for FCE to differ between treatment groups between day 21-49 (P=0.062) reflecting greater FCE in the COMB group (254 ± 39 g kgDM$^{-1}$) and lowest in INF (112 ± 24 g kgDM$^{-1}$) and CON (121 ± 19 g kgDM$^{-1}$).

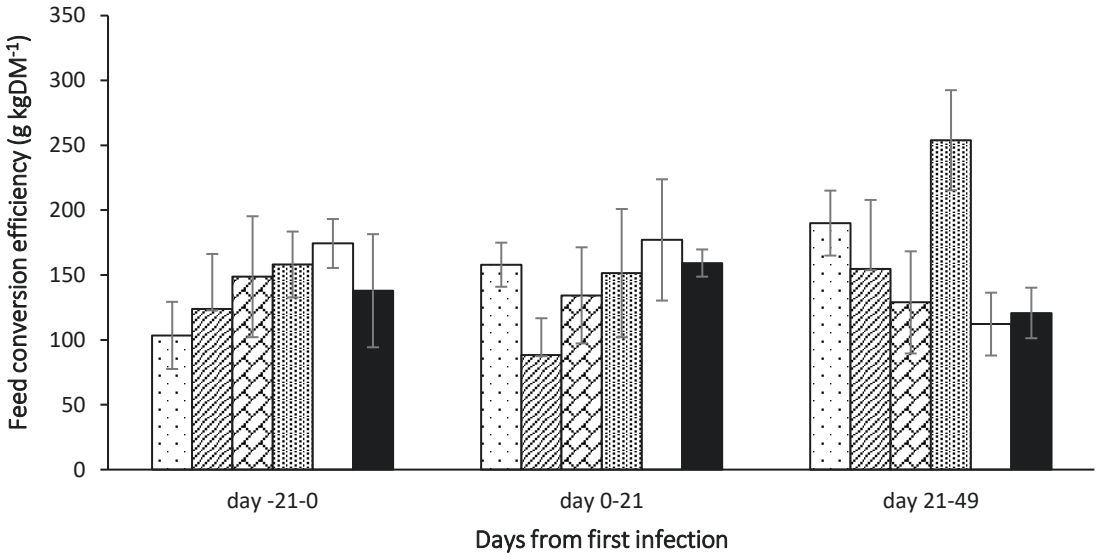


Figure 3.5: Feed conversion efficiency (± SEM) of lambs infected with 14 000 *T. colubriformis* week$^{-1}$ (INF □), infected and injected with *Tcol*-L3 (TL3 ⊡), *Tcol*-L4 (TL4 ▨), *Tcol*-L5 (TL5 ⊠), or *Tcol*-L3/L4/L5 antigen (COMB ▩), and non-infected controls (CON ■).

### 3.3.6 Carcass composition

Table 3.2 shows carcass composition estimated by computed tomography on days -2 and 46. There was no difference in liveweight gain (P=0.568), carcass weight gain (P=0.851), bone deposition (P=0.439), fat deposition (P=0.480) and lean tissue deposition (P=0.532) between the groups on day 46. Similarly, there was no difference in total ME intake (P=0.978), total NE stored (P=0.554) and NE: ME ratio (P=0.583) between treatments.

Table 3.2: Carcass composition (± SEM) estimated by computed tomography and energy utilisation of lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF), infected and injected with *Tcol*-L3 (TL3), *Tcol*-L4 (TL4), *Tcol*-L5 (TL5), or *Tcol*-L3/L4/L5 antigen (COMB), and non-infected controls (CON). There was no significant difference between the groups (P>0.05).

| | Treatment group (n=6) | | | | | |
|---|---|---|---|---|---|---|
| Initial body composition on day -2 | TL3 | TL4 | TL5 | COMB | INF | CON |
| Live weight (kg) | 33.90 ± 1.23 | 33.27 ± 1.74 | 34.48 ± 0.72 | 34.23 ± 2.25 | 34.13 ± 1.96 | 35.60 ± 2.13 |
| Carcass weight (kg) | 15.59 ± 0.81 | 15.69 ± 1.03 | 16.06 ± 0.72 | 15.50 ± 0.83 | 15.94 ± 0.83 | 17.00 ± 1.44 |
| Bone (kg) | 2.01 ± 0.09 | 1.71 ± 0.11 | 1.99 ± 0.09 | 1.85 ± 0.13 | 1.98 ± 0.18 | 2.12 ± 0.17 |
| Fat (kg) | 3.48 ± 0.38 | 3.73 ± 0.49 | 4.14 ± 0.38 | 3.83 ± 0.26 | 3.79 ± 0.40 | 4.07 ± 0.45 |
| Lean tissue (kg) | 10.10 ± 0.45 | 10.24 ± 0.44 | 9.92 ± 0.35 | 9.85 ± 0.53 | 10.18 ± 0.92 | 10.78 ± 0.48 |
| | | | | | | |
| Tissue deposition on day 46 | | | | | | |
| Liveweight gain (kg) | 8.55 ± 1.12 | 7.66 ± 2.23 | 10.90 ± 1.91 | 10.88 ± 1.12 | 8.03 ± 1.78 | 10.43 ± 0.84 |
| Carcass weight gain (kg) | 3.56 ± 0.60 | 3.85 ± 0.63 | 3.19 ± 0.94 | 3.70 ± 0.73 | 3.23 ± 1.02 | 3.99 ± 1.54 |
| Bone (kg) | -0.04 ± 0.11 | 0.19 ± 0.09 | -0.09 ± 0.05 | 0.09 ± 0.07 | 0.14 ± 0.21 | -0.07 ± 0.17 |
| Fat (kg) | 1.62 ± 0.22 | 1.27 ± 0.37 | 1.46 ± 0.35 | 2.12 ± 0.38 | 2.33 ± 0.42 | 2.09 ± 0.75 |
| Lean tissue (kg) | 1.43 ± 0.37 | 0.59 ± 0.27 | 1.21 ± 0.60 | 1.49 ± 0.43 | 1.58 ± 0.55 | 0.64 ± 0.65 |
| | | | | | | |
| Energy utilisation | | | | | | |
| Total ME intake (MJ) | 987 ± 72.17 | 956 ± 93.28 | 1,000 ± 54.16 | 956 ± 60.91 | 972 ± 92.64 | 1,058 ± 122.45 |
| Total NE Stored (MJ) | 68.91 ± 10.10 | 53.90 ± 15.68 | 61.22 ± 16.28 | 98.81 ± 16.31 | 99.23 ± 18.80 | 83.54 ± 33.43 |
| NE:ME | 0.09 ± 0.01 | 0.08 ± 0.03 | 0.09 ± 0.02 | 0.13 ± 0.03 | 0.15 ± 0.04 | 0.10 ± 0.04 |

### 3.3.7 Parasitology

#### 3.3.7.1 Faecal egg count

Mean back-transformed (log 10 (count +10)) faecal egg counts (FEC, epg) are given in Figure 3.6. Overall, FEC increased with time (P<0.001) in all infected groups starting from day 21 and peaking on day 35 with the highest peak FEC in INF at 1,533 epg and the lowest peak in TL3 and COMB at 720 and 730 epg, respectively, before declining to less than 700 epg in INF and less than 400 epg in the remaining groups on day 49. There was no difference between the infected groups (P=0.291) nor interaction between treatment and time (P=0.212).

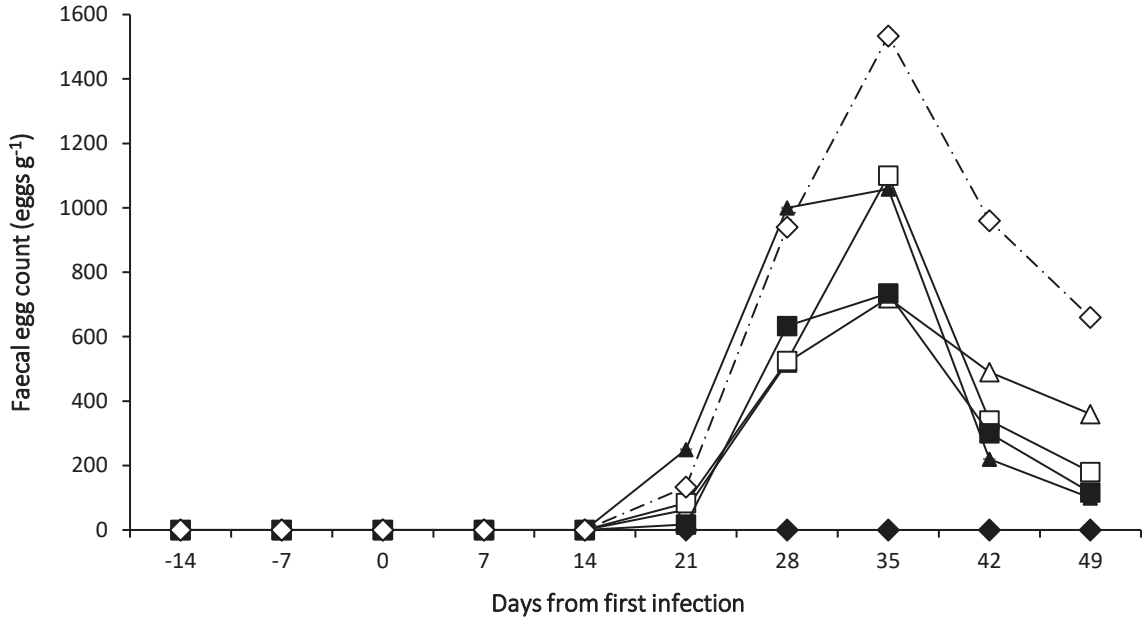


Figure 3.6: Mean back-transformed (log 10 (count +10)) FEC of lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

#### 3.3.7.2 Worm burden

Small intestinal worm counts and worm length are given in Table 3.3. Total worm burden was lowest in the CON group (15) compared with the infected groups. Within the infected animals, the INF had the highest worm burden (11,820) and COMB the lowest (5,975) (P<0.001). Adult worms contributed 97-99% of the total worm burden. Worm length differed between the infected treatment groups (P<0.001) being longest in INF *viz.* 8.35 ± 0.13 mm and 6.38 ± 0.08 mm for females and males, respectively, and shortest in COMB (6.50 ± 0.10 mm and 5.43 ± 0.07 mm) representing a reduction of 22% and 15% of female and male worms, respectively. Within the antigen treated groups the COMB had proportionally 15% shorter female and 9% shorter male worms than the other groups.

Table 3.3: Mean log transformed (log 10 (count+10)) small intestinal worm burden and worm length (mm) (± SEM) of lambs infected with 14,000 *T. colubriformis* larvae week$^{-1}$ (INF), infected and injected with *Tcol*-L3 (TL3), *Tcol*-L4 (TL4), *Tcol*-L5 (TL5), or *Tcol*-L3/L4/L5 antigen (COMB), and non-infected controls (CON). Back-transformed values are given in parenthesis. Means with different superscript are significantly different (P<0.05).

| | Treatment group | | | | | |
|---|---|---|---|---|---|---|
| Larval stage | TL3 | TL4 | TL5 | COMB | INF | CON |
| L3 | 1 ± 0$^{a}$ (0) | 1 ± 0$^{a}$ (0) | 1 ± 0$^{a}$ (0) | 1.18 ± 0.18$^{a}$ (5) | 0 ± 0$^{a}$ (0) | 1 ± 0 (0) |
| L4 | 1.79 ± 0.37$^{a}$ (51) | 1.52 ± 0.33$^{a}$ (23) | 1.55 ± 0.30$^{a}$ (68) | 1.28 ± 0.22$^{a}$ (5) | 1.12 ± 0.12$^{a}$ (3) | 1 ± 0 (0) |
| L5/ adult | 3.86 ± 0.07$^{ab}$ (7,271) | 3.94 ± 0.04$^{ab}$ (8,674) | 3.85 ± 0.10$^{ab}$ (7,001) | 3.77 ± 0.04$^{a}$ (5,924) | 4.07 ± 0.03$^{b}$ (11,813) | 1.39 ± 0.27 (14) |
| Total | 3.88 ± 0.07$^{ab}$ (7,527) | 3.95 ± 0.04$^{ab}$ (8,803) | 3.85 ± 0.10$^{ab}$ (7,115) | 3.78 ± 0.04$^{a}$ (5,975) | 4.07 ± 0.03$^{b}$ (11,820) | 1.40 ± 0.28 (15) |
| Female worm length | 7.60 ± 0.09$^{b}$ | 7.56 ± 0.12$^{b}$ | 7.68 ± 0.11$^{b}$ | 6.50 ± 0.10$^{a}$ | 8.35 ± 0.13$^{c}$ | |
| Male worm length | 5.84 ± 0.06$^{b}$ | 5.85 ± 0.07$^{b}$ | 6.09 ± 0.07$^{b}$ | 5.43 ± 0.07$^{a}$ | 6.38 ± 0.08$^{c}$ | |

## 3.3.8 Serum antibodies

### 3.3.8.1 IgA

Mean absorbance (OD) of serum IgA antibodies to *T. colubriformis* L3-specific antigen (*Tcol*-L3) is given in Figure 3.7. Overall, there was an effect of time (P<0.001) reflecting increasing IgA followed by a decline around day 35-42. There was no difference in IgA profiles between treatment groups (P=0.506) nor interaction between treatment and time (P=0.984).

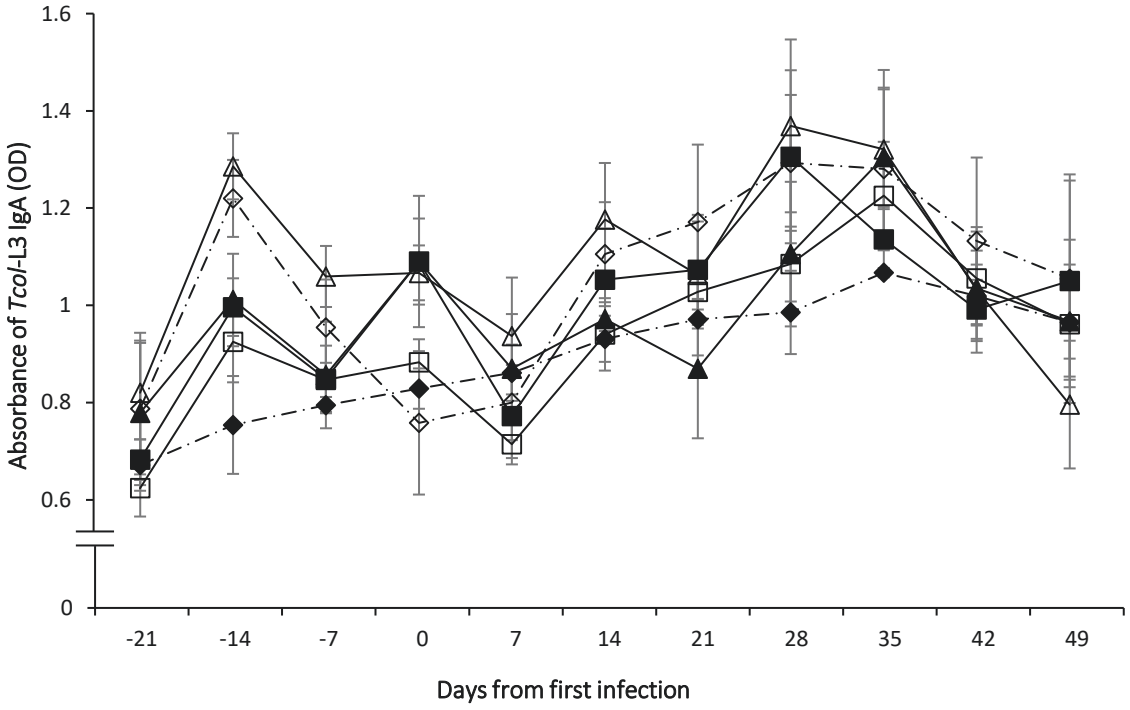


Figure 3.7: Mean absorbance (± SEM) of *T. colubriformis* L3 IgA antibody levels for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

Mean absorbance (OD) of serum IgA antibodies to *T. colubriformis* L4-specific antigen (*Tcol*-L4) is given in Figure 3.8. Overall, there was an effect of time (P<0.001) with increasing IgA in all infected groups until day 35 followed by a decline. There was an effect of treatment with IgA levels in CON remaining relatively steady throughout the trial (P=0.016). There was no interaction between treatment and time (P=0.679).

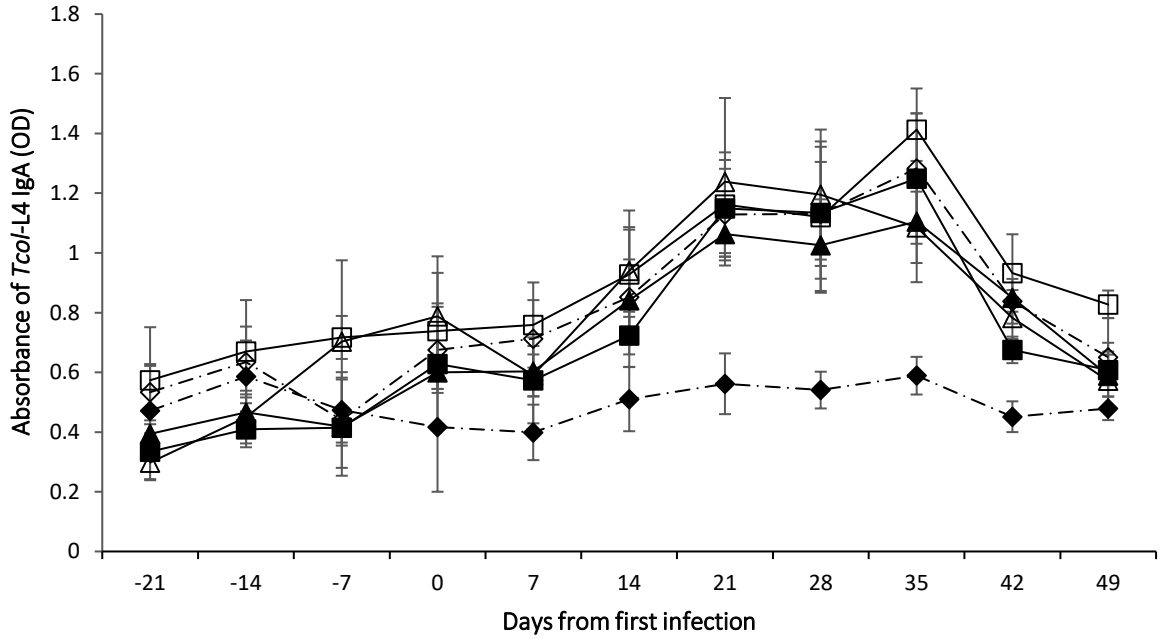


Figure 3.8: Mean absorbance (± SEM) of *T. colubriformis* L4 IgA antibody levels for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

Mean absorbance (OD) of serum IgA antibodies to *T. colubriformis* L5-specific antigen (*Tcol*-L5) is given in Figure 3.9. Overall, there was a treatment x time interaction (P=0.021) reflecting similar IgA profiles in all infected groups which increased in all infected groups to reach peaks on day 35 except in the CON group that remained relatively steady throughout the trial.

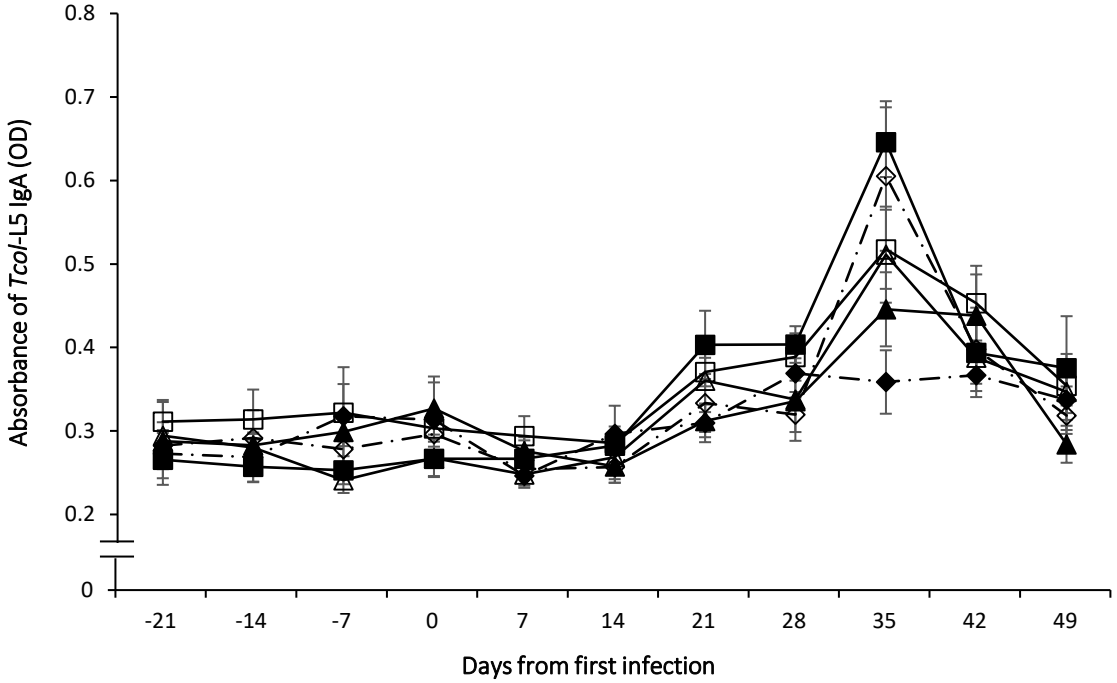


Figure 3.9: Mean absorbance (± SEM) of *T. colubriformis* L5 IgA antibody levels for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

### 3.3.8.2 IgG

Mean absorbance (OD) of serum IgG antibodies to *T. colubriformis* L3-specific antigen (*Tcol*-L3) is given in Figure 3.10. Overall, there was an effect of time (P<0.001) reflecting increasing IgG with time in all infected groups and peaking on day 28. There was an effect of treatment (P=0.002) with consistently lower IgG in the CON group. There was no interaction between treatment and time (P=0.174).

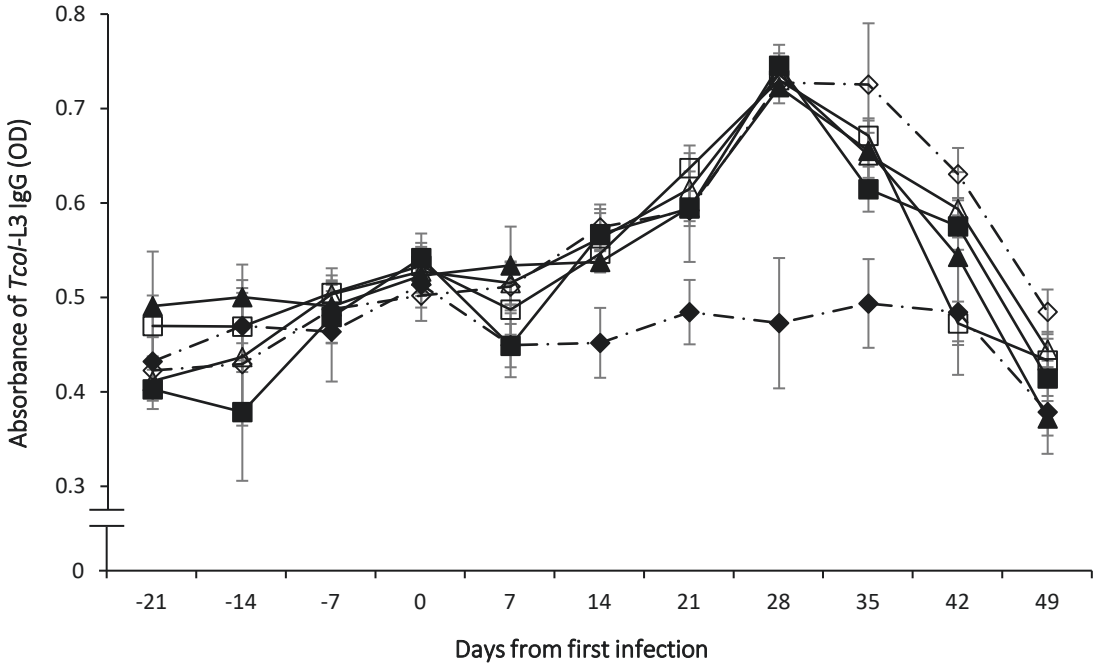


Figure 3.10: Mean absorbance (± SEM) of *T. colubriformis* L3 IgG antibody levels for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

Mean absorbance (OD) of serum IgG antibodies to *T. colubriformis* L4-specific antigen (*Tcol*-L4) is given in Figure 3.11. Overall, there was an effect of treatment (P<0.001) and time on IgG (P<0.001) reflecting similar and increasing IgG profiles in all groups with lower absorbance in CON. There was no interaction between treatment and time (P=0.848).

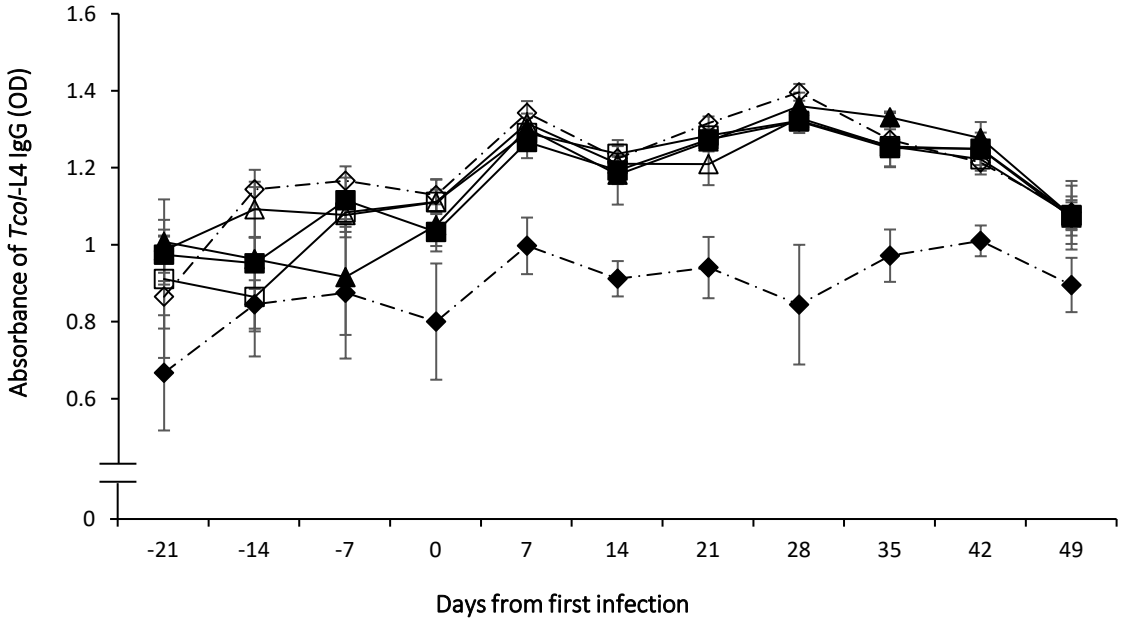


Figure 3.11: Mean absorbance (± SEM) of *T. colubriformis* L4 IgG antibody levels for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

Mean absorbance (OD) of serum IgG antibodies to *T. colubriformis* L5-specific antigen (*Tcol*-L5) is given in Figure 3.12. Overall, there was an effect of time (P<0.001) with IgG profiles being similar in all infected groups with an initial increase followed by decreased absorbance from day 28 in TL3, TL4 and COMB and day 35 in TL5 and INF. There was an effect of treatment (P=0.005) reflecting lower absorbance in CON than the infected groups. There was no interaction between treatment and time (P=0.824).

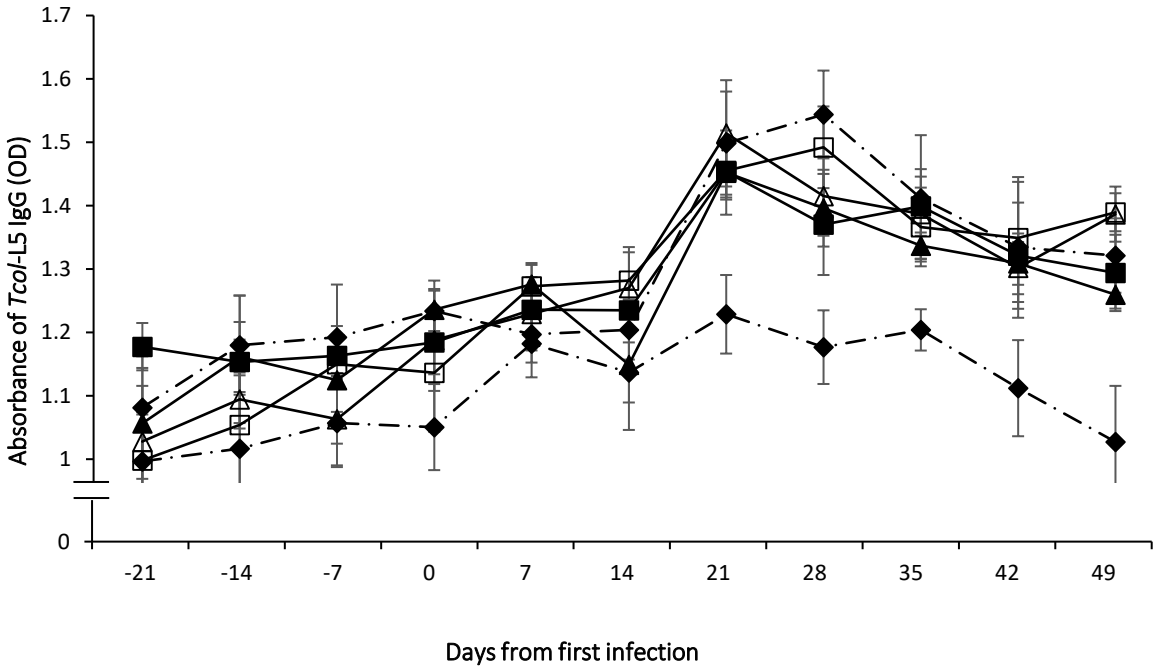


Figure 3.12: Mean absorbance (± SEM) of *T. colubriformis* L5 IgG antibody levels for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

### 3.3.8.3 IgE

Mean absorbance (OD) of serum IgE antibodies to *T. colubriformis* L3-specific antigen (*Tcol*-L3) is given in Figure 3.13. There was an interaction between treatment and time in IgE (P<0.001) reflecting a consistently low absorbance in CON compared with the infected groups which all had similar profiles increasing to peak on day 35 before declining to levels similar to CON animals by day 49.

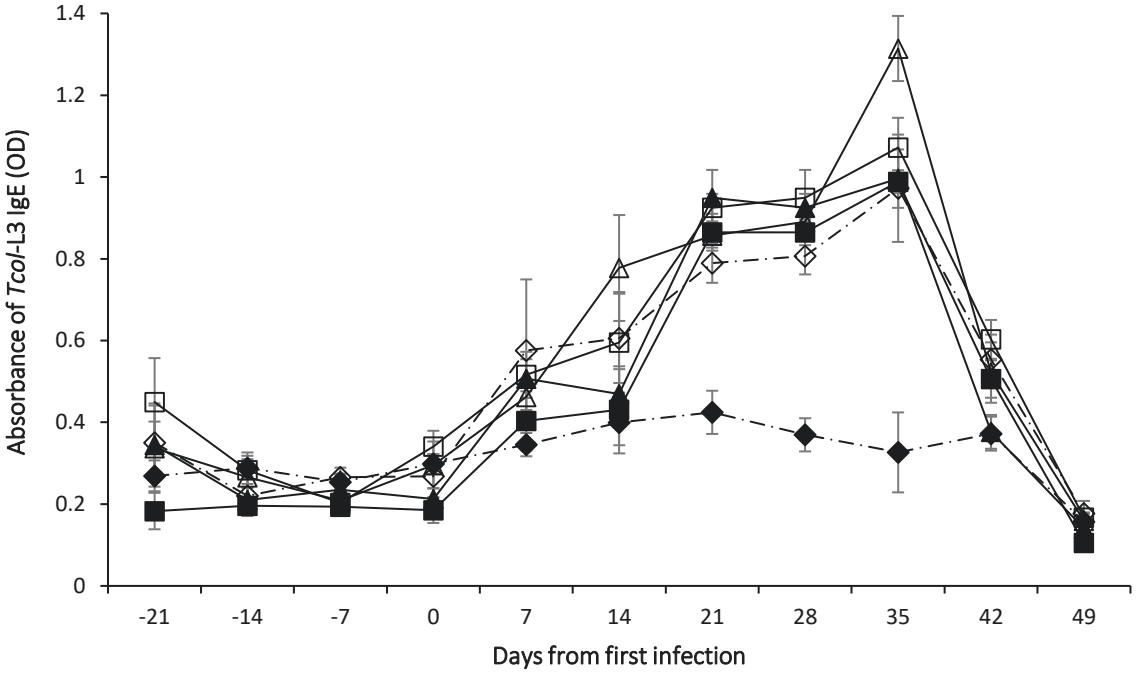


Figure 3.13: Mean absorbance (± SEM) of *T. colubriformis* L3 IgE antibody levels for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

Mean absorbance (OD) of serum IgE antibodies to *T. colubriformis* L4-specific antigen (*Tcol*-L4) is given in Figure 3.14. Overall, IgE was consistently lower in CON than in the infected groups (P=0.003). The IgE increased with time in the infected groups, peaking at day 28 (P<0.001). There was no interaction between treatment and time (P=0.506).

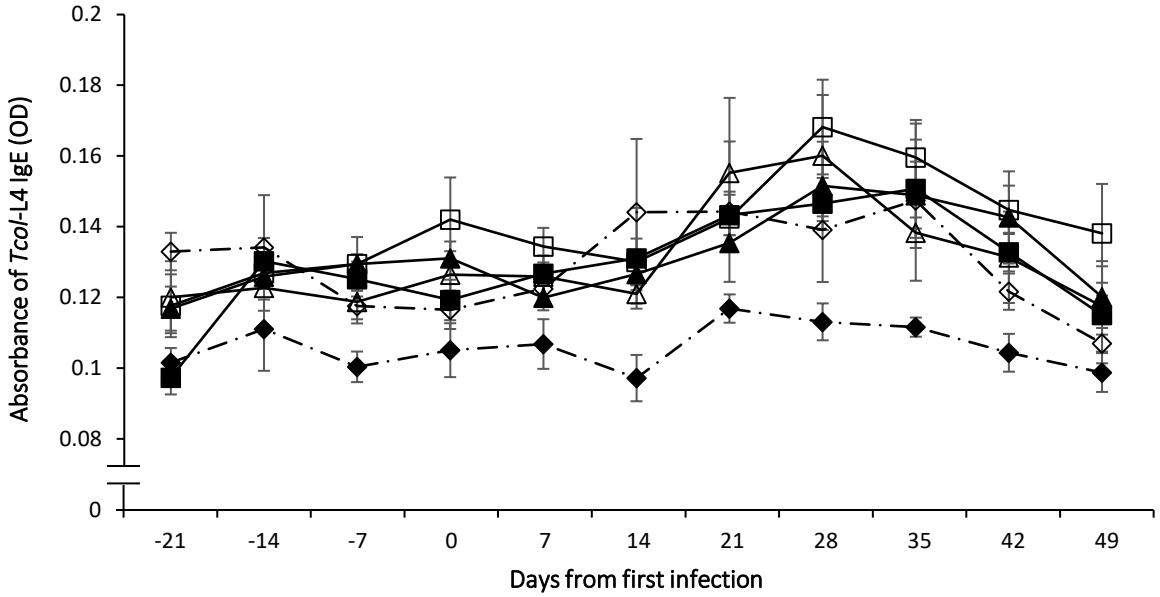


Figure 3.14: Mean absorbance (± SEM) of *T. colubriformis* L4 IgE antibody levels for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

Mean absorbance (OD) of serum IgE antibodies to *T. colubriformis* L5-specific antigen (*Tcol*-L5) is given in Figure 3.15. Overall, there was a treatment by time interaction (P<0.001) reflecting consistently low absorbance in CON compared with the infected groups which all had similar profiles peaking on days 28, except COMB that peaked on day 21.

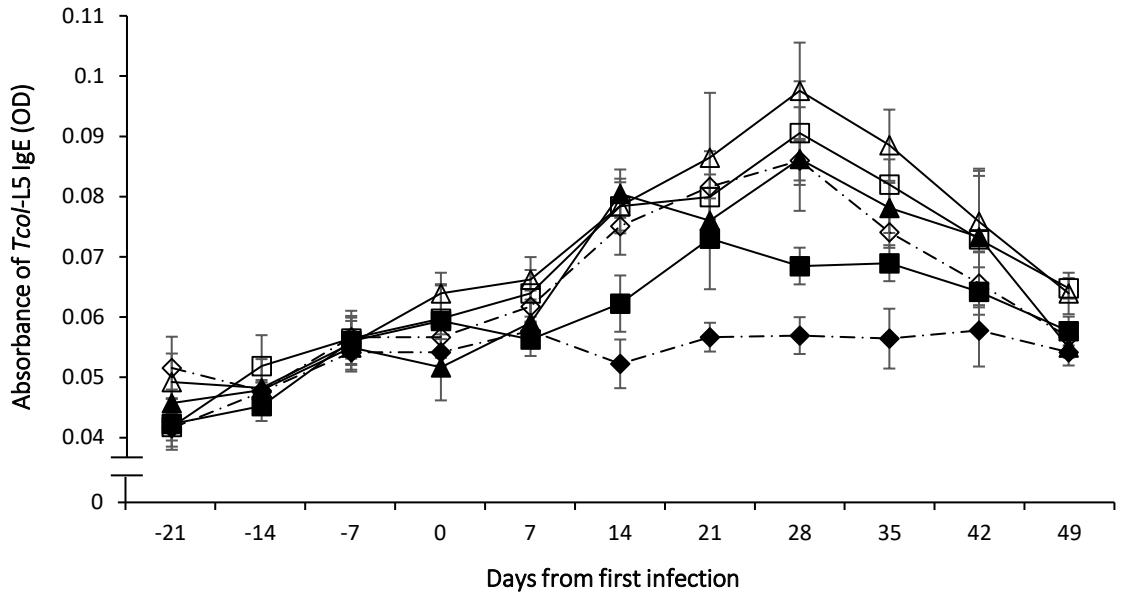


Figure 3.15: Mean absorbance (± SEM) of *T. colubriformis* L5 IgE antibody levels for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

## 3.3.9 Cytokine production

### 3.3.9.1 IL-4

Mean IL-4 concentration (pg $ml^{-1}$) produced by white blood cells (WBC) stimulated with *T. colubriformis*-L3/L4/L5 antigen is given in Figure 3.16. There was an interaction between treatment and time (P<0.001) reflecting an increase in IL-4 production in all infected groups but with a greater concentration in the COMB and TL4 groups at peak on day 35, *viz.* 44, 46, 42, 72, 36 and 29 pg $ml^{-1}$ in TL3, TL4, TL5, COMB, INF, and CON respectively.

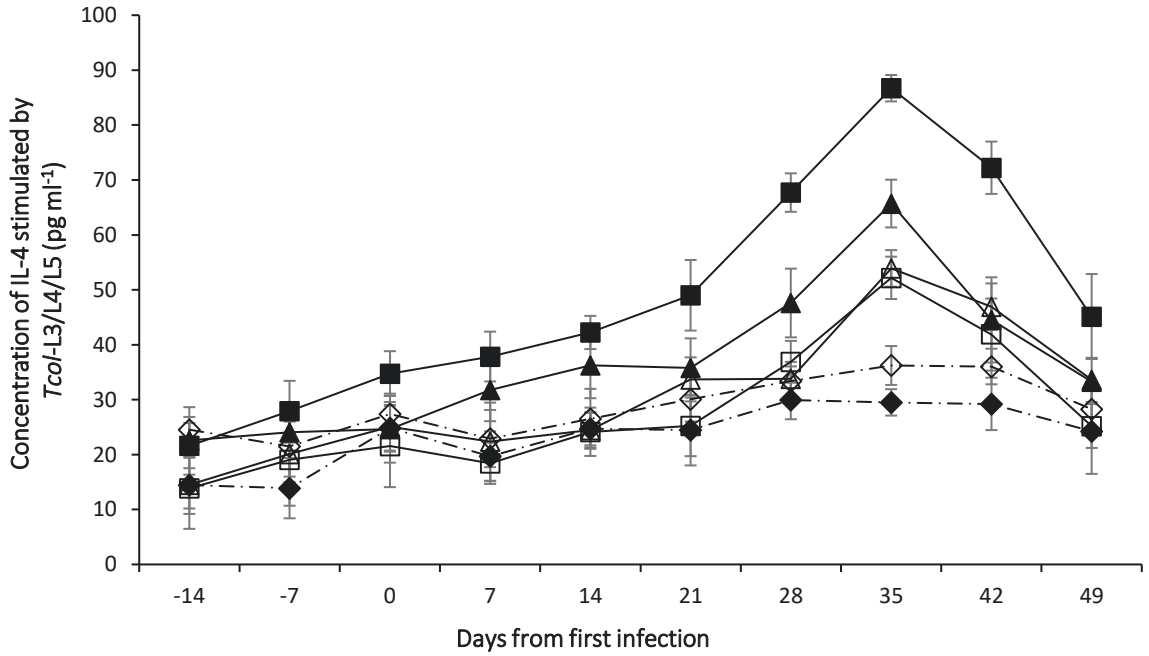


Figure 3.16: Mean IL-4 concentration (± SEM) produced by WBC stimulated with *T. colubriformis* L3/L4/L5 antigen for lambs infected with 14,000 *T. colubriformis* $week^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

### 3.3.9.2 IFN-Y

Mean IFN-γ concentration (pg $ml^{-1}$) produced by WBC stimulated with *T. colubriformis*-L3/L4/L5 antigen is given in Figure 3.17. There was no effect of treatment (P=0.764) but there was an effect of time (P<0.001) reflecting increasing IFN-γ concentration in all infected groups over time. There was no interaction between treatment and time (P=1.000).

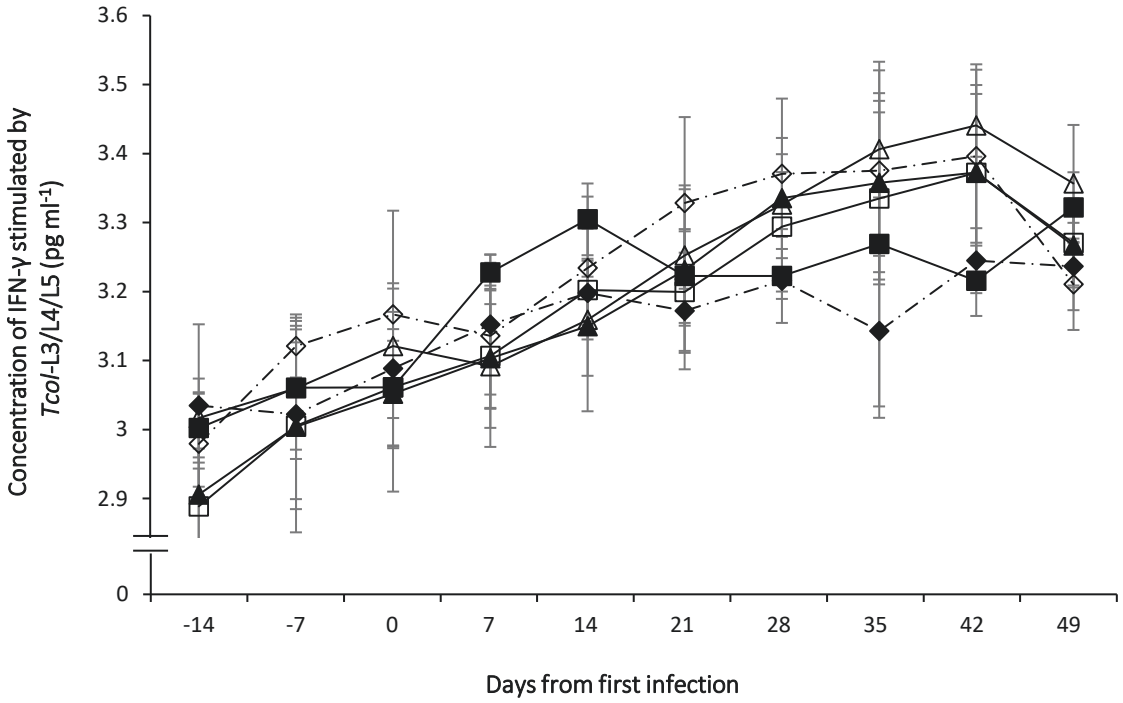


Figure 3.17: Mean IFN-γ concentration (± SEM) produced by WBC stimulated with *T. colubriformis* L3/L4/L5 antigen for lambs infected with 14,000 *T. colubriformis* $week^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

## 3.3.10 Serum phosphorous

Mean serum phosphorous (P) concentrations (mmol $l^{-1}$) are given in Figure 3.18. Overall, there was an effect of time (P<0.001) with initially increasing concentrations followed by a decline in serum P values from day 7 in all groups. There was no effect of treatment (P=0.990) and no interaction between treatment and time (P=0.830).

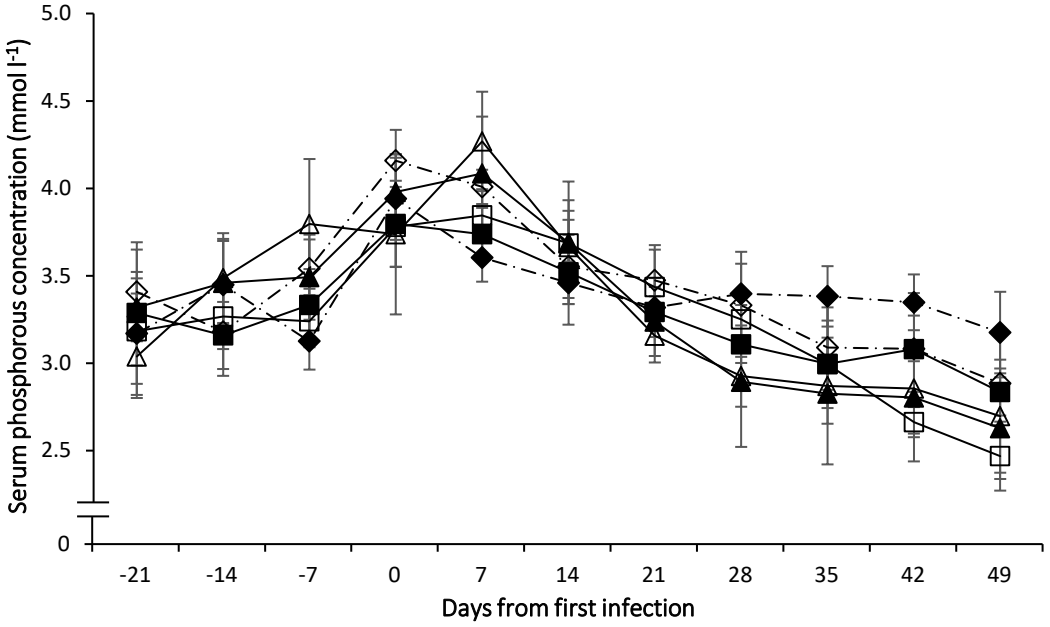


Figure 3.18: Mean serum P concentrations (± SEM) for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

## 3.3.11 Serum urea

Mean serum urea concentrations (mmol $l^{-1}$) are given in Figure 3.19. There was an effect of time ($P<0.001$) reflected by initially increasing serum urea concentrations in all groups from day -21 to day 0 thereafter declining in all groups starting earlier in INF (day-7). There was no effect of treatment ($P=0.622$) nor interaction between treatment and time ($P=0.988$).

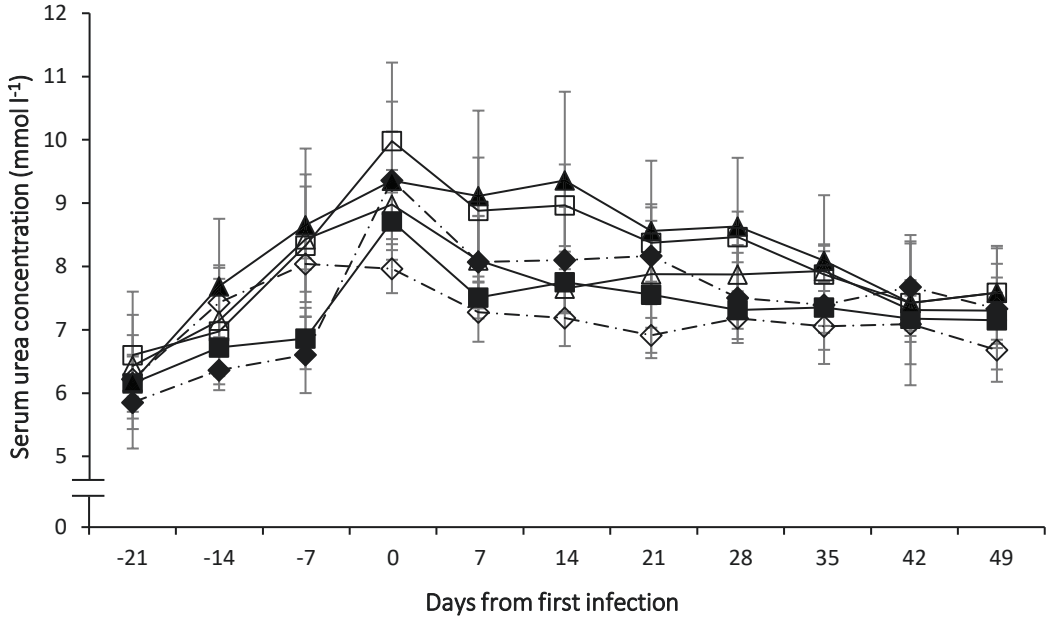


Figure 3.19: Mean serum urea concentrations (± SEM) for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

## 3.3.12 Serum total protein

Mean total protein concentrations (g $l^{-1}$) are given in Figure 3.20. Overall, there was an effect of time ($P<0.001$) reflecting decreasing protein concentrations from day 21 until the end of the study. There was no difference between the groups ($P=0.275$) nor treatment x time interaction ($P=0.518$).

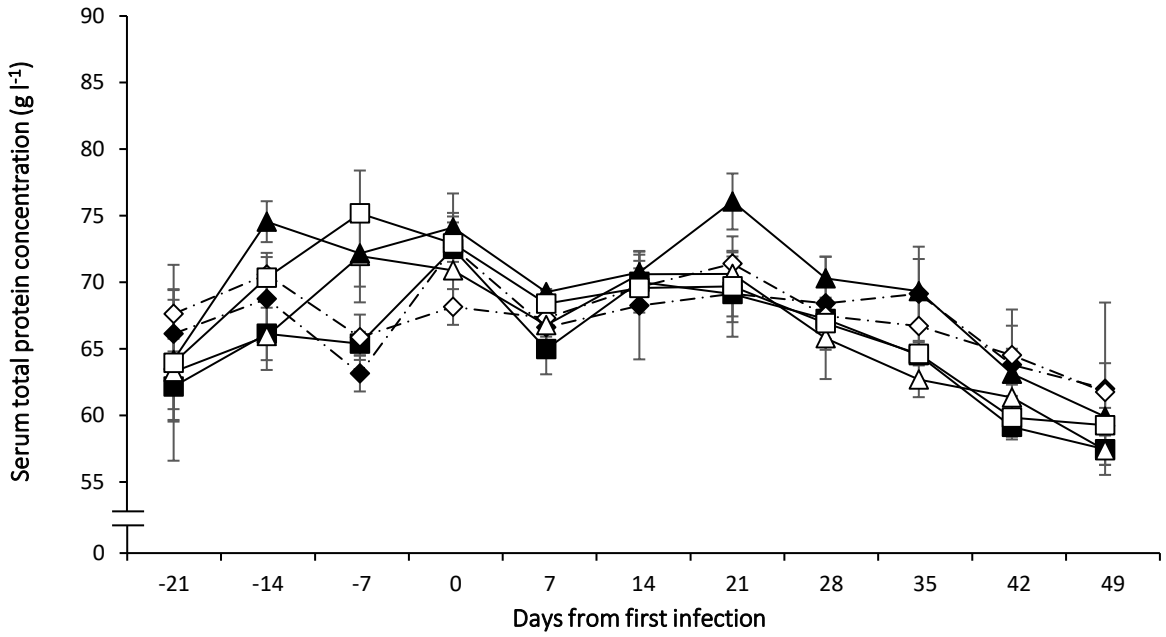


Figure 3.20: Mean serum total protein concentrations (± SEM) for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

## 3.3.13 Serum albumin

Mean serum albumin concentrations (g $l^{-1}$) are shown in Figure 3.21. There was an effect of time ($P<0.001$) reflecting decreasing albumin concentrations in all groups from day 42. The albumin concentrations did not vary between the groups ($P=0.433$) nor was there treatment x time interaction ($P=0.120$).

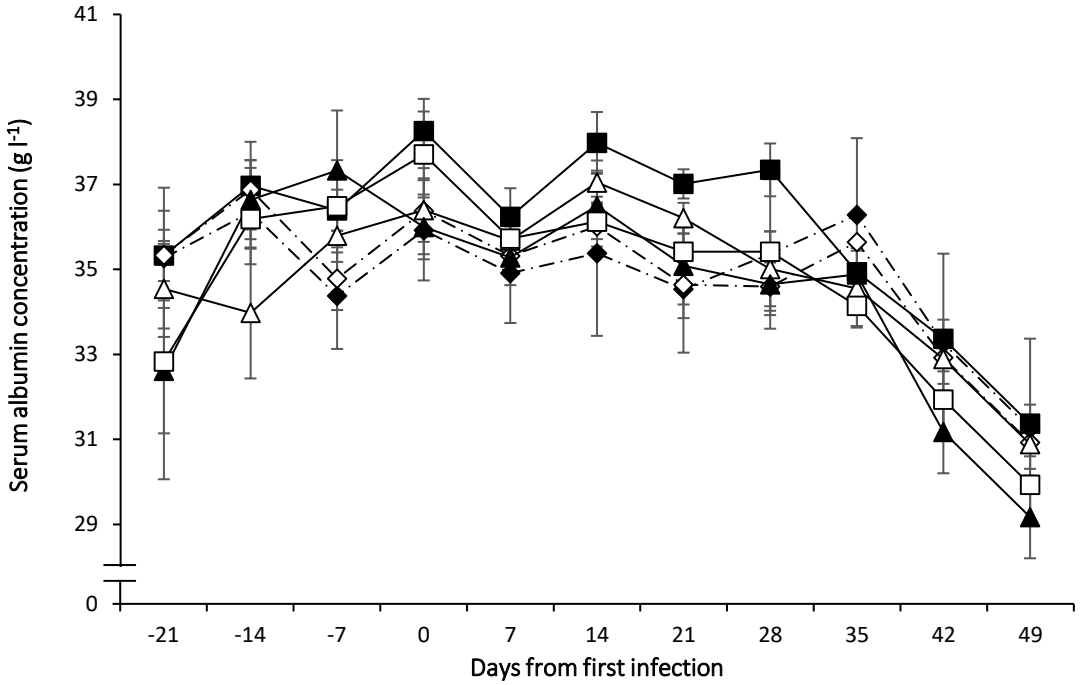


Figure 3.21: Mean serum albumin concentrations (± SEM) for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected with *Tcol*-L3 (TL3, solid line open triangle), *Tcol*-L4 (TL4, solid line filled triangle), *Tcol*-L5 (TL5, solid line open square), or *Tcol*-L3/L4/L5 antigen (COMB, solid line closed square), and non-infected controls (CON, dashed line closed diamond).

## 3.4 Discussion

These findings suggest that repeated injections with a somatic *T. colubriformis* antigen combination appears to invoke a Th2 immune response in lambs. The greater rise in IL-4 cytokine production in all the antigen injected animals compared with the INF group and the elevated serum antibodies suggest that the antigen treatment boosted the Th2-based immunological effect and immunity towards *T. colubriformis* with the greatest response in the COMB group. Furthermore, the worm burden data (Table 3.3) revealed that the antigen treatment lowered the worm numbers in all the antigen treated groups with the shortest worms observed in the lambs injected with the antigen combination *T. colubriformis*-L3/L4/L5 (COMB). As such, rather than providing a desensitising effect, as intended, the treatment regime appeared to stimulate some immune functions.

The rationale for administrating the antigen without an adjuvant was to avoid a Th2 immune response skewed towards immunity. Adjuvants are generally known to improve the immunostimulatory effect of an antigen and it was hypothesised that the antigen treatment would induce tolerance in the lambs. Greer et al. (2014) observed improved performance in antigen treated lambs without the use of an adjuvant using a similar antigen regime to the one used in the present study, where the rationale for using different doses of antigen for each larval stage was the limitation of available antigen, supporting the suggestion that an adjuvant would not be required. While the antigen doses used by Greer et al. (2014) was twice the amount of the doses used in the current study, data from a dose-response study (A.W. Greer, unpublished data) showed that 100% and 50% of the antigen doses were equally effective, thus the smaller amount was chosen here to minimise the amount of antigen required for the study. However, there was no evidence that the antigen treatment induced tolerance in the current study. The elevated IL-4 concentrations are in line with observations by Pernthaner et al. (2006), that Th2 cytokines are dominant in the intestinal lymph of sheep infected with *T. colubriformis* and that IL-4 is crucial for the immune response following challenge. While a Th2 response is associated with both immunity and tolerance to nematodes (Andronicos, Hunt, & Windon, 2010; Strobel, 2001; Weiner, 1997; Williams, Palmer, et al., 2010) the elevated serum antibodies post-infection and lower worm burdens suggest that the current regime appeared to provoke immunity rather than tolerance. However, the lack of increase in IL-4 in the INF group was surprising considering the decrease in FEC which indicated that the lambs had developed some degree of immunity to the nematodes. While IFN-γ is known to be linked to priming of Th1 immune responses and has been observed before the establishment of tolerance (Weiner, 1997), the apparent lack of difference in IFN-γ production between INF and the antigen treated lambs further suggests that tolerance was not

induced. The rise in IFN-γ in all groups, including CON, implies that the IFN-γ production in the present study was not related to the nematode infection per se. The reason for the increase remains unknown.

Development of immunity was indicated by the declining serum IgA antibody concentrations along with the reduction in FEC around days 35 and 42. Although immunity was not the intention of the current treatment regime, timings are in agreement with Sykes (2008) who described a decline in FEC and a return of IgA to levels at pre-infection in sheep infected with *T. colubriformis* and *T. circumcincta* when immunity had been established. It is further supported by Bisset and Morris (1996), Douch et al. (1994) and Shaw et al. (2012) who showed that elevated IgG1 and IgA were negatively correlated with FEC. However, the declining IgG concentrations towards the end of the study was unexpected and it is unknown what caused the decline. Moreover, reduced FEC has been associated with increased IgE responses (Bendixsen et al., 2004; Gossner, Wilkie, Joshi, & Hopkins, 2013; Murphy et al., 2010; Shaw et al., 1999). Since IL-4 is the major inducer of B cells switching to IgE production (Abbas, Murphy, & Sher, 1996) and IgE plays a significant role in parasite expulsion (Huntley et al., 2001; Miller, 1996; Shaw et al., 1998) it is suggested that IL-4 may have served as a component in the reduction of the worm establishment in the present study considering the greater IL-4 secretion in the antigen treated groups and the corresponding lower worm burdens.

The lack of difference in IgE between INF and the antigen treated lambs was surprising given the lower worm burden in the antigen treated lambs. The similar serum antibody levels between INF and the antigen treated groups indicates that the antigen treatment did not further increase peripheral antibody responses. However, it remains unknown whether the treatment triggered local IgA antibody production at mucosal sites since this was not measured in the present study. The prominent reduction of length of both male and female worms in the antigen treated groups suggest that IgA may have been secreted in the small intestine, given that local IgA secretion is associated with a developing immune response and has been linked to reduction of female worm length (Stear et al., 1995; Stear et al., 1999a; Strain et al., 2002; Sykes, 2008). Haneberg et al. (1994) found that serum IgA reflected local IgA secretion in mice following oral, gastric and rectal immunisation with cholera toxin. It was further noticed that rectal immunisation induced distant IgA secretion in the small intestine. On the contrary, Sinski et al. (1995) observed only a moderate link between IgA responses in the abomasal mucus and plasma of sheep infected with *Ostertagia circumcincta.*

The three different *T. colubriformis* larval antigens were observed to be equally efficient at reducing worm numbers and worm length and more potent when used in combination. This is indicated by the greatest reduction in COMB and the lack of difference between the individual antigen groups TL3, TL4

and TL5, and is further supported by the higher IL-4 production in COMB. While the different doses of antigen used for the different larval stages may have had an impact on the results, the absence of between-treatment difference in IgA, IgG and IgE antibody responses when stimulated by the separate antigens, *Tcol*-L3, *Tcol*-L4 and *Tcol*-L5 further suggests that this was not present in the current study. Further, it suggests that each larval stage had similar immune-stimulatory properties. The rationale for using different doses of antigen was that the same antigen and dose rates had previously been used in a dose-response study with positive results including a lack of a reduction in voluntary feed intake and increased performace for the first seven weeks of infection (A.W. Greer, unpublished data). Moreover, the tendency for a greater FCE in COMB between days 21-49 suggests that the antigen combination had a positive effect on performance which was not observed when antigens from each larval stage were used individually although CON animals had low FCE during this period, so firm conclusions cannot be drawn.

Despite the tendency for greater FCE and liveweight gain in COMB post-infection, the absence of effect of infection on appetite and performance in INF indicate that either the larvae were not pathogenic enough to cause a reduction in feed intake and subsequent weight loss or that the lambs were resilient to the adverse impact on the host of infection. Feed intake has been estimated to be responsible for 60-90% of the performance losses during nematode infections (Coop & Holmes, 1996; Houtert & Sykes, 1996) and since it was not reduced in the present study it is perhaps not surprising that performance was not affected. In contrast, Greer et al. (2005) observed a 30% depression in feed intake in sheep infected with 2,000 *T. colubriformis* day$^{-1}$. Similar to Greer et al. (2005), Houtert and Sykes (1996) and Sykes (2008) described a 15-30% reduction in feed intake two to four weeks post-infection in naïve lambs around three to four months of age which was restored once immunity was acquired. Greer et al. (2005) also noted a 20% reduction in gross efficiency of use of metabolisable energy for net energy deposition during the acquisition period, a figure supporting the estimates by Sykes (1994) that a production cost of 15% is required in sheep to maintain immunity to parasites. Further, Kyriazakis et al. (1994a) showed a 30% lower growth rate in sheep challenged with 2,500 *T. colubriformis* day$^{-1}$. Additionally, the lack of reduction in liveweight gain and the elevated IgE in the infected groups, are contradictive to findings of Shaw et al. (1999), who reported unfavourable effects on production linked to elevated IgE responses to *T. colubriformis* antigen.

The lack of impact on the host by the parasites is further supported by not only a lack of reduction in serum albumin but also the absence of a difference in carcass composition which suggests that little energy was invested into repair of damage to the gastrointestinal tract. However, it is worth noting an apparent inconsistency between liveweight gain and carcass weight, as predicted by computed

tomography, was apparent. This can possibly be explained by a poor correlation between fat and live weight on day -2. Moreover, the pre-infection rise in serum urea levels and the lack of difference between the infected lambs and the control lambs in serum urea suggests that the rise was not associated with infection. Reduced serum albumin concentrations are commonly associated with nematode infections of the small intestine due to leakage of plasma proteins into the alimentary tract and decreased albumin synthesis in the liver (Coop et al., 1976; Steel et al., 1980; Sykes & Coop, 1976). Although serum albumin levels declined post-infection in the present study it was not different between infected and non-infected lambs suggesting either a lack of pathogenicity or ability to replace endogenous loss, which may have been helped by a lack of effect on feed intake. The reason for reduced serum albumin concentrations in CON animals is not clear. While another feature of nematode infections is disrupted N balance, which is considered to be caused by endogenous loss of N due to increased secretion of plasma proteins into the gastrointestinal tract and sloughing of epithelial cells (Bown et al., 1991; Poppi et al., 1986), it is considered unlikely that the reduced serum albumin in the present study was an effect of damage to the intestinal epithelium since there was no difference between the infected lambs and their uninfected counterparts. All groups, including the uninfected controls, displayed reductions in serum protein and serum P concentrations post-infection. While hypophosphataemia is typically seen in sheep infected with *T. colubriformis* due to impaired absorption of serum P (Bown et al., 1989; Poppi et al., 1985; Wilson & Field, 1983), the reductions in the present study are unlikely caused by the parasites but possibly a consequence of insufficient levels in the feed.

The lack of difference in performance may have been confounded with Cu toxicity in the present study due to the excess Cu in the feed. While attempts were made to prevent further harm by supplementing the lambs with 50 mg $day^{-1}$ ammonium molybdate and 500 mg $day^{-1}$ sodium sulphate when the first symptoms were detected, it cannot be dismissed that the Cu toxicity may have affected the outcome of the study. Feed analysis revealed Cu levels 10-fold the toxic levels for sheep. This was reflected in serum Cu levels three times the normal range (Underwood & Suttle, 1999) which confirmed acute Cu toxicity (Hefnawy & El-Khaiat, 2015). Apart from liver damage, severe gastroenteritis, diarrhoea and anorexia are symptoms of acute Cu toxicity (Radostits, 1994). The excess Cu in the feed may have suppressed growth of the lambs in the present study which may explain the observation that the performance of the CON lambs was no different than in the infected groups. Cu has been found to have anti-parasitic properties, proven effective against abomasal nematodes but less effective against intestinal nematode species (Burke, Miller, Olcott, Olcott, & Terrill, 2004; Knox, 2002; Leal et al., 2014; Soli et al., 2010). Bang, Familton, and Sykes (1990) noted a 96% protection against *H. contortus* and 56% protection against *T. circumcincta* using Cu oxide wire particles (COWP).

However, no significant effect of Cu was reported on the establishment of *T. colubriformis.* Similarly, Chartier et al. (2000) described Cu oxide needles to be effective against *H. contortus* in goats but saw no effect on *T. colubriformis.* Burke, Orlik, Miller, Terrill, and Mosjidis (2010) observed some reduction on the number of *T. colubriformis* eggs excreted by ewes treated with COWP but reported a greater effect on *H. contortus*. Further, given the worm establishment in the INF group was similar to data presented by Greer et al. (2005), who used a similar infection regime, it appears unlikely that the excess Cu in the feed reduced the worm population in the present study. However, it remains possible that the pathogenicity of the worms could have been affected by the Cu levels given that the carcass composition showed no reductions in tissue deposition or energy utilisation as a consequence of infection which indicates that the nematodes did not cause metabolic distress. In contrast to Greer et al. (2014), who showed enhanced performance in lambs with an increase of 2.03 kg carcass weight in antigen treated lambs, there was no difference in carcass composition between the antigen treated lambs and their non-antigen treated counterparts in the present study.

There were some unexpected inconsistensies in the serum results in the present study which reduced the clarity of the results. While the high L3 IgA pre-treatment could have indicated an initial response to the antigen injections, this is contradicted by the elevated serum IgA concentrations in the untreated INF group. Although the response of serum L3 IgA in the CON group was greater than expected, elevated IgA levels have been seen in control animals in pen studies. It may also be related to the Cu toxicity as rises in serum IgA concentrations have been noted in patients with liver damage (Woof & Kerr, 2006). Furthermore, it is unknown why the serum L3 and L5 IgG concentrations decreased towards the end of the study whereas the L4 IgG and L4 IgE remained steady but with greater initial concentrations in the infected animals despite not being exposed to nematode challenge at this stage. The declining serum P and serum albumin concentrations in CON were also surprising. Considering the poor condition of the lambs at delivery from the sheep milking facility it is possible that this has confounded some of the results and the outcome of the study.

In summary, the present study demonstrates that the antigen treatment reduced the worm establishment and worm length with the greatest effect in the lambs injected with the antigen combination. Moreover, there was evidence that the treatment induced a Th2 response and boosted the immunity in the antigen treated lambs rather than induced tolerance to infection with *T. colubriformis*.

# Chapter 4
# Different delivery routes for *Trichostrongylus colubriformis* antigen

## 4.1 Introduction

The development of immunity against gastrointestinal nematodes is considered to have nutritional costs for the host animal and the majority of production losses associated with temperate nematode infections in lambs are attributed to the immune system (Greer et al., 2008; Greer et al., 2005). The immune response against *T. colubriformis* infections has been reported to be responsible for up to 75% of the total cost of infection (Dever et al., 2016) with the acquisition phase of immunity being most costly since it is thought to have a higher priority than growth in young naïve lambs. It is associated with reduced feed intake and nutrient imbalance due to a redirection of nutrients away from production tissues towards the liver and gastrointestinal tract for the production of acute phase proteins (Colditz, 2002). Immunosuppression of sheep with corticosteroids have been shown to reduce the production losses associated with *T. colubriformis* infections (Dever et al., 2016; Greer et al., 2005). Therefore, sheep that do not mount an immune response when challenged with non-haematophagic nematodes are suggested to have a greater productivity than animals that develop immunity.

Vaccines that enhance the immune-mediated resistance of the host have previously been investigated as an alternative strategy for control of gastrointestinal nematode infections (Douch, 1989; McClure, 2009; Windon, Dineen, Gregg, Griffiths, & Donald, 1984). Most vaccines available on the market are administered via the systemic route, i.e. intramuscular or subcutaneous injection and are strong inducers of systemic immune responses with production of serum antibodies but often induce weak mucosal immunity and only few mucosal vaccines have been commercially available (McGhee et al., 1992; Mestecky, 1987). Mucosal tolerance is a local and systemic immune unresponsiveness induced by administration of antigens to mucosal surfaces without adjuvants. While little is known about mucosal tolerance in ruminants, delivery of nematode antigen via the subcutaneous route has been effective at reducing FEC and worm burdens in sheep with various effect on antibody production (Nisbet et al., 2013; Wedrychowicz et al., 1995). There are yet a number of challenges to overcome for viable production of nematode immunogens, including determining the optimal administration route which takes into account effectivity and practicality. This study investigated the intra-rectal and subcutaneous delivery routes of a somatic *T. colubriformis* antigen for induction of mucosal tolerance or immunity in lambs, bearing in mind the practical limitations when and if used on farm.

## 4.2 Methods and Materials

The study was carried out at Johnstone Memorial Laboratory (JML), Lincoln University, with approval from and in accordance with Lincoln University Animal Ethics Committee LUAEC# 2017-14. Thirty-five Coopworth lambs were born and raised on lucerne to minimise larval exposure before being housed indoors in individual pens at four months of age to investigate two different administration routes, the intra-rectal and subcutaneous routes, of a somatic nematode antigen cocktail previously used in the trial described in Chapter 3. At housing (day -28), the lambs were drenched with 1 ml 5 kg live weight$^{-1}$ Trio®Sheep combination drench (1.0 g l$^{-1}$ abamectin, 40.0 g l$^{-1}$ levamisole hydrochloride, 25.0 g l$^{-1}$ albendazole, Ravensdown New Zealand Ltd, New Zealand), fitted with electronic ear tags (Allflex, New Zealand) and allocated hierarchically by sex and live weight (mean 35.73 ± 1.32 kg) into one of five treatment groups (n=7).

### 4.2.1 Treatment

Thirty-five lambs were allocated into one of five groups; RE (injected with antigen intra-rectally), SC (injected with antigen subcutaneously), IS (injected with corticosteroids), INF (infected) and CON (non-infected control). Two groups; RE and SC, were injected with a somatic antigen combination on days -21, -14 and -7. The amount of protein in each dose was identical to previous indoor studies for each fraction where a positive response had been observed and that used in Chapter 3, viz, 7.5 µg of L3 somatic antigen; 5.5 µg of L4 somatic antigen; 10.5 µg of L5 somatic antigen. Doses were contained in 200 µl PBS and injected either subcutaneously (SC group) in the neck area or into the rectal submucosa (RE group) with the use of a speculum as mentioned in Chapter 3. The remaining groups; IS, INF and CON were injected with 200 µl PBS to the rectal submucosa. When injecting intra-rectally, care was taken to ensure a blister was visually observed at the site of injection reflecting administration of the antigen cocktail correctly into the rectal submucosa. Antigen was prepared prior to the trial by infecting sheep with L3 *T. colubriformis* larvae and retrieving L4 and L5 larvae on day 10 and day 21 post infection as described in Chapter 3. One group (IS) was injected intramuscularly with the immunosuppressant methylprednisolone acetate 40 mg ml$^{-1}$ (Depo-Medrol, Zoetis New Zealand Ltd, New Zealand) once a week between day 0 and day 42 at a dose of 1 ml 30 kg live weight$^{-1}$. Four groups; RE, SC, IS and INF were challenged with 21,000 *T. colubriformis* L3 larvae week$^{-1}$, administered three times a week from day 0 until the end of the trial. Larvae were distributed onto filter paper which was rolled and administered orally by the use of a balling gun. The INF group was the control group for the level of infection and CON was the positive growth rate control and the only non-infected group.

Table 4.1: Experimental design.

| Group (n=7) | Treatment | Dose rate | Challenge from day 0 |
|---|---|---|---|
| RE | Intra-rectal *Tcol*-L3/L4/L5 | 3x on day -21, - 14, -7 | 21,000 *T. col* L3 week$^{-1}$ * |
| SC | Subcutaneous *Tcol*-L3/L4/L5 | 3x on day -21, - 14, -7 | 21,000 *T. col* L3 week$^{-1}$ * |
| IS | Immunosuppressant + 200 µl PBS | 1x week$^{-1}$ from day 0-35 | 21,000 *T. col* L3 week$^{-1}$ * |
| INF | Intra-rectal 200 µl PBS | NA | 21,000 *T. col* L3 week$^{-1}$ * |
| CON | Intra-rectal 200 µl PBS | NA | No challenge |

*administered in split doses three times week$^{-1}$

### 4.2.2 Feed

Animals had *ad libitum* access to fresh water and were offered *ad libitum* feed daily consisting of lucerne pellets containing 10.26 MJME kgDM$^{-1}$ and 214 g CP kgDM$^{-1}$ and *ad libitum* access to a salt lick. Individual feed refusals were collected and weighed weekly. Subsamples of the refusals were collected and dried at 90 °C for seven days for determination of dry matter (DM) percentage.

### 4.2.3 Live weight

Live weights were recorded weekly from day -21 using a Prattley weigh crate fitted with Tru-Test load bars. Animal identification was recorded using an Edit Display Wand and Tru-Test XR3000 (Tru-Test Ltd, New Zealand) with a sensitivity of 0.2 kg.

### 4.2.4 Parasitology

#### 4.2.4.1 Faecal egg count

Faecal samples were collected weekly directly from the rectum of the lambs starting on day -21. Determination of faecal egg counts (FEC) was performed using a modified McMaster method as described in Chapter 3. Each egg counted was equivalent to 100 eggs gram$^{-1}$ of faeces (epg).

#### 4.2.4.2 Total worm burden

Total worm burden analysis was performed on the small intestine from all animals at the end of the trial. Slaughter was performed by stunning the animal with a captive bolt gun and severing the carotid arteries and jugular veins. The procedures for total worm counts and worm length are described in Chapter 3.

### 4.2.5 Blood samples

Blood samples were collected weekly from all animals by jugular venipuncture using 2 x 10 ml lithium heparin and non-heparinised vacutubes (Becton Dickinson Vacutainer®, Belliver Industrial Estate, Plymouth, UK) starting on day -21. The non-heparinised tubes were set to coagulate overnight and then centrifuged at 1,200 x g for 10 minutes at 4 ˚C. Serum was collected in 1.2 ml tubes and stored at -20 °C until examined for IgA, IgG and IgE antibodies by ELISA. The heparinised blood samples were prepared immediately for leukocyte stimulation assays. The techniques for antibody determination and leukocyte stimulation assays are described in Chapter 3.

Serum albumin, total protein, urea and P concentrations were analysed by colourimetric assays on a Randox Rx Daytona analyser (Randox Headquarters Co. Antrim, UK) with concentrations determined photometrically as described in Chapter 3.

### 4.2.6 Body composition

To determine changes in the tissue deposition that may result from treatment, body composition at day -2 and day 45 of infection was assessed using computed tomography following the same procedure as described in Chapter 3.

### 4.2.7 Statistical analysis

Statistical analyses were carried out using the GENSTAT statistical package (GENSTAT 2018. GenStat Eighteenth edition, VSN International Limited, United Kingdom). Blood analyses, live weight and FEC were analysed for repeated measures by REML with treatment groups and time included as factors and with missing values estimated. Liveweight gain, feed intake, feed conversion efficiency, worm length and worm burdens were analysed separately for each sampling time by a one way ANOVA using Minitab ®18 Statistical Software (Minitab Inc. Eighteenth edition, USA). FEC and worm burdens were log-transformed (log10 (count+10)) before analysis and uninfected control animals were excluded from the analysis. $P \leq 0.05$ was regarded as significant and all values are group means and expressed as mean ± SEM unless otherwise specified.

## 4.3 Results

### 4.3.1 Clinical observations

Six animals (three INF, one IS and one RE) were treated for footrot on days 1 and 2 with Dicholorophen Footrot Aerosol (GEA FIL, New Zealand). One lamb in CON suffered from epididymitis between day 13 and day 22 and was treated with 1 ml 35 $kg^{-1}$ Rimadyl® (carprofen 50 mg $ml^{-1}$, Zoetis UK Ltd, UK) and 7 mg $kg^{-1}$ Betamox (amoxicillin 150 mg $ml^{-1}$ Norbrook Laboratories Ltd, UK) daily from day 13 to day 16. Data from this animal was included in the statistical analysis using missing value estimates from sampling days 14 and 21. There were no clinical signs of parasitism observed in any animals throughout the study.

### 4.3.2 Live weight

Mean live weight (kg) is given in Figure 4.1. There was an effect of time (P<0.001) reflecting increasing live weights in all groups over time. There was no effect of treatment (P=0.564) nor interaction between treatment and time (P=0.139).

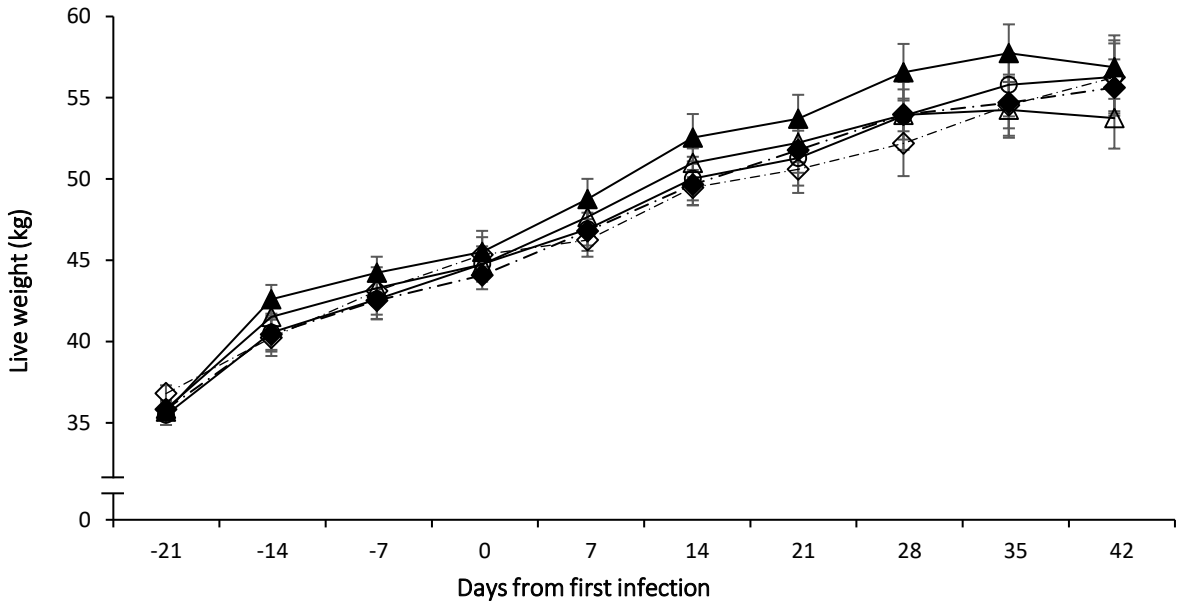


Figure 4.1: Mean live weights (± SEM) of lambs infected with 21,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected intra-rectally (RE, solid line open triangle) or subcutaneously (SC, solid line closed triangle) with *Tcol*-L3/L4/L5, infected and immunosuppressed (IS, solid line open circle), and non-infected controls (CON, dashed line closed diamond).

### 4.3.3 Liveweight gain

Mean liveweight gain (g day$^{-1}$) is given in Figure 4.2. There was no difference in liveweight gain between treatment groups pre-infection (day -21 to day 0, P=0.862), nor during the first 21 days of infection (day 0 to 21, P=0.308). However, there was a difference in liveweight gain between RE and INF the last 21 days of infection (day 21 to 42, P<0.05 Tukey pairwise comparisons).

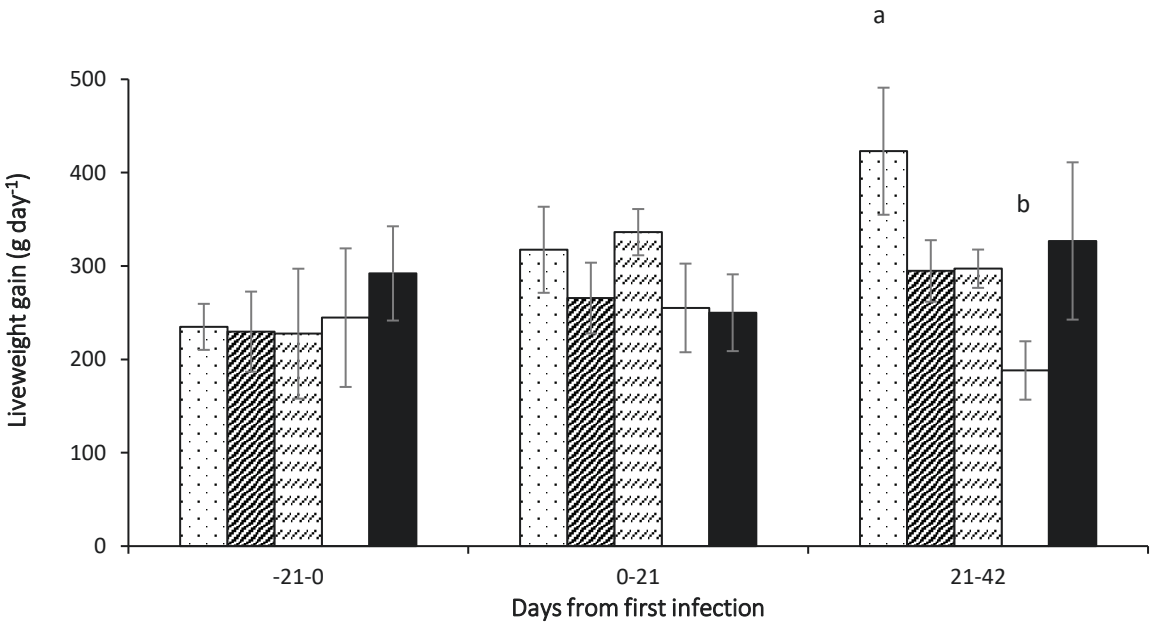


Figure 4.2: Mean liveweight gain (± SEM) of lambs infected with 21,000 *T. colubriformis* week$^{-1}$ (INF ▭), infected and injected intra-rectally (RE ⁘) or subcutaneously (SC ▨) with *Tcol*-L3/L4/L5, infected and immuno-suppressed (IS ▨), and non-infected controls (CON ■). Means with different superscript are significantly different (P<0.05).

### 4.3.4 Feed intake

Mean voluntary feed intake (kgDM day$^{-1}$) is given in Figure 4.3. There was no difference in mean voluntary feed intake between treatment groups pre-infection (day -21 to day 0, P=0.800) nor during the first 21 days of infection (day 0 to 21, P=0.482), but there was a difference in feed intake during last 21 days of infection (day 21 to 42, P<0.05 Tukey pairwise comparisons) reflecting lower intake in INF compared with RE.

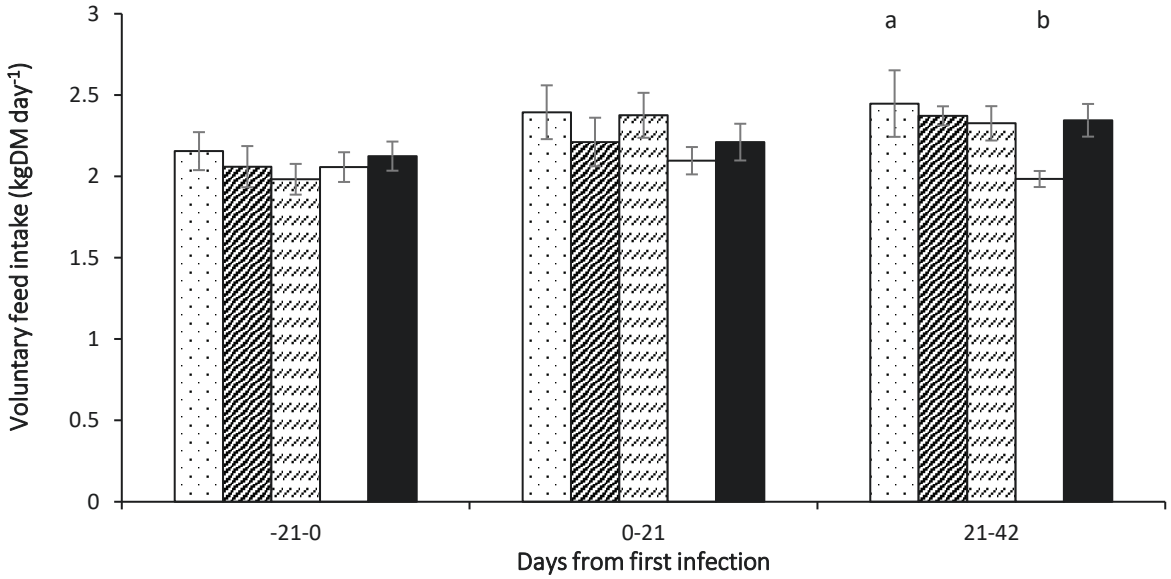


Figure 4.3: Mean voluntary feed intake (± SEM) of lambs infected with 21,000 *T. colubriformis* week$^{-1}$ (INF ), infected and injected intra-rectally (RE ) or subcutaneously (SC ) with *Tcol*-L3/L4/L5, infected and immunosuppressed (IS ), and non-infected controls (CON ). Means with different superscript are significantly different (P<0.05).

### 4.3.5 Feed conversion efficiency

Mean feed conversion efficiency (FCE, g live weight gained $kgDM^{-1}$ of feed consumed), is displayed in Figure 4.4. There was no difference in FCE between treatment groups during pre-infection (P=0.931) nor during the first 21 days of infection (P=0.301). However, the FCE differed between the RE and INF groups post-infection (P<0.05 Tukey pairwise comparisons) with the greatest FCE in the RE group (178 ± 30 g $kgDM^{-1}$) and the lowest FCE in INF (82 ± 22 g $kgDM^{-1}$).

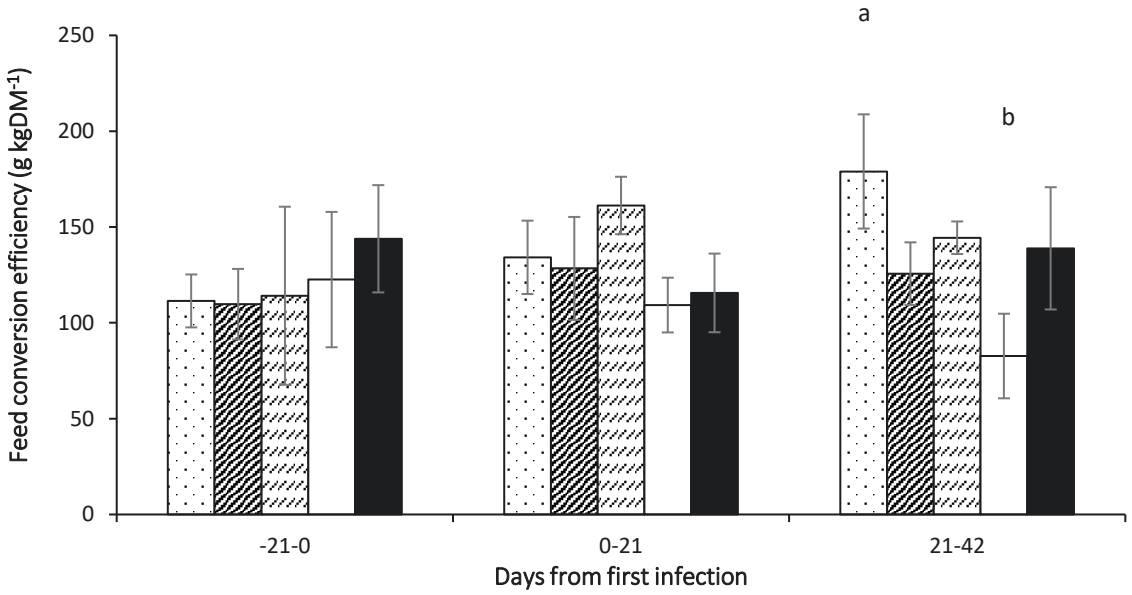


Figure 4.4: Mean feed conversion efficiency (± SEM) of lambs infected with 21,000 *T. colubriformis* $week^{-1}$ (INF □), infected and injected intrarectally (RE ⁘) or subcutaneously (SC ▨) with *Tcol*-L3/L4/L5, infected and immunosuppressed (IS ▨), and non-infected controls (CON ■). Means with different superscript are significantly different (P<0.05).

### 4.3.6 Carcass composition

Carcass composition estimated by computed tomography on day -2 and day 45 is given in Table 4.2. There was no difference in carcass weight gain (P=0.803), bone deposition (P=0.389), fat deposition (P=0.240) and lean tissue deposition (P=0.116) between the treatments. Similarly, there was no difference in total ME intake (P=0.568), total NE stored (P=0.394) and NE: ME ratio (P=0.435) between treatments.

Table 4.2: Carcass composition (± SEM) estimated by computed tomography and energy utilisation of lambs infected with 21,000 *T. colubriformis* week$^{-1}$ (INF), infected and injected intra-rectally (RE) or subcutaneously (SC) with *Tcol*-L3/L4/L5 antigen, infected and immunosuppressed (IS), and non-infected controls (CON). There was no significant difference between the treatment groups (P>0.05).

| | Treatment group (n=7) | | | | |
|---|---|---|---|---|---|
| | RE | SC | IS | INF | CON |
| **Initial body composition, day -2** | | | | | |
| Live weight (kg) | 44.74 ± 0.81 | 45.51 ± 0.71 | 44.77 ± 0.99 | 44.08 ± 1.03 | 44.34 ± 0.77 |
| Carcass weight (kg) | 14.86 ± 0.46 | 15.27 ± 0.26 | 14.69 ± 0.23 | 14.78 ± 0.30 | 14.51 ± 0.50 |
| Bone (kg) | 1.78 ± 0.24 | 1.73 ± 0.24 | 1.65 ± 0.26 | 1.64 ± 0.16 | 1.72 ± 0.28 |
| Fat (kg) | 3.35 ± 0.30 | 3.13 ± 0.23 | 3.09 ± 0.24 | 3.45 ± 0.26 | 3.06 ± 0.44 |
| Lean tissue (kg) | 9.73 ± 0.18 | 10.41 ± 0.05 | 9.95 ± 0.06 | 9.69 ± 0.04 | 9.73 ± 0.08 |
| **Tissue deposition, day 45** | | | | | |
| Liveweight gain (kg) | 21.23 ± 0.93 | 17.91 ± 0.99 | 20.82 ± 1.05 | 19.81 ± 1.02 | 20.86 ± 1.05 |
| Carcass weight gain (kg) | 7.19 ± 0.89 | 6.80 ± 0.76 | 6.63 ± 0.76 | 7.12 ± 1.22 | 8.07 ± 0.61 |
| Bone (kg) | 0.41 ± 0.15 | 0.49 ± 0.10 | 0.33 ± 0.10 | 0.54 ± 0.12 | 0.65 ± 0.11 |
| Fat (kg) | 3.33 ± 0.01 | 3.09 ± 0.01 | 4.22 ± 0.01 | 3.38 ± 0.02 | 3.44 ± 0.01 |
| Lean tissue (kg) | 3.44 ± 0.56 | 3.22 ± 0.47 | 2.08 ± 0.44 | 3.20 ± 0.57 | 3.97 ± 0.40 |
| **Energy utilisation** | | | | | |
| Total ME intake (MJ) | 1,366 ± 44 | 1,484 ± 67 | 1,362 ± 41 | 1,306 ± 45 | 1,442 ± 115 |
| Total NE stored (MJ) | 149 ± 13 | 139 ± 12 | 177 ± 145 | 151 ± 26 | 158 ± 10 |
| NE: ME | 0.09 ± 0.01 | 0.11 ± 0.01 | 0.13 ± 0.01 | 0.12 ± 0.02 | 0.11 ± 0.01 |

## 4.3.7 Parasitology

### 4.3.7.1 Faecal egg count

Mean back-transformed (log 10 (count +10)) faecal egg counts (FEC, epg) are given in Figure 4.5. There was an interaction between treatment and time (P<0.001) with increasing FEC in all groups from day 14 to day 35 whereafter FEC declined but was higher in IS from day 35-42.

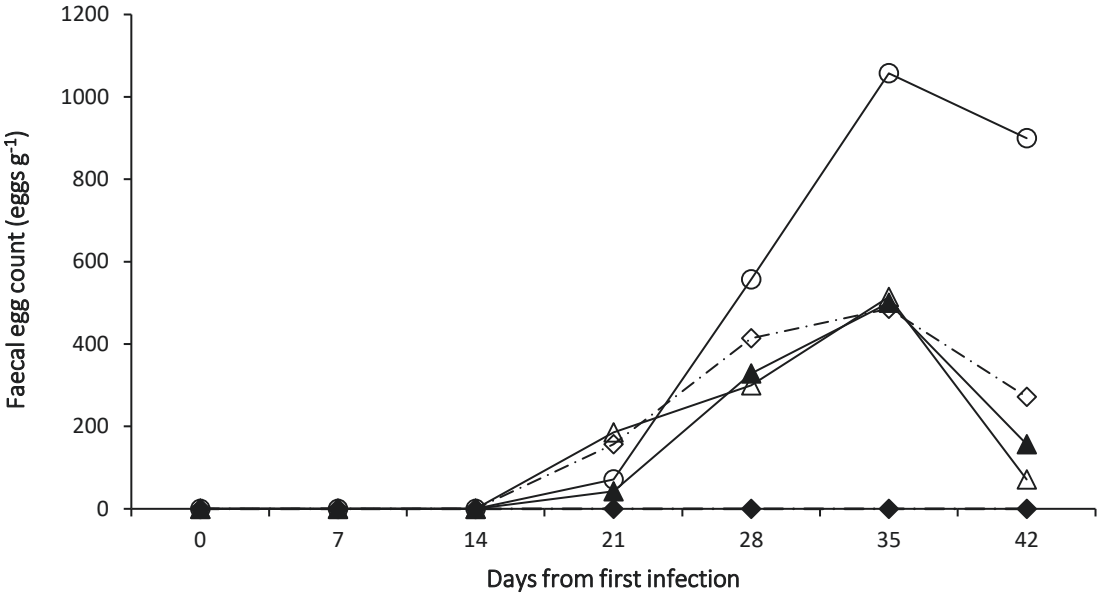


Figure 4.5: Mean back-transformed (log 10 (count +10)) FEC of lambs infected with 21,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected intra-rectally (RE, solid line open triangle) or subcutaneously (SC, solid line closed triangle) with *Tcol*-L3/L4/L5, infected and immunosuppressed (IS, solid line open circle), and non-infected controls (CON, dashed line closed diamond).

### 4.3.7.2 Worm burden

Small intestinal worm counts and worm length are given in Table 4.3. Total worm burden was lowest in the CON group (0) compared with the infected groups. Within the infected animals, IS had the highest worm burden (39,514) and RE the lowest (20,330) (P<0.001). Adult worms contributed 89%, 91%, 93% and 94% of the total worm burden for RE, SC, IS and INF, respectively.

Female worm length differed between the infected treatment groups (P<0.001) being longest in INF (8.20 ± 0.09 mm) and shortest in RE (6.62 ± 0.08 mm). The female worms in RE were 20% shorter than in the INF group.

Table 4.3: Mean log transformed (log 10 (count+10)) small intestinal worm burden and worm length (mm) (± SEM) of lambs infected with 21,000 *T. colubriformis* larvae week$^{-1}$ (INF), infected and injected intra-rectally (RE) or subcutaneously (SC) with *Tcol*-L3/L4/L5, infected and immunosuppressed (IS), and non-infected controls (CON). Back-transformed values are given in parenthesis. Means with different superscript are significantly different (P<0.05).

| | Treatment group | | | | |
|---|---|---|---|---|---|
| Larval stage | RE | SC | IS | INF | CON |
| L3 | 1.31 ± 0.20$^{a}$ (10) | 1.15 ± 0.15$^{a}$ (4) | 1.91 ± 0.25$^{a}$ (71) | 1.19 ± 0.19$^{a}$ (6) | 1 ± 0 (0) |
| L4 | 3.08 ± 0.18$^{a}$ (1,206) | 3.20 ± 0.15$^{a}$ (1,575) | 3.28 ± 0.16$^{a}$ (1,888) | 3.20 ± 0.10$^{a}$ (1,579) | 1 ± 0 (0) |
| L5/ adult | 4.26 ± 0.06$^{a}$ (18,158) | 4.41 ± 0.05$^{ab}$ (25,551) | 4.57 ± 0.05$^{b}$ (36,938) | 4.51 ± 0.05$^{b}$ (32,194) | 1 ± 0 (0) |
| Total | 4.31 ± 0.04$^{a}$ (20,330) | 4.45 ± 0.04$^{ab}$ (27,940) | 4.60 ± 0.05$^{b}$ (39,514) | 4.54 ± 0.05$^{b}$ (34,320) | 1 ± 0 (0) |
| Female worm length | 6.62 ± 0.08$^{a}$ | 7.05 ± 0.09$^{b}$ | 8.03 ± 0.08$^{c}$ | 8.20 ± 0.09$^{c}$ | |
| Male worm length | 5.56 ± 0.06$^{a}$ | 5.52 ± 0.08$^{a}$ | 5.66 ± 0.08$^{a}$ | 5.65 ± 0.07$^{a}$ | |

## 4.3.8 Serum antibodies

### 4.3.8.1 IgA

Mean L3 *T. colubriformis*-specific IgA responses (OD) are given in Figure 4.6. There was a treatment x time interaction (P<0.001) reflecting increasing greater IgA production in all infected groups with greater absorbance in RE from day 21 and lower absorbance in CON.

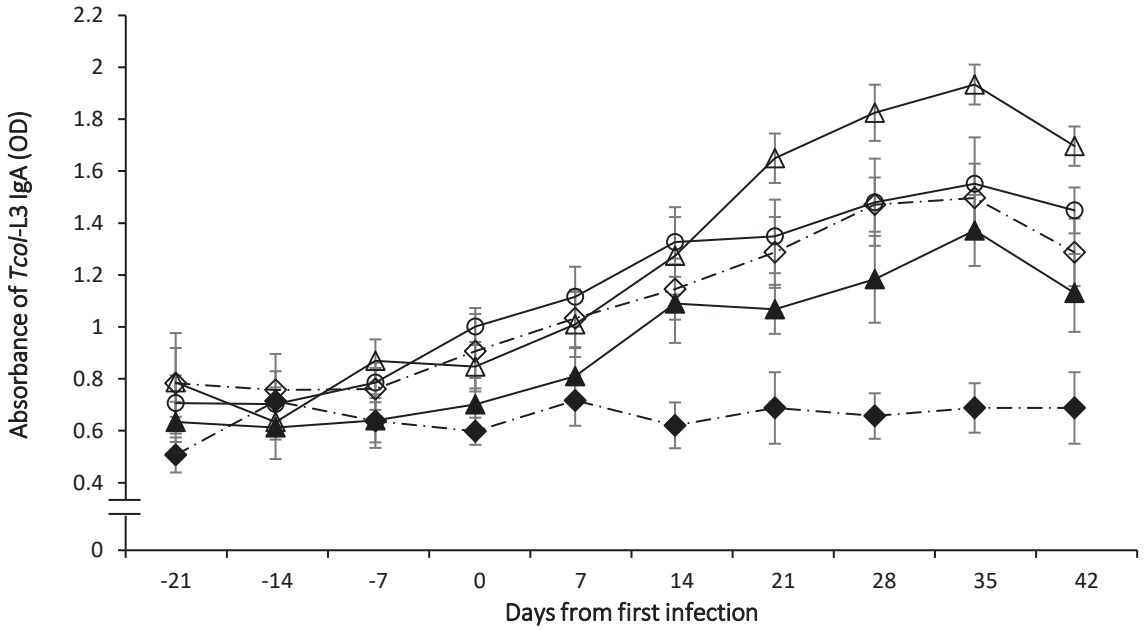


Figure 4.6: Mean absorbance (± SEM) of L3 *T. colubriformis* IgA antibody levels for lambs infected with 21,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected intra-rectally (RE, solid line open triangle) or subcutaneously (SC, solid line closed triangle) with *Tcol*-L3/L4/L5, infected and immunosuppressed (IS, solid line open circle), and non-infected controls (CON, dashed line closed diamond).

### 4.3.8.2 IgG

Mean L3 *T. colubriformis*-specific IgG responses (OD) are given in Figure 4.7. Overall, there was a treatment x time interaction (P<0.001) showing similar IgG profiles that increased with time in the antigen treated and infected lambs with lower absorbance in CON and IS.

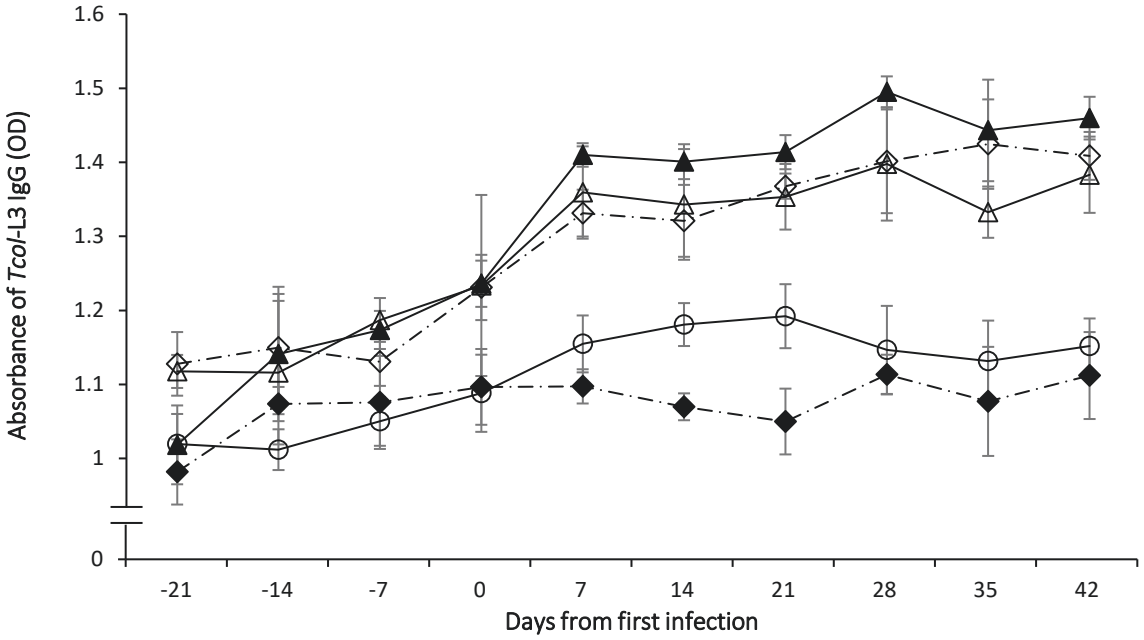


Figure 4.7: Mean absorbance (± SEM) of L3 *T. colubriformis* IgG antibody levels for lambs infected with 21,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected intra-rectally (RE, solid line open triangle) or subcutaneously (SC, solid line closed triangle) with *Tcol*-L3/L4/L5, infected and immunosuppressed (IS, solid line open circle), and non-infected controls (CON, dashed line closed diamond).

#### 4.3.8.3 IgE

Mean L5 *T. colubriformis*-specific IgE responses (OD) are given in Figure 4.8. There was an interaction between treatment and time (P=0.019) reflecting increasing absorbance in RE, SC and INF followed by a decrease on day 42 when IgE levels in all groups rapidly declined. CON and IS antibody responses remained lower than the infected groups.

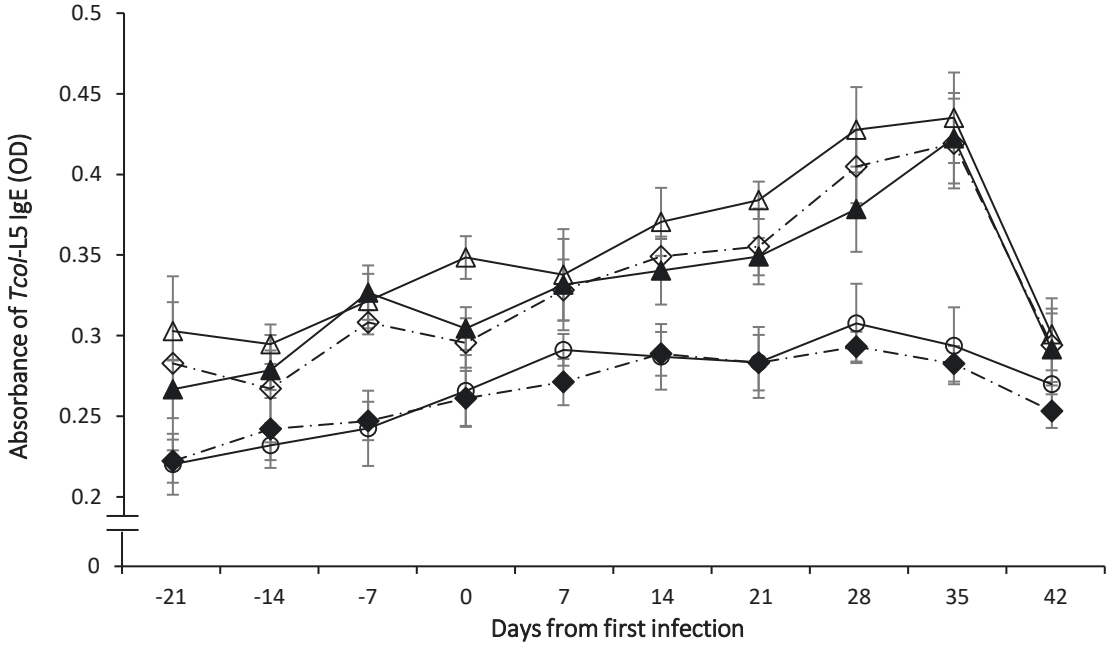


Figure 4.8: Mean absorbance (± SEM) of L5 *T. colubriformis* IgE antibody levels for lambs infected with 21,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected intra-rectally (RE, solid line open triangle) or subcutaneously (SC, solid line closed triangle) with *Tcol*-L3/L4/L5, infected and immunosuppressed (IS, solid line open circle), and non-infected controls (CON, dashed line closed diamond).

## 4.3.9 Cytokine production

### 4.3.9.1 IL-4

Mean concentration of IL-4 (pg $ml^{-1}$) produced by WBC when stimulated with *T. colubriformis* L3/L4/L5 antigen is given in Figure 4.9. There was treatment x time interaction (P<0.001) reflecting increasing IL-4 concentrations in all infected groups, except IS, peaking on day 35 and being greater in RE. The IL-4 concentration in CON and IS remained low throughout the study.

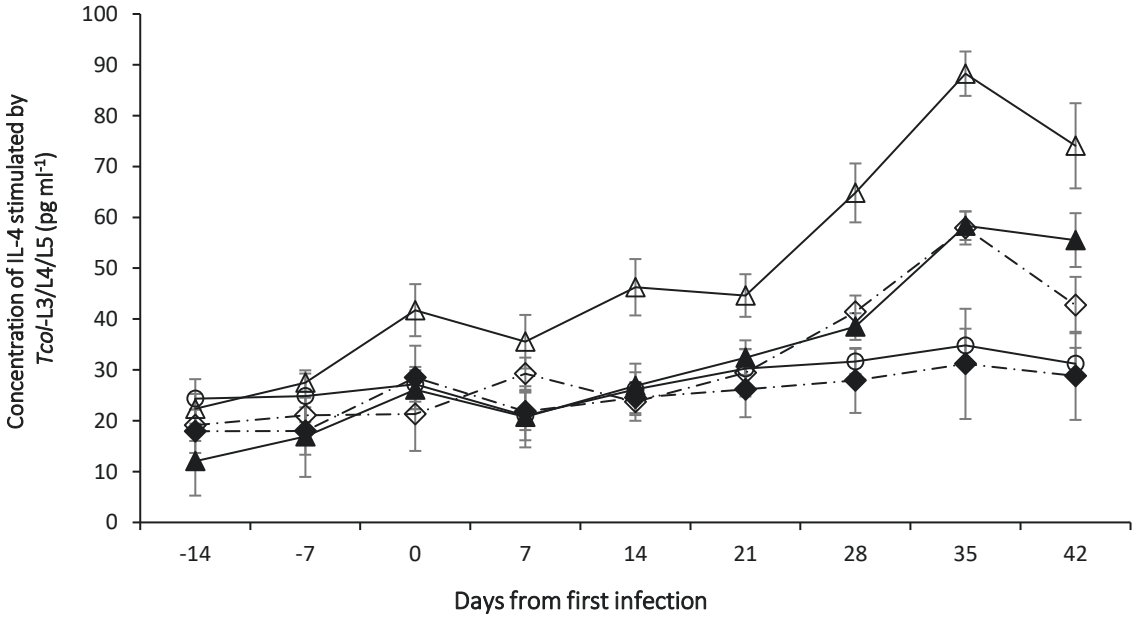


Figure 4.9: Mean IL-4 concentration (± SEM) produced by WBC stimulated with *T. colubriformis* L3/L4/L5 antigen for lambs infected with 21,000 *T. colubriformis* $week^{-1}$ (INF, dashed line open diamond), infected and injected intra-rectally (RE, solid line open triangle) or subcutaneously (SC, solid line closed triangle) with *Tcol*-L3/L4/L5, infected and immunosuppressed (IS, solid line open circle), and non-infected controls (CON, dashed line closed diamond).

### 4.3.9.2 IFN-γ

Mean concentration of IFN-γ (pg $ml^{-1}$) produced by WBC when stimulated with *T. colubriformis* L3/L4/L5 antigen is given in Figure 4.10. The IFN-γ concentration increased with time (P<0.001) and was lower in IS (P=0.048). There was no interaction between treatment and time (P=1.000).

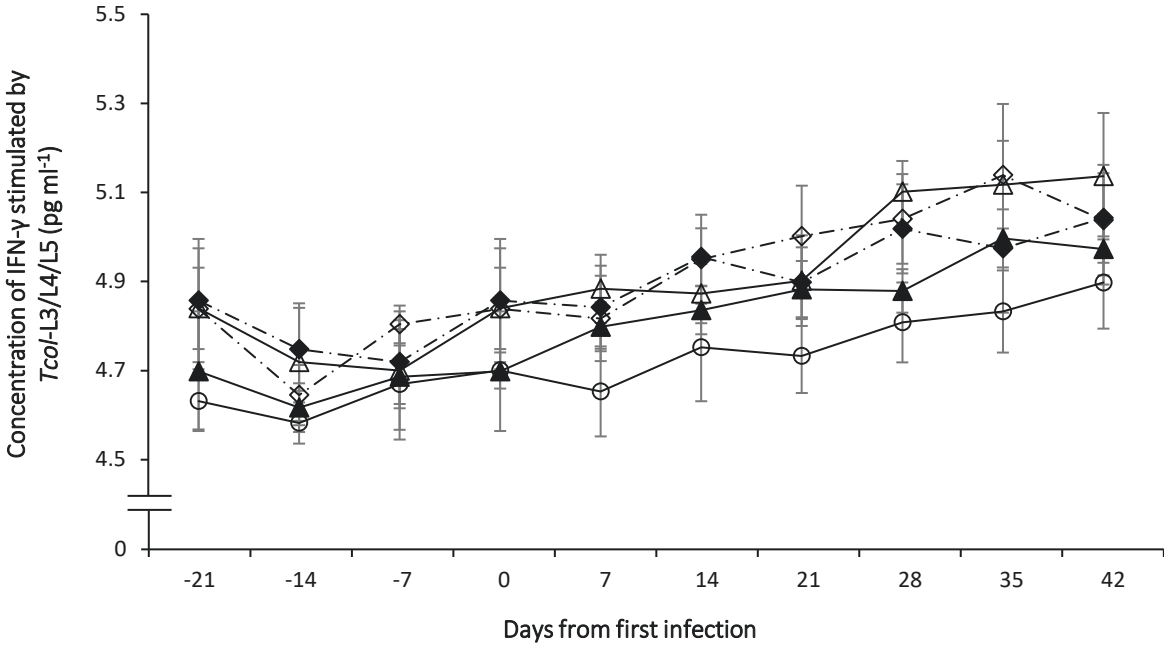


Figure 4.10: Mean IFN-γ concentration (± SEM) produced by WBC stimulated with *T. colubriformis* L3/L4/L5 antigen for lambs infected with 21,000 *T. colubriformis* $week^{-1}$ (INF, dashed line open diamond), infected and injected intra-rectally (RE, solid line open triangle) or subcutaneously (SC, solid line closed triangle) with *Tcol*-L3/L4/L5, infected and immunosuppressed (IS, solid line open circle), and non-infected controls (CON, dashed line closed diamond).

## 4.3.10 Serum phosphorous

Mean serum phosphorous (P) concentrations (mmol $l^{-1}$) are given Figure 4.11. Overall, there was an effect of time (P<0.001) reflecting decreasing serum P concentrations in all groups. There was no effect of treatment (P=0.188) nor a treatment x time interaction (P=0.416).

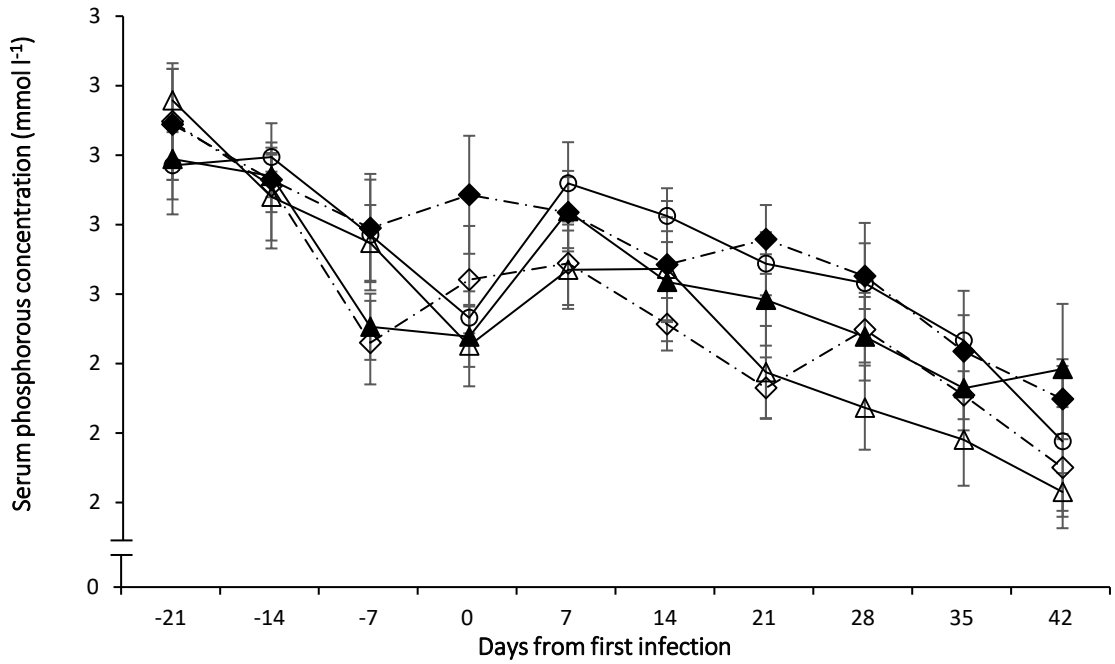


Figure 4.11: Mean serum P concentrations (± SEM) for lambs infected with 21,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected intra-rectally (RE, solid line open triangle) or subcutaneously (SC, solid line closed triangle) with *Tcol*-L3/L4/L5, infected and immunosuppressed (IS, solid line open circle), and non-infected controls (CON, dashed line closed diamond).

## 4.3.11 Serum urea

Mean serum urea concentrations (mmol $l^{-1}$) are given in Figure 4.12. There was an effect of time ($P<0.001$) reflecting initially decreasing serum urea concentrations in all groups followed by plateauing concentrations. There was no difference in concentrations between the groups ($P=0.125$) nor a treatment x time interaction ($P=0.362$).

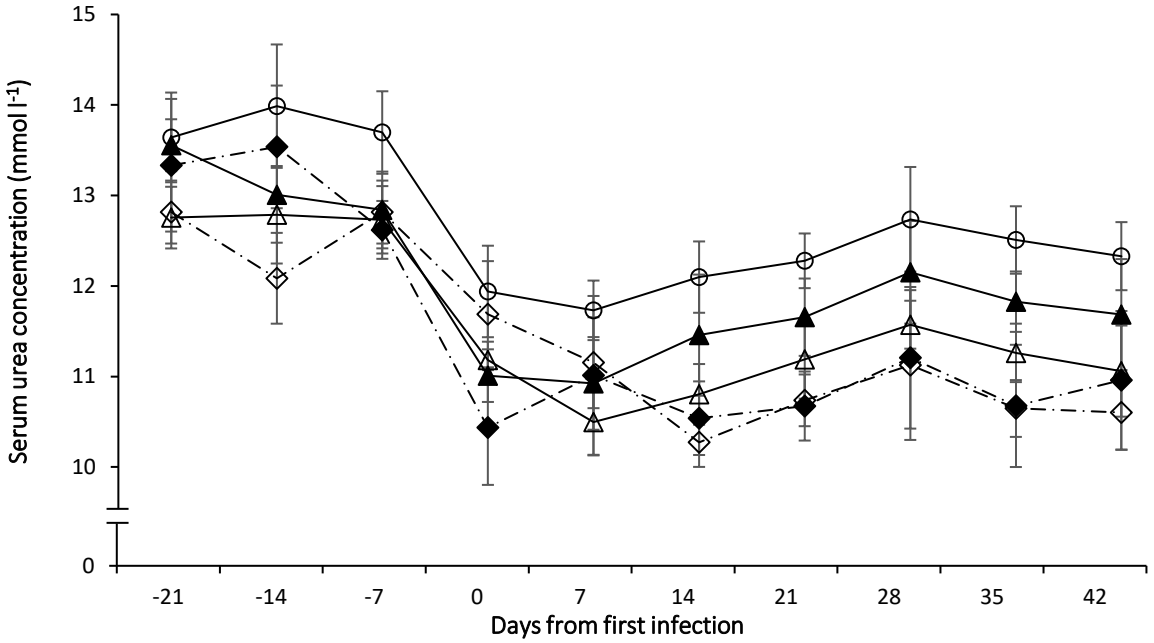


Figure 4.12: Mean serum urea concentrations (± SEM) for lambs infected with 21,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected intra-rectally (RE, solid line open triangle) or subcutaneously (SC, solid line closed triangle) with *Tcol*-L3/L4/L5, infected and immunosuppressed (IS, solid line open circle), and non-infected controls (CON, dashed line closed diamond).

## 4.3.12 Serum total protein

Mean serum total protein concentrations (g $l^{-1}$) are given in Figure 4.13. There was a tendency for a treatment x time interaction (P=0.058) reflected in lower concentrations in INF from day 21.

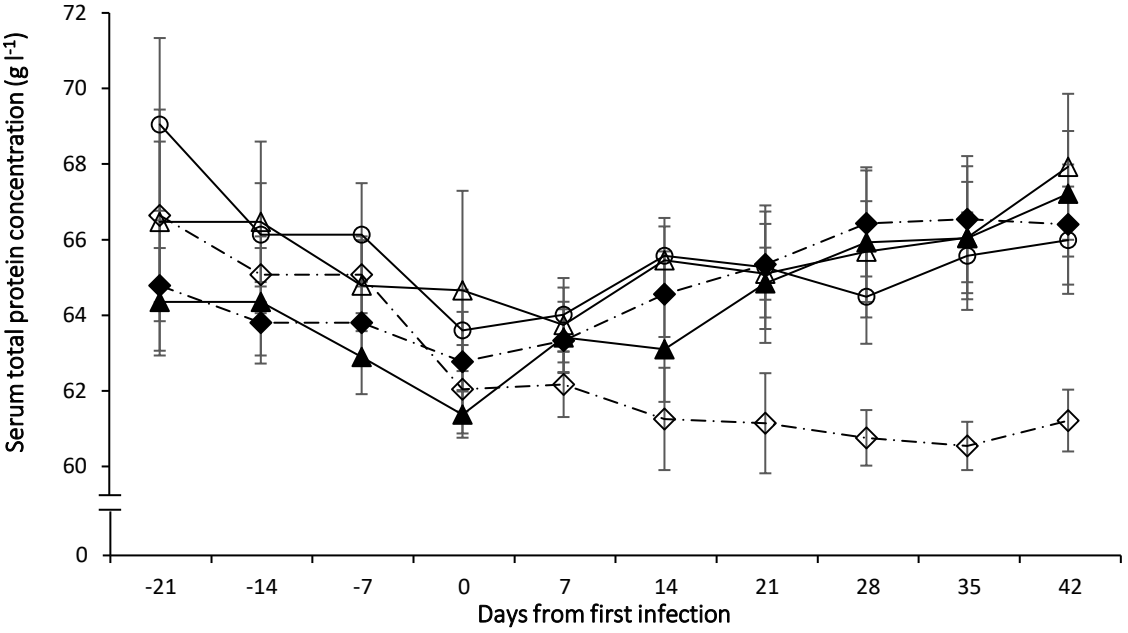


Figure 4.13: Mean serum total protein concentrations (± SEM) for lambs infected with 21,000 *T. colubriformis* $week^{-1}$ (INF, dashed line open diamond), infected and injected intra-rectally (RE, solid line open triangle) or subcutaneously (SC, solid line closed triangle) with *Tcol*-L3/L4/L5, infected and immunosuppressed (IS, solid line open circle), and non-infected controls (CON, dashed line closed diamond).

## 4.3.13 Serum albumin

Mean serum albumin concentrations (g $l^{-1}$) are shown in Figure 4.14. There was a treatment x time interaction (P=0.045) reflecting similar initial levels but a decrease in only INF from day 21 onwards.

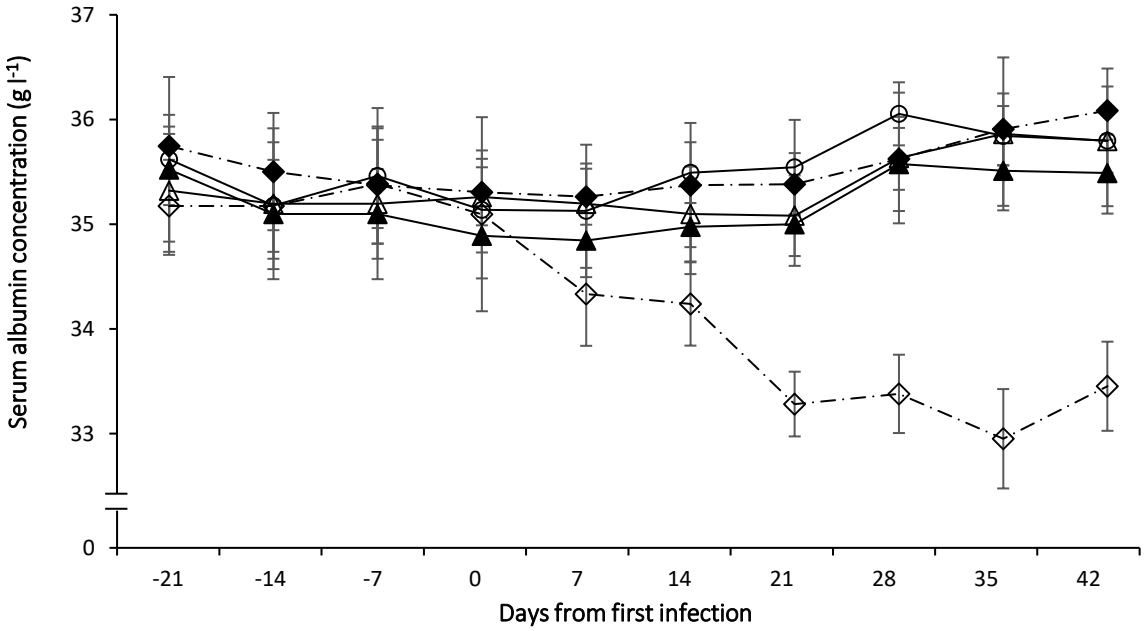


Figure 4.14: Mean serum albumin concentrations (± SEM) for lambs infected with 21,000 *T. colubriformis* week$^{-1}$ (INF, dashed line open diamond), infected and injected intra-rectally (RE, solid line open triangle) or subcutaneously (SC, solid line closed triangle) with *Tcol*-L3/L4/L5, infected and immunosuppressed (IS, solid line open circle), and non-infected controls (CON, dashed line closed diamond).

## 4.4 Discussion

These findings support the observations outlined in Chapter 3 that repeated antigen injections induced a Th2 response in the lambs which was skewed towards immunity against *T. colubriformis*. Treatment enhanced performance in the intra-rectally injected lambs and reduced worm burden and female worm length with the greatest effect achieved when the antigen was delivered via the intra-rectal route.

The effect of the infection regime in the present study was smaller than anticipated with no apparent difference in performance between untreated infected lambs (INF) and the control group. Despite high FEC and worm burden in the INF group there was a lack of reduction in feed intake, liveweight gain and feed conversion efficiency compared with the controls. Bearing in mind the lack of difference in performance between INF and CON in Chapter 3 the infection rate was increased from 2,000 to 3,000 *T. colubriformis* $day^{-1}$ to enhance the pathogenicity of infection in the present study. In part, this was effective as indicated by the reduction in serum albumin and serum total protein indicating there was some damage to the intestinal mucosa by the parasites. However, this was not sufficient to have a significant effect on performance in the INF group supported by the lack of difference in carcass composition and energy utilisation. In contrast, Bown et al. (1991) reported no difference in plasma nitrogen loss at 42 days post-infection but a fourfold increase in infected lambs at day 84 compared with controls, whereas the albumin reduction occurred around 21 days post-infection in the present study. The worm burden of 34,000 in INF in the present study was considerably greater than the 7,900 reported by Bown et al. (1991) after six weeks with a comparable infection regime. In contrast, these authors observed reductions in feed intake up to 30% in infected lambs compared with only a 16% decrease in feed intake seen in the present study. While Bown et al. (1991) reported the greatest feed reduction between days 42 and 84 post-infection with a worm burden around 39,000 at day 84, it is considered unlikely that a greater effect of the parasites on feed intake or growth would have been observed had the study continued given the indication of returning serum total protein and serum albumin levels and the drop in FEC on day 42 indicating development of immunity. Although serum P concentrations in the present study decreased post-infection, reductions were observed in all groups and were not different between the infected groups and the controls. While reduced absorption and retention of P and reduced plasma P concentrations are features of nematode infections due to villus atrophy in the small intestine caused by the nematodes (Bown et al., 1989; Poppi et al., 1985) this indicates that the reductions were not associated with the nematode infection. This finding is contradictive to those of Wilson and Field (1983) who observed 30% lower absorption of exogenous P

and increased losses of endogenous P following infection with *T. colubriformis* at the same dose rate of 3,000 L3 larvae day$^{-1}$ and possibly indicate a dietary insufficiency in the current study.

It appears that the antigen treatment improved performance when delivered via the intra-rectal route indicated by the differences in liveweight gain, feed intake and feed conversion efficiency 21-42 days post-infection between the RE and INF groups. It also appeared treatment had an effect on the parasites which was reflected in lower worm burdens and shorter female worms. Furthermore, the lack of reduction in serum albumin and serum total protein concentrations in the antigen treated groups may be a result of less direct damage to the epithelium of the small intestine by the parasites or as a result of immunopathology. It may also be due to a greater ability of the antigen treated animals to cope with the albumin and protein leakage to the gastrointestinal tract or indicate that leakage did not occur since immunopathological changes, such as increased production of acute phase proteins in the liver to facilitate tissue repair, may have been less severe. Many of the amino acids used in the acute phase protein synthesis are derived from muscle degradation mediated by the pro-inflammatory cytokines IL-1, TNF-α, IL-6, which is typical for a developing immune response (Basset et al., 2003; Colditz, 2002; Johnson, 1997, 1998).

The reduced worm burden in the RE group is in agreement with findings of Jacobs et al. (1999) who reported reduced worm burdens in sheep that were infected with *H. contortus,* following intra-rectal injection of 20 µg Hc-sL3 antigen and 250 µg aluminium hydroxide adjuvant, although the reduction was smaller in the present study, i.e. 41% compared with the 67-69% reported by those authors. Similarly, Wedrychowicz et al. (1995) found that 25 µg kg live weight$^{-1}$ ovalbumin or L3 surface proteins of *T. circumcincta* administered with 4 mg kg live weight$^{-1}$ beryllium hydroxide subcutaneously reduced worm burdens by 54% and 72%, respectively, compared with only a 19% reduction in the SC group here. While there was no difference in FEC between antigen treated lambs and controls in the present study, Jacobs et al. (1999) observed decreases in FEC of 52%. Nisbet et al. (2013) immunised lambs with 400 µg of an antigen combination comprising eight recombinant proteins from *T. circumcincta* injected subcutaneously with 10 mg Quil A adjuvant. In two separate trials, these authors described a 75% and 56% lower worm burden in immunised lambs than controls injected with adjuvant only. Moreover, vaccinated lambs had a 70% and 58% mean reduction in egg output compared with unvaccinated counterparts. Unlike the studies by Jacobs et al. (1999), Wedrychowicz et al. (1995) and Nisbet et al. (2013), no adjuvant was used in the present study as the intention was to avoid an immune response, and it cannot be discarded that delivery of the antigen with an adjuvant could potentially have aided further reduction of worm establishment and increased the FEC reduction rate.

The elevated serum IgA and the reduced worm burden and female worm length in RE could be an indication of IgA production in the small intestinal mucosa since local IgA is associated with reduced female fecundity and worm length (Stear et al., 1995; Stear et al., 1999a; Strain et al., 2002) and considering that mucosal immunisation at one site can lead to IgA secretion in other mucosal tissues (Mestecky, 1987). Although serum IgA levels do not necessarily reflect local IgA production (Sinski et al., 1995), these findings are supported by (Greer et al., 2005) who recorded low worm burdens of 224 *T. colubriformis* 77 days post-infection with 2,000 L3 larvae day$^{-1}$ which were associated with elevated serum IgA concentrations in infected lambs. In contrast, (Jacobs et al., 1999) observed no effect of immunisation on worm length despite elevated levels of serum IgA antibodies and an approximate 50% reduction in FEC following intra-rectal immunisation. The 20% reduction in female worm length in RE was comparable with the 22% in COMB in Chapter 3 suggesting that the combination larval antigen is most efficient when administered intra-rectally.

The purpose of the immunosuppressed group was to enable detectable differences in performance between this group and the infected lambs since immunosuppression has been shown to improve production in lambs challenged with *T. colubriformis* (Dever et al., 2016; Greer et al., 2005) and *T. circumcincta* (Greer et al., 2008). The immunosuppressing regime of weekly intramuscular injections with 1.3 mg methylprednisolone acetate kg live weight$^{-1}$ used in the present study has previously been reported to alleviate the reduction in voluntary feed intake in *T. colubriformis*-infected lambs by 30% (Greer et al., 2005). However, no differences in liveweight gain, feed intake or feed conversion efficiency between immunosuppressed and infected lambs were observed in the present study. The lack of difference in carcass composition between the immunosuppressed group and any of the other groups is in agreement with Dever et al. (2016) who found little effect of immunosuppression and infection on carcass composition in grazing lambs that were infected with 2,000 or 4,000 *T. colubriformis* week$^{-1}$. In contrast, Greer et al. (2005) reported 20% lower gross efficiency of use of metabolisable energy for net energy deposition in infected lambs compared with corticosteroid treated counterparts during the acquisition period of immunity. While there was no statistically significant differences in the carcass composition and energy utilisation between the immunosuppressed lambs and the remaining groups in the current study, there was an indication of lower lean tissue deposition which presumably reflects the slightly greater serum urea concentrations and suggests a catabolic effect from the corticosteroid treatment. The lack of reductions in serum total protein and serum albumin concentrations in the immunosuppressed group, despite harbouring a greater worm burden than the INF group, is supported by Greer et al. (2005) and Vaughan et al. (2006) who found that corticosteroid treatment possibly prevented a reduction in plasma albumin concentrations in immunosuppressed lambs through an enhanced ability to replace lost proteins.

While the FEC, serum IgG, IgE and worm burden in the present study indicate successful immunosuppression, there was surprisingly an increase in serum IgA production. The elevated serum IgA in the immunosuppressed lambs along with the unaffected worms suggest that components of the immune system that regulate worm length and worm burden were not suppressed by the corticosteroid treatment. This has been observed previously by Greer, Sedcole, et al. (2009) with the same immunosuppressing regime and drug used here whereby incomplete immunosuppression giving rise to increases in IgA were associated with lower production.

The antigen injections did not appear to induce tolerance in the lambs; although there was evidence of a Th2 immune response skewed towards immunity following treatment. This was indicated by the elevated antibody responses post-infection and the declining FEC which are indicators of developing immunity. The superior serum IgA profile in RE compared with SC suggests that the rectal delivery route induced a stronger systemic immune response. Although, the rise in serum antibodies confirms a systemic immunity in all the infected lambs, it is unknown whether the antigen treatment induced mucosal immunity since local antibody production was not measured in the present study. Contradictory to the serum IgA, there was a lack of difference in serum IgG and IgE profiles between antigen and non-antigen treated lambs. While a majority of vaccines are given parenterally and induce systemic immunisation but often are poor inducers of mucosal immunity (Mestecky, 1987) it has also proven difficult to stimulate strong mucosal immune responses in practise through administration via the mucosal route. However, McClure (2009) demonstrated mucosal protection associated with lower worm burdens and enhanced antibody production in the small intestine in lambs immunised with a *T. colubriformis* antigen that was delivered to the rectal submucosa without an adjuvant.

Additional support for the Th2-based immunity is the increased production of IL-4 cytokines which are typically produced during a Th2 immune response. The greater IL-4 concentrations in the antigen treated groups confirm the finding in Chapter 3 that the treatment caused a vaccine-like effect which boosted the IL-4 production in the antigen treated lambs with a greater effect in RE than SC. The low IFN-γ concentrations further support this finding since IFN-γ cytokines are associated with a Th1 response and typically suppressed by a Th2 response (Korn et al., 2009). Since Th2 responses are normally enhanced following nematode infections, elevated IFN-γ concentrations can compromise the host's ability to expel nematodes (Else & Finkelman, 1998). In contrast, Coltman, Wilson, Pilkington, Stear, and Pemberton (2001) suggested that the expression of IFN-γ could enhance the activity of Th2 responses. Previous research has shown that peripheral immunisation with aluminium hydroxide as an adjuvant is an effective way of inducing a Th2 immune response characterised by an increase in IL-4 production and increased IgE production (Cox & Coulter, 1997). Although the serum IgE

concentrations were inconsistent with the IL-4 production it can be speculated that delivering the larval antigen with a suitable adjuvant could perhaps have induced a stronger immune response in the immunised lambs.

In summary, findings from this study confirm the previous observations in Chapter 3 that the antigen treatment induced a Th2 immune response in the lambs and enhanced the development of immunity rather than inducing tolerance. Worm burden and female worm length were reduced in the antigen treated lambs with a greater reduction via the intra-rectal delivery route. Furthermore, the lambs injected intra-rectally had improved performance compared with the infected controls. It is suggested that the intra-rectal route is a more effective route than the subcutaneous.

# Chapter 5
# Field study investigating the importance of age on injection with *Trichostrongylus colubriformis* antigen to induce mucosal tolerance in new-born and weaned lambs

## 5.1 Introduction

Gastrointestinal nematode infections are a major animal health constraint causing production losses in ruminants worldwide. The control of the nematodes relies heavily on anthelmintics. However, with increasing anthelmintic resistance there is a demand for alternative treatment methods for parasite control. Enhancing the immune response is one such method, and although many attempts have been made to enhance the immune system of the host by the use of conventional vaccines these have been unsuccessful at mitigating the negative effects of the nematodes (Waller, 1999). In part, this may be attributed to production losses associated with temperate nematode infections being mostly a consequence of a strong immune response to the parasite in the host animal rather than direct effects of the parasites (Greer, 2008; Williams, Palmer, et al., 2010). Alternative control methods, specifically those that aim to reduce the immune responses, may therefore hold potential for novel approaches to reduce production losses related to parasitism. It has been shown that nematode infections in very young lambs, up to 12 weeks of age, have no measurable impact on production (Iposu et al., 2008). This may reflect immunological hyporesponsiveness and a lack of ability of young lambs to acquire immunity to parasites and its associated impacts (Colditz et al., 1996; Greer, 2008; Greer et al., 2005). The maximum benefit of a desensitising regime aimed at reducing the effects of immune responses can therefore be expected to occur on grazing animals post-weaning (approximately three to four months of age). With this in mind, theories of mucosal tolerance also propose that the optimum timing for desensitising animals occurs in the neonate, particularly during the first 24 h of life when the immune response is naïve to a number of potential pathogens and in the process of developing an ability to determine self and non-self. Thus, the period immediately after birth may provide an optimum opportunity to expose animals to gastrointestinal nematode antigens in order to train the immune system not to respond to such challenge later in life. Indoor studies to date have shown beneficial effects of desensitising parasite-naïve four-month-old lambs with improvement in animal growth and voluntary feed intake (Greer et al., 2014), although, such benefits have not been repeated in Chapters 3 and 4. However, it is yet to be evaluated if these benefits can be observed in lambs younger than three to four months old and in lambs on pasture. This study was designed to investigate the impact of age on the ability to induce mucosal tolerance in lambs exposed to the gastrointestinal nematode *T. colubriformis* in a field situation.

## 5.2 Methods and Materials

### 5.2.1 Animals and experimental design

The study was conducted as a field study on Lincolnsheep research unit from September 2014 to April 2015 with approval from and in accordance with Lincoln University Animal Ethics Committee (LUAEC#2014-577) to investigate the importance of animal age on the ability to induce mucosal tolerance in a field situation. Seventy two sets of crossbred mixed-sex twin lambs were born on low-contaminated newly sown pasture between 10$^{th}$ and 12$^{th}$ September 2014 and trickle-infected with parasites one month post-weaning from four months of age. At birth, the lambs were fitted with electronic ear tags (Allflex, New Zealand) and sequentially allocated into one of six treatment groups (n=24) based on birth order, and balanced for sex, table 5.1.

Two groups were injected with a somatic antigen, each animal receiving a rectal submucosal injection weekly for three weeks starting within 24 h of birth (NBDES) and weaning (WDES), respectively. One of the twins from each set was used as a control for the respective treatment, NBCON and WCON were controls of NBDES and WDES, respectively, and received sham injections with 200 µl PBS. The antigen treatment regime comprised of injections of an antigen mix containing 7.5 µg of L3 somatic antigen; 5.5 µg of L4 somatic antigen and 10.5 µg L5 somatic antigen. Doses were contained in 200 µl PBS and injected three times into the rectal submucosa as previously described in Chapter 3. Care was taken to ensure a blister was visually observed at the site of injection reflecting administration of the antigen cocktail correctly into the rectal submucosa with the use of a rectal speculum which in the case of new-born lambs consisted of a modified 5 ml syringe barrel. CON was injected subcutaneously with 1 mg kg$^{-1}$ of the long-acting anthelmintic Cydectin (20 g l$^{-1}$ Moxidectin, Zoetis New Zealand Ltd, New Zealand) on days 0 and 63 of infection and used for positive growth control. INF was injected with PBS at weaning and acted as control for the level of pathogenicity of infection. The lambs grazed with their dams until weaning at three months of age and after weaning were rotationally grazed following the ewes and shifted weekly to minimise pasture contamination. The NBDES and NBCON were grazed separately from the other four groups until weaning due to ease of management.

All groups except the CON were challenged three times weekly with the equivalent of 14,000 *T. colubriformis* L3 larvae week$^{-1}$ for seven weeks from day 0 (at the age of four months). Larvae from a solution of a known concentration were pipetted onto filter paper which was rolled and administered orally by use of a balling gun as described by Donaldson et al. (2001). Additionally, six tracer lambs were used for determining pasture contamination, grazing two at a time for four weeks on three occasions; day -56 to -28, day 0 to 28 and day 56 to 84.

Table 5.1: Experimental design.

| Group (n=24) | Treatment | Challenge from day 0 |
|---|---|---|
| NBDES | Antigen treated at birth with *Tcol*-L3/L4/L5 | 14,000 *T. col* L3 week$^{-1}$ * |
| NBCON | PBS at birth | 14,000 *T. col* L3 week$^{-1}$ * |
| WDES | Antigen treated at weaning with *Tcol*-L3/L4/L5 | 14,000 *T. col* L3 week$^{-1}$ * |
| WCON | PBS at weaning | 14,000 *T. col* L3 week$^{-1}$ * |
| CON | Cydectin long-acting anthelmintic | No challenge |
| INF | PBS at weaning | 14,000 *T. col* L3 week$^{-1}$* |

*administered in split doses three times week$^{-1}$

### 5.2.2 Live weight

Live weights were recorded at birth and every four weeks until weaning using Tru-Test XR3000 (Tru-Test Ltd, New Zealand) and a Prattley autodrafter with a sensitivity of 0.2 kg. At weaning, on day -28, full live weights and fasted live weights (24 h fasting) were recorded. Full live weights were recorded on a weekly basis from weaning onwards with fasted live weights recorded at the start of infection (four months of age) and every four weeks thereafter. Animals that suffered a 15% or greater loss of body weight between two weight recordings were drenched with combination drench ComboSheep (37.5 g l$^{-1}$ levamisole hydrochloride, 24.0 g l$^{-1}$ albendazol, Ravensdown New Zealand Ltd, Christchurch, New Zealand), 1 ml 5 kg live weight$^{-1}$.

### 5.2.3 Parasitology

#### 5.2.3.1 Antigen preparation

Soluble somatic parasite antigen was prepared prior to the trial by infecting three sheep with *T. colubriformis* as described by Knox and Jones (1990) and described in Chapter 3.

#### 5.2.3.2 Faecal egg count

Faecal samples were collected fortnightly directly from the rectum of the lambs. Determination of faecal egg counts (FEC) was performed using a modified McMaster method as previously described in Chapter 3. Each egg counted was equivalent to 100 eggs gram$^{-1}$ of faeces (epg).

#### 5.2.3.3 Total worm burden

Post-mortem worm recovery from abomasum and small intestine was performed in accordance with the method described by Donaldson et al. (2001). The abomasum and small intestine were collected at slaughter from six tracer animals throughout the study and from 18 animals at the end of the study, six

from each of the groups NBDES, WDES and INF. The tracer lambs were removed from pasture after four weeks of grazing and kept indoors for 10 days before being sacrificed. Slaughter was performed by stunning the animal with a captive bolt gun and severing the carotid arteries and jugular veins. The procedure for total worm counts is described in Chapter 3 with the exception that abomasum was also ligated and removed. The larvae were counted and identified for sex, larval stage and species.

### 5.2.4 Blood samples

Blood samples were collected monthly from animals in the NBDES, NBCON, WDES and CON groups by jugular venipuncture using 2 x 10 ml lithium heparin and non-heparinised vacutubes (Becton Dickinson Vacutainer®, Belliver Industrial Estate, Plymouth, UK), starting from birth in NBDES and NBCON and at weaning in the other groups. NBCON, WCON and INF received the same treatment therefore only one of the groups, NBCON, was sampled to minimise animal usage. The non-heparinised tubes were set to coagulate overnight and then centrifuged at 1,200 x g for 10 minutes at 4 ˚C. Serum was collected and stored at -20 °C until examined for IgA antibodies by ELISA. The heparinised blood samples were prepared immediately for leukocyte stimulation assays. The techniques for antibody determination and leukocyte stimulation assays are described in Chapter 3.

### 5.2.5 Statistical analysis

Statistical analyses were carried out using the GENSTAT statistical package (GENSTAT 2016, GenStat Sixteenth edition, VSN International Limited, UK). Blood measurements, live weight, liveweight gain and FEC were analysed as repeated measures by REML with treatment groups and time included as factors with missing values estimated. FEC and antibodies were log transformed (log10 (count+10)) prior to analysis. Worm burdens and worm length were analysed by analysis of variance (ANOVA) and also log transformed (log10 (count+1)) prior to analysis. $P \leq 0.05$ was regarded as significant all values are group means and expressed as mean ± SEM unless otherwise specified.

## 5.3 Results

### 5.3.1 Clinical observations

Three animals (one NBCON and two CON) suffered postnatal death and another four lambs died suddenly on day -14 (one NBDES, one CON), day 7 (one NBDES) and day 28 (one NBCON). The cause of death could not be determined by autopsy. Data from these animals was excluded from the trial.

Eight animals suffered a 15% or greater loss of body weight between two weight recordings and were salvage drenched with 1 ml 5 kg live weight$^{-1}$ of ComboSheep Combination Drench (37.5 g l$^{-1}$

levamisole hydrochloride, 24.0 g $l^{-1}$ albendazol, Ravensdown New Zealand Ltd, New Zealand) on day 42 (one NBCON), day 49 (one CON), day 56 (one WDES), day 63 (one WCON), day 77 (one WDES), and day 84 (one CON and two WCON). All data from these animals was included in the analysis.

Thirty nine animals were affected by myiasis throughout the trial on days 2, 49, 56, 63, 69, 76, and 79, due to failure of a preventive program. In total four NBDES, five NBCON (one animal affected three times), 10 WDES (one affected three times), 10 WCON (three affected twice), two CON and eight INF (two affected twice) were affected. The infested area was shorn and treated topically with Maggo (16 g $l^{-1}$ propetamphos, 400 g $l^{-1}$ paradichlorobenzene and 510 g $l^{-1}$ hydrocarbon liquids, Bayer New Zealand Ltd, New Zealand).

### 5.3.2 Live weight

Mean live weights for NBDES and NBCON are given in Figure 5.1. Live weights increased with time ($P<0.001$) but were not different between the two groups ($P=0.990$). There was no treatment x time interaction between the NBDES and NBCON ($P=0.949$). Figure 5.2 shows the live weights for WDES, WCON, CON, and INF. There was an interaction between treatment and time ($P<0.001$) reflecting initial similar live weights in all four groups but from day 28 CON had a greater rate of increase.

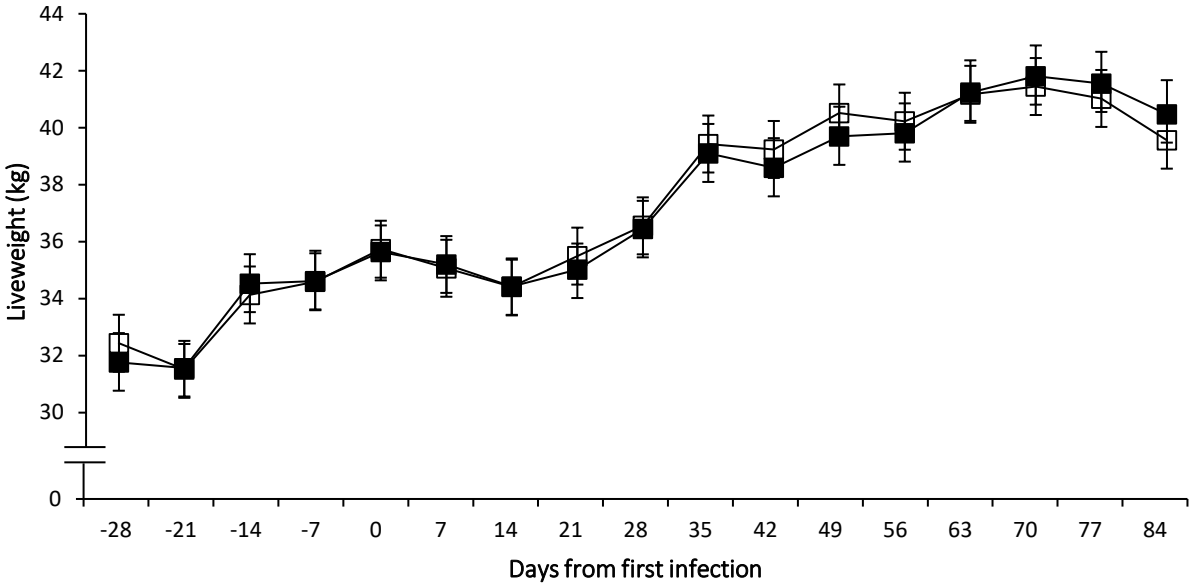


Figure 5.1: Mean live weight (± SEM) of lambs infected with 14,000 *T. colubriformis* larvae week$^{-1}$ (NBCON, solid line open square), infected and additionally injected with a somatic antigen at birth (NBDES, solid line filled square). NBDES and NBCON were grazed separately from the other four groups until day -28 due to ease of management, thus data is presented seperately.

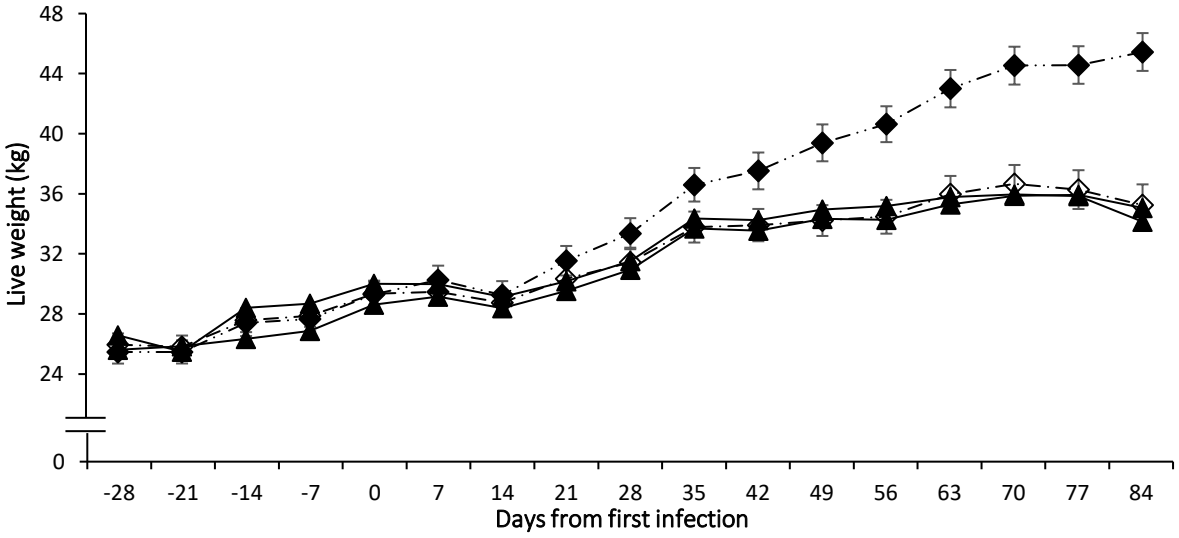


Figure 5.2: Mean live weight (± SEM) of lambs infected with 14,000 *T. colubriformis* larvae week$^{-1}$ (WCON, solid line open triangle and INF, dashed line open diamond), infected and additionally injected with a somatic antigen at weaning (WDES, solid line filled triangle), and drenched with a long-acting anthelmintic (CON, dashed line filled diamond).

### 5.3.3 Liveweight gain

Cumulative mean liveweight gains (kg) are given in Figures 5.3 and 5.4. There was no difference in liveweight gain between NBDES and NBCON (P=0.998) but there was an effect of time (P<0.001) reflecting an initial increase in liveweight gain until day 0 followed by a decrease and gradually increasing liveweight gain from day 21 to day 70 (Figure 5.3). There was no interaction between treatment and time (P=0.910). For the remaining groups (Figure 5.4) there was a treatment x time interaction which reflected greater liveweight gain in CON from day 28 compared with WDES, WCON, and INF (P<0.001) and being 60% greater on day 84. There was no difference in liveweight gain within the infected groups (WDES, WCON and INF) (P=0.684).

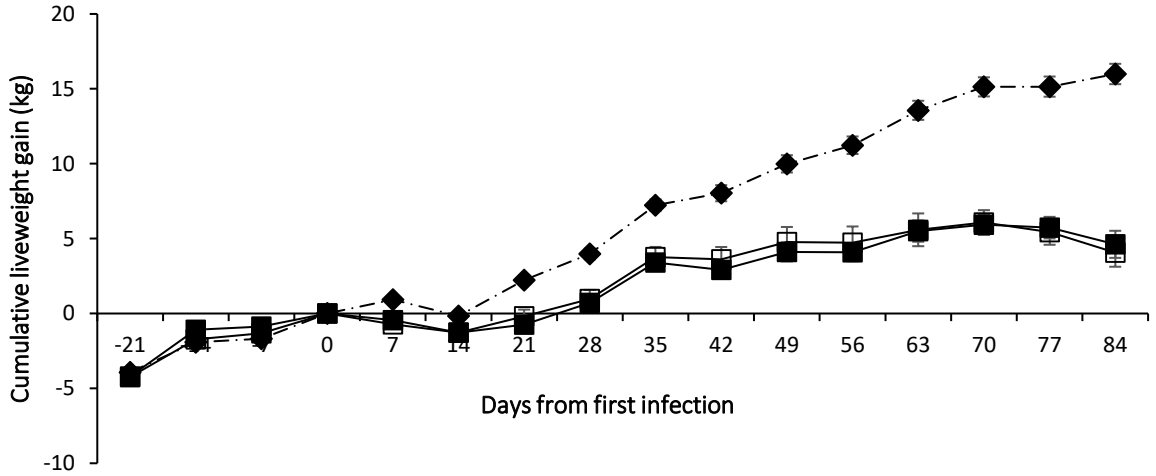


Figure 5.3: Mean cumulative liveweight gain (± SEM) of lambs infected with 14,000 *T. colubriformis* larvae week$^{-1}$ (NBCON, solid line open square, infected and additionally injected with a somatic antigen at birth (NBDES, solid line filled square), and drenched with a long-acting anthelmintic (CON, dashed line filled diamond) for comparison. NBDES and NBCON were grazed separately from the other four groups until day -28 due to ease of management, thus data is presented seperately.

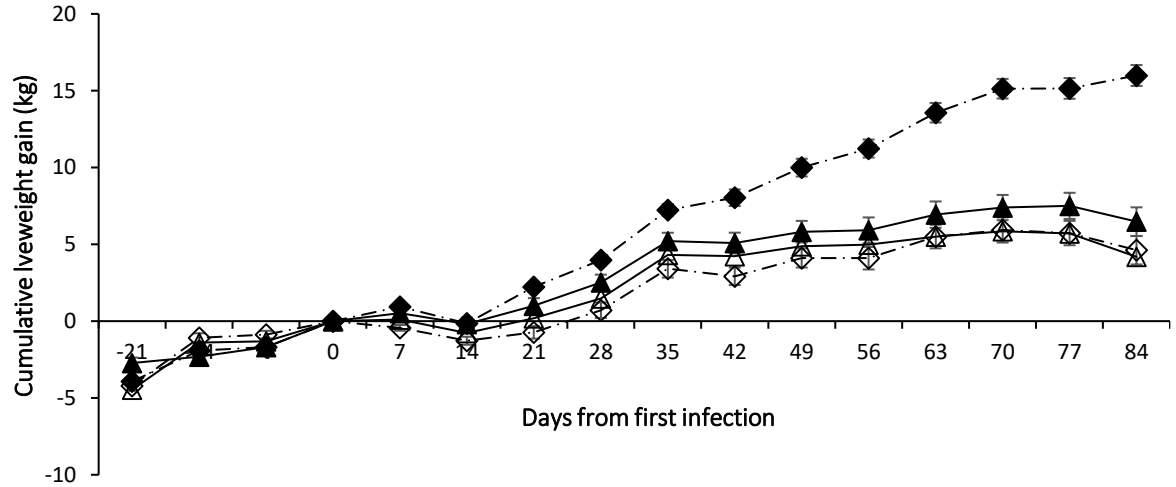


Figure 5.4: Mean cumulative liveweight gain (± SEM) of lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (WCON, solid line open triangle and INF, dashed line open diamond), infected and additionally injected with a somatic antigen at weaning (WDES solid line filled triangle), and drenched with a long-acting anthelmintic (CON, dashed line filled diamond) for comparison.

## 5.3.4 Parasitology

### 5.3.4.1 Faecal egg count

Mean back-transformed (log 10 (count + 10)) faecal egg counts (FEC, epg) are given in Figures 5.5 and 5.6. There was interaction between treatment and time (P=0.003) in the NBDES and NBCON groups reflecting an increase over time in both groups with declining FEC on day 84 (Figure 5.5) with a temporary decrease in NBCON on day 56. For the remaining groups (Figure 5.6) there was also treatment x time interaction (P=0.011) reflecting similar values in all groups up to day 42 from when INF showed an increased FEC compared with WDES and WCON. NBDES, NBCON and INF peaked at 3,500, 3,200 and 3,300 epg, respectively, on day 70. The WDES and WCON peaked at 2,500 and 2,400 epg on day 42 and day 56 respectively.

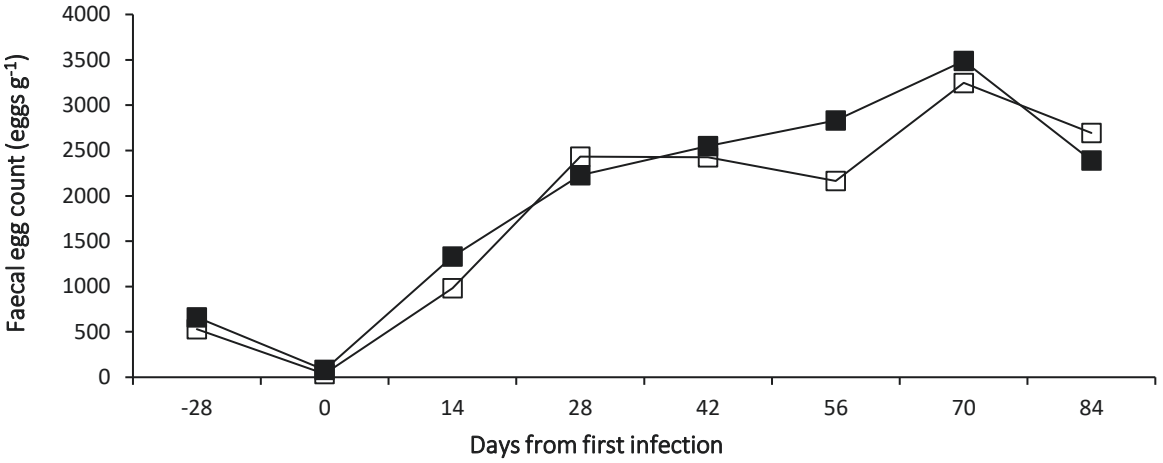


Figure 5.5: Mean back-transformed log 10 (count +10) FEC of lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (NBCON, solid line open square and infected and additionally injected with a somatic antigen at birth (NBDES, solid line filled square). NBDES and NBCON were grazed separately from the other four groups until day -28 due to ease of management, thus data is presented seperately.

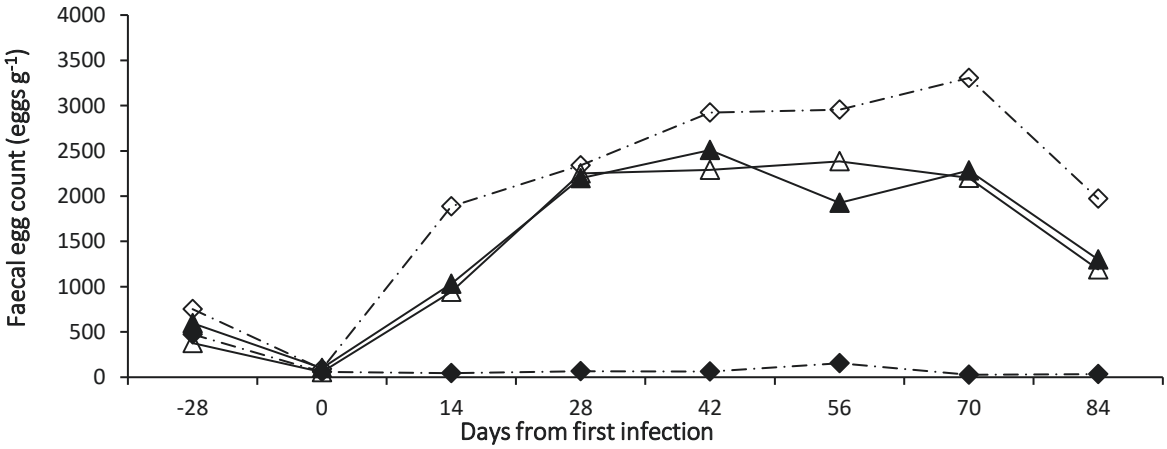


Figure 5.6: Mean back-transformed log 10 (count +10) FEC of lambs infected with 14,000 *T. colubriformis* week-$^{1}$ (WCON, solid line open triangle and INF, dashed line open diamond), infected and additionally injected with a somatic antigen at weaning (WDES, solid line filled triangle), and drenched with a long- acting anthelmintic (CON, dashed line filled diamond).

### 5.3.4.2 Post mortem worm burden

The mean worm burdens from the small intestine and abomasum of six tracer lambs on day -28, day 28, and day 84 are given in Table 5.2. There was an increase in worm numbers over time. *T. colubriformis* contributed 41%, 98% and 95% of total worm burdens on days -28, 28 and 84, respectively.

Table 5.2: Mean small intestinal worm count (a) and abomasal worm count (b) (± SEM) for tracer lambs on days -28, 28 and 84. Numbers of log10 (count+10) worms with back-transformed values in parenthesis.

| Worm species | Day | | |
|---|---|---|---|
| a) | -28 | 28 | 84 |
| *T. colubriformis* | 2.28 ± 0.47 (180) | 2.38 ± 0.21 (230) | 4.51 ± 0.09 (31,985) |
| *Cooperia* | 1.18 ± 0.15 (5) | 1.00 ± 0 (0) | 2.72 ± 0.26 (515) |
| *Nematodirus* | 2.42 ± 0.70 (255) | 1.18 ± 0.15 (5) | 3.10 ± 0.21 (1,245) |
| *Total* | 2.65 ± 0.57 (440) | 2.39 ± 0.20 (235) | 4.53 ± 0.07 (33,745) |

| Worm species | Day | | |
|---|---|---|---|
| b) | -28 | 28 | 84 |
| *T. circumcincta* | 2.11 ± 0.37 (120) | 1.93 ± 0.44 (75) | 3.87 ± 0.11 (7,405) |
| *T. axei* | 1.93 ± 0.19 (75) | 2.08 ± 0.68 (110) | 3.31 ± 0.04 (2,030) |
| *Total* | 2.31 ± 0.31 (195) | 2.29 ± 0.63 (185) | 3.98 ± 0.09 (9,435) |

Mean abomasal worm burdens of six animals from each of the groups INF, NBDES and WDES are given in Table 5.3. Total worm burden was not different between the three groups (P=0.664) or varied between different species, *T .circumcincta* (P=0.869), and *T.axei* (P=0.652). Adult worms contributed 95%, 96% and 92% of total worm burden in INF, NBDES and WDES, respectively.

Table 5.3: Mean abomasal worm burden (± SEM) of lambs infected with 14,000 *T. colubriformis* larvae week$^{-1}$ (INF), infected and injected with a somatic antigen at birth (NBDES) or at weaning (WDES). Numbers of log10 (count+10) worms with back-transformed values in parenthesis. There was no significant difference between treatment groups (P>0.05).

| Worm species | Treatment group (n) | | |
|---|---|---|---|
| *T. circumcincta* | INF (6) | NBDES (6) | WDES (6) |
| L3 | 2.24 ± 0.30 (165) | 1.45 ± 0.18 (18) | 1.18 ± 0.10 (5) |
| L4 | 2.20 ± 0.31 (148) | 2.24 ± 0.36 (165) | 1.74 ± 0.20 (45) |
| L5/ adult | 3.87 ± 0.12 (7,778) | 3.68 ± 0.59 (4,732) | 3.59 ± 0.33 (3,898) |
| Total | 3.89 ± 0.12 (8,091) | 3.76 ± 0.60 (5,717) | 3.66 ± 0.31 (4,590) |
| *T. axei* | | | |
| L3 | 1.86 ± 0.36 (63) | 1.67 ± 0.23 (37) | 1.00 ± 0.36 (0) |
| L4 | 2.40 ± 0.27 (238) | 2.50 ± 0.34 (303) | 2.46 ± 0.21 (278) |
| L5/ adult | 3.80 ± 0.48 (6,352) | 3.78 ± 0.17 (6,055) | 3.57 ± 0.10 (3,715) |
| Total | 3.83 ± 0.49 (6,773) | 3.81 ± 0.17 (6,397) | 3.62 ± 0.12 (4,168) |
| *Total* | | | |
| L3 | 2.55 ± 0.28 (348) | 1.81 ± 0.26 (55) | 2.39 ± 0.33 (233) |
| L4 | 2.59 ± 0.19 (387) | 2.57 ± 0.39 (358) | 2.29 ± 0.22 (287) |
| L5/ adult | 4.15 ± 0.78 (14,130) | 3.83 ± 0.21 (10,787) | 2.32 ± 0.12 (6,311) |
| Total | 4.17 ± 0.08 (14,865) | 4.03 ± 0.21 (11,707) | 3.94 ± 0.12 (8,710) |

Mean small intestinal worm burdens of six animals from each of the groups INF, NBDES, and WDES are given in Table 5.4. There was no difference in total worm burden between the three groups (P=0.740) or between different species, *Cooperia* (P=0.385), *Nematodirus* (P=0.287), and *T. colubriformis* (P=0.611). Adult worms contributed 85%, 91% and 85% of total worm burden in INF, NBDES and WDES, respectively, with *T. colubriformis* contributing 93-99% of total worm burden.

Table 5.4: Mean small intestinal worm burden (± SEM) of lambs infected with 14,000 *T. colubriformis* larvae week$^{-1}$ (INF), infected and injected with a somatic antigen at birth (NBDES) or at weaning (WDES). Numbers of log10 (count+10) worms with back-transformed values in parenthesis. There was no significant difference between treatment groups (P>0.05).

| Worm species | Treatment group (n) | | |
|---|---|---|---|
| *Cooperia* | INF (6) | NBDES (6) | WDES (6) |
| L3 | 1.00 ± 0 (0) | 1.00 ± 0 (0) | 2.27 ± 0.33 (176) |
| L4 | 2.08 ± 0.32 (110) | 1.48 ± 0.18 (20) | 1.88 ± 0.27 (66) |
| L5/ adult | 2.08 ± 0.30 (110) | 2.14 ± 0.31(128) | 2.61 ± 0.29 (402) |
| Total | 2.36 ± 0.32 (220) | 2.20 ± 0.32 (148) | 2.82 ± 0.19 (644) |
| *Nematodirus* | | | |
| L3 | 1.68 ± 0.22 (38) | 1.93 ± 0.27 (75) | 1.48 ± 0.18 (20) |
| L4 | 2.01 ± 0.28 (92) | 1.95 ± 0.27 (78) | 1.50 ± 0.19 (22) |
| L5/ adult | 2.37 ± 0.37 (223) | 2.29 ± 0.35 (187) | 1.93 ± 0.27 (75) |
| Total | 2.43 ± 0.10 (261) | 2.54 ± 0.36 (340) | 2.10 ± 0.28 (117) |
| *T. colubriformis* | | | |
| L3 | 2.40 ± 0.30 (240) | 2.08 ± 0.28 (110) | 2.44 ± 0.28 (268) |
| L4 | 3.11 ± 0.18 (1,268) | 3.12 ± 0.16 (1,310) | 3.38 ± 0.21 (2,698) |
| L5/ adult | 4.35 ± 0.14 (22,462) | 4.26 ± 0.18 (18,072) | 4.22 ± 0.18 (16,596) |
| Total | 4.38 ± 0.14 (23,987) | 4.29 ± 0.17 (19,491) | 4.28 ± 0.18 (19,138) |
| *Total* | | | |
| L3 | 2.56 ± 0.30 (352) | 2.33 ± 0.29 (203) | 2.56 ± 0.21 (354) |
| L4 | 3.17 ± 0.15 (1,470) | 3.15 ± 0.15 (1,408) | 3.39 ± 0.21 (2,442) |
| L5/ adult | 4.32 ± 0.19 (20,668) | 4.26 ± 0.17 (18,460) | 4.23 ± 0.18 (16,898) |
| Total | 4.38 ± 0.14 (24,248) | 4.27 ± 0.17 (19,608) | 4.28 ± 0.18 (19,225) |

### 5.3.5 Serum antibodies

Mean absorbance (OD) of serum IgA antibodies to L3 *T. colubriformis* antigen (*Tcol*-L3) in NBDES and NBCON is given in Figure 5.7. IgA levels rapidly decreased (P<0.001) from day -112 to day -84 followed by an increase up to day -28. There was no difference between the groups (P=0.647) nor interaction between treatment and time (P=0.789).

From day -28 there was an interaction between treatment and time (P=0.028) with an initial decrease in all groups followed by an increase in all groups but remained lower in CON from day 28 until day 84.

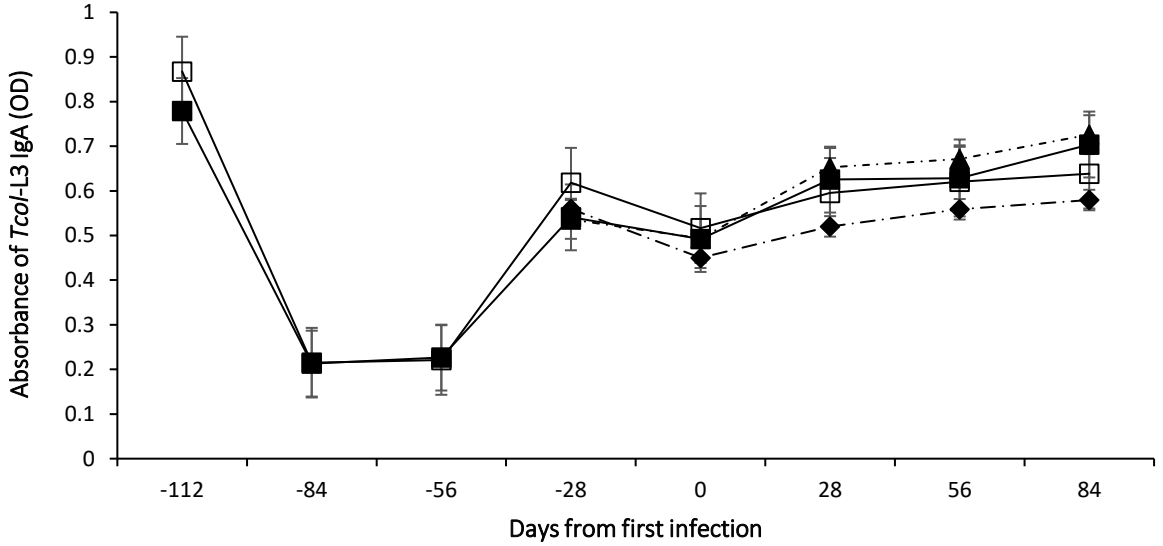


Figure 5.7: Mean absorbance (± SEM) of L3 *T. colubriformis* IgA antibody levels for lambs infected with 14,000 *T. colubriformis* larvae week$^{-1}$ (NBCON, solid line open square), infected and additionally injected with a somatic antigen at birth (NBDES, solid line filled square) sampled from day -112, infected and additionally injected with a somatic antigen at weaning (WDES, dashed line filled triangle), drenched with a long-acting anthelmintic (CON, dashed line filled diamond) sampled from day -28.

### 5.3.6 Cytokine production

#### 5.3.6.1 IL-4

Mean concentrations (pg $ml^{-1}$) of IL-4 produced by WBC stimulated with *Tcol*-L3, *Tcol*-L4, and *Tcol*-L5 antigen are given in Figure 5.8. For *Tcol*-L3 (Figure 5.8 a) there was an interaction between treatment and time (P<0.001) reflecting an initial increase in all groups which was greater in the NBDES and WDES groups than the NBCON and CON groups, before declining and being similar on day 56.

For *Tcol*-L4 (Figure 5.8 b) there was an effect of time (P<0.001) reflecting an increase in cytokine production from day -28 until day 28. The IL-4 production was not different between the groups (P=0.087) nor was there interaction between treatment and time (P=0.295).

For *Tcol*-L5 (Figure 5.8 c) there was an effect of treatment (P=0.024) with lower IL-4 production in WDES on day 56. In addition, there was an increase in cytokine production from day -28 until the end of the study (P<0.001). There was no interaction between treatment and time (P=0.693).

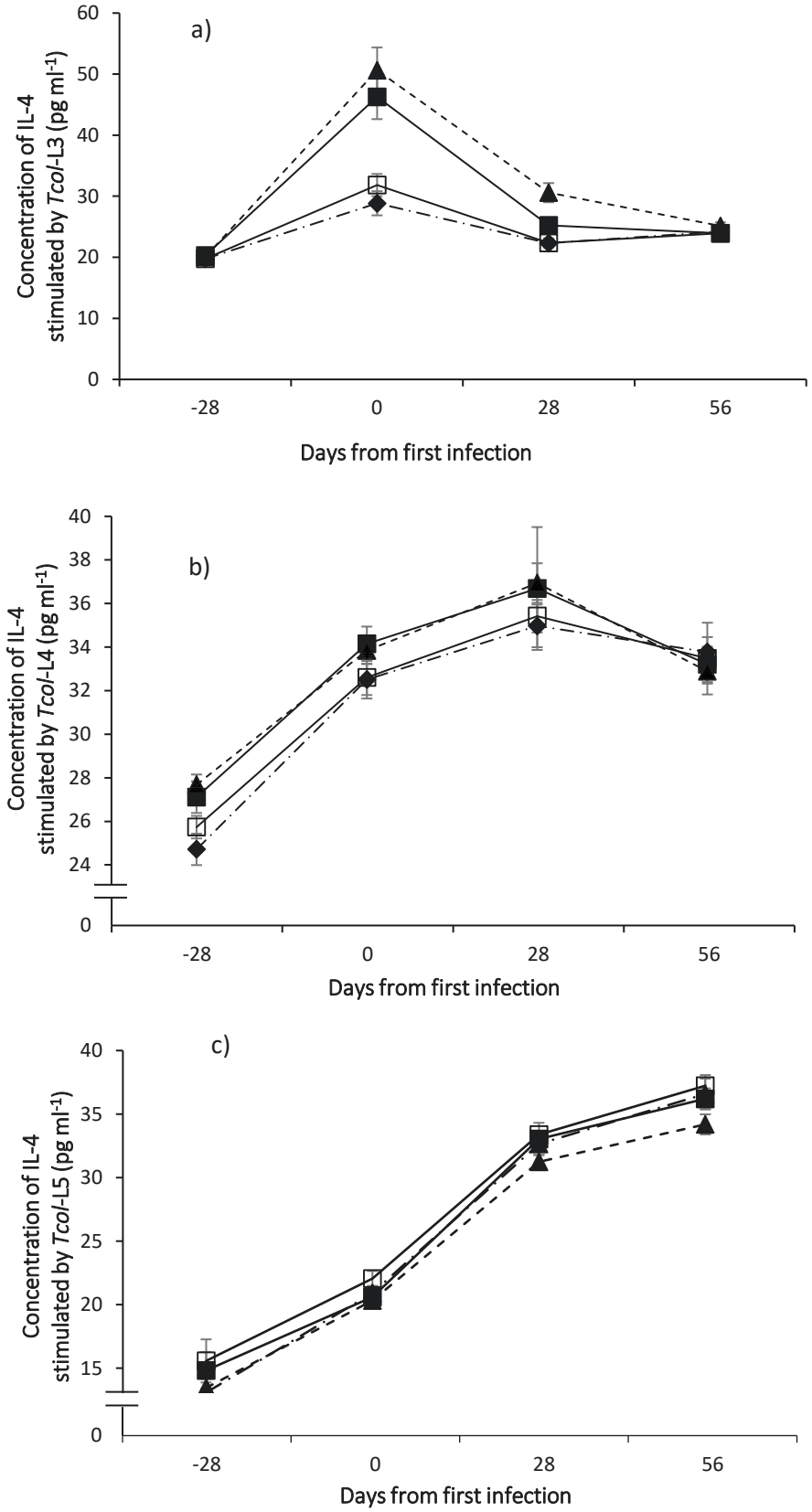


Figure 5.8: Mean concentration (± SEM) of IL-4 produced by WBC stimulated with a) *Tcol*-L3, b) *Tcol*-L4, and c) *Tcol*-L5 antigen for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (NBCON, solid line open square), infected and additionally injected with a somatic antigen at birth (NBDES, solid line filled square) or at weaning (WDES, dashed line filled triangle), drenched with a long acting anthelmintic (CON, dashed line filled diamond).

#### 5.3.6.2 IFN-γ

Mean concentrations (pg $ml^{-1}$) of IFN-γ produced by WBC stimulated with *Tcol*-L3, *Tcol*-L4, and *Tcol*-L5 antigen are given in Figure 5.9. For *Tcol*-L3 (Figure 5.9 a) there was interaction between treatment and time (P=0.019) with initially higher IFN-γ production in the NBDES group on day -28 which subsequently decreased and remained similar to the other groups.

The IFN-γ production in response to *Tcol*-L4 (Figure 5.9 b) decreased over time (P<0.001) and was not different between the treatment groups (P= 0.527). There was no interaction between treatment and time (P= 0.407).

For *Tcol*-L-5 (Figure 5.9 c) there was similarly an effect of time (P<0.001) reflecting increasing values throughout the study. There was no effect of treatment (P=0.550) nor interaction between treatment and time (P=0.281).

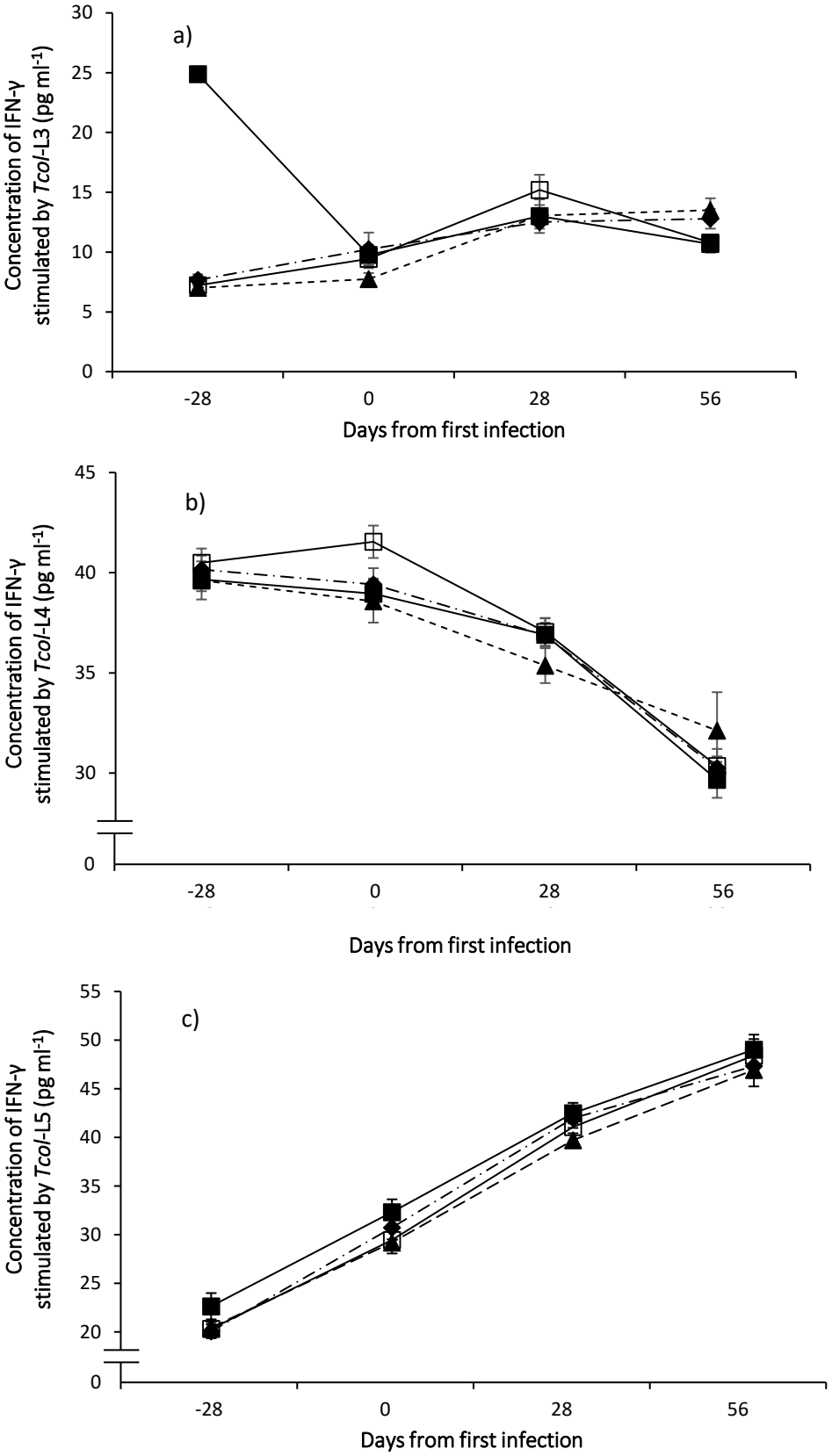


Figure 5.9: Mean concentration (± SEM) of IFN-γ produced by WBC stimulated with a) *Tcol*-L3, b) *Tcol*-L4, and c) *Tcol*-L5 antigen for lambs infected with 14,000 *T. colubriformis* week$^{-1}$ (NBCON, solid line open square), infected and additionally injected with a somatic antigen at birth (NBDES, solid line filled square) or at weaning (WDES, dashed line filled triangle), drenched with a long acting anthelmintic (CON, dashed line filled diamond).

## 5.4 Discussion

Immunological findings in this study were that repeated injections with a somatic *T. colubriformis* antigen induced a Th2 immune response in the lambs, although this did not appear to successfully induce tolerance or confer protection against further challenge. It was hypothesised that exposing neonates to gastrointestinal nematode antigens in order to train the immune system may cause a non-responsiveness to such challenge later in life however, there was no data in the present study that supports this hypothesis.

The larval infection was pathogenic enough to cause a reduced growth with the infected animals having a 60% reduction in liveweight gain compared with the drenched control group. This is consistent with findings of Sykes and Coop (1976) who reported a 50% growth reduction in sheep infected with *T. colubriformis* at a similar initial dose rate that was used in the present study, although it is clear from the worm burdens of the tracer lambs that challenge was greater with time presumably reflecting recycling of contamination. However, the antigen treatment did not mitigate the negative impacts of parasites on growth which was indicated by the liveweight gain between the antigen treated animals and their twins not being significantly different. Considering the lambs also harboured an abomasal worm burden (Table 5.3), this is likely to have contributed to the reduced growth, supported by Greer et al. (2008) who observed reduced efficiency of energy utilisation by 20% in lambs challenged with 4,000 *T. circumcincta* day$^{-1}$ and worm numbers similar to the abomasal worm burdens in the present study. Moreover, Bown et al. (1989) noted lower liveweight gain in lambs receiving concurrent infections of 3,000 *T. circumcincta* and 3,000 *T. colubriformis* day$^{-1}$ compared with uninfected controls. The WDES received their first challenge a week following the last treatment, with timing and infection rate comparable with the study by Greer et al. (2014) who reported an increase in carcass weight of antigen treated lambs. In contrast to the findings of these authors, there was no effect of treatment on liveweight gain, antibody levels and parasitological parameters in the antigen treated lambs in the present study. The pressure of the other nematode species on pasture may have confounded the results given the antigen treatment was *T. colubriformis*-specific, thus may have been directed only towards this nematode species. While, cross-reactive immune responses occur between different nematode antigens (Fairlie-Clarke, Shuker, & Graham, 2009), it remains unclear whether this was present in the current study as there was no evidence of treatment mitigating the effects of the parasites.

All pastures in the present study were newly sown at the beginning of the study, hence expected to be parasite free. The total worm burden data from the tracer lambs, 400 and 600 larvae on day -28 and day 28 respectively, confirms that the pasture contamination in the early stages of the study was low

and it is considered unlikely that the low number of worms had an impact on the treatment as there was no difference in liveweight gain between the controls and the infected animals until day 28. At this stage, it was expected that there would be an apparent effect of the trickle infection which is confirmed by the greater liveweight gain in CON from day 28 onwards. While a decrease in feed intake around 15-30% is commonly seen in subclinical intestinal parasitism (Poppi et al., 1990; Steel et al., 1980; Sykes, 2008; Sykes, Poppi, & Elliot, 1988) it is uncertain whether any lambs reduced their feed intakes in the present study since it was not feasible to measure the feed intake due to the outdoor grazing situation. Although feed intake was not measured the study was designed with the intention to allow the lambs *ad libitum* access to feed. Whereas rotational grazing was applied to help the pastures recover from being grazed there was insufficient irrigation around day -14 to day 7 leading to poor pasture quantity and the decreasing liveweight gain in all groups around day 0-14 is likely a reflection of this, supported by the average liveweight gain in the CON of -0.5 kg at this time. Despite pasture larval counts not being performed to confirm the level of pasture contamination, the lowest abomasal worm numbers were comparable with Greer et al. (2008) who recorded a reduction in feed intake of 17% in lambs after 56 days of trickle infection with 4,000 *T. circumcincta* $day^{-1}$. It is possible that the outcome is confounded by the lack of feed to enable any benefit to be expressed and it remains possible the effect of the antigen treatment if present had worn off once the pastures were properly irrigated again. Considering that the present study was conducted in the field, this makes it challenging to draw comparisons with the findings from the indoor trials by Greer et al. (2014) and in Chapters 3 and 4 since the environmental factor is an element likely to be accountable for some of the inconsistency in the results. The problem with fly strike has undoubtedly caused the lambs some stress despite several attempts to control and prevent fly infestations. All treatment groups were represented amongst the affected animals with larger numbers in the WDES, WCON and INF group and it is unknown whether the fly strike outbreaks during the study has had an impact on performance or the outcome of the study.

There was no indication from the antibody profiles that tolerance was induced. Despite this, there was evidence of treatment inducing some immunological change. Production of IL-4 cytokines in response to *T. colubriformis* L3 antigen in the antigen treated lambs at the time of weaning indicates that there was an immunological effect skewed towards a Th2 immune response and that antigen treatment caused a booster effect as a stronger immune response was noted in the antigen treated lambs. While IL-4 is responsible for inducing differentiation of Th2 cells and production of antibodies by B cells, which play an essential role in the host's defence against parasites (Kimura & Kishimoto, 2010; Korn et al., 2009), it is considered unlikely that the IL-4 levels were a response to nematodes since the lambs had not been exposed to nematode infection at this point of the study and worm burdens in the tracer

lambs indicated insignificant pasture contamination. The minor peak in the drenched and uninfected group around the same time supports this assumption. Although the rise occurred after the final antigen treatment in the WDES and may have been a response to the antigen the peak in the NBDES at the same time is unexpected considering this group received the antigen treatment three months earlier. Moreover, this pre-infection rise is contradictive to the observations in Chapters 3 and 4 where IL-4 response peaked post-infection and coincided with a drop in FEC indicating development of immunity. The reason for the rise in IL-4 concentrations is unclear but the increase may possibly be associated with stress when the lambs were first separated from their mothers, although typically stress suppresses immune development (Emery et al., 1993).

There were inconsistent effects of larval antigens on IL-4 production. While there was a difference in IL-4 production between antigen treated and control lambs when stimulated with the *Tcol*-L3 antigen, there was no evidence to suggest that the IL-4 production to *Tcol*-L4 and *Tcol*-L5 antigen differed between the treatment groups. Further, the response did not appear to be related to nematode infection. Since the surface of L3 larvae is the main site for host-parasite interaction much research has been focused on developing antigen from L3 larvae (Maass et al., 2007; McClure, 2009; Newton & Meeusen, 2003; Wedrychowicz et al., 1995). Harrison, Pulford, Hein, Severn, and Shoemaker (2003) showed that nematode infected sheep produced an immune response against the carbohydrate larval surface antigen CarLA found on the exsheathed larvae and on the inside of the sheath on the L3 of all trichostrongyloid nematode species. Unlike the L4 and L5 larval stages, L3 is covered by a sheath which remains as a protecting cuticle until the larvae enter the host (Bruere & West, 1993). Since the CarLA antigen was only found on L3 and not L4 and L5 larvae (Harrison et al., 2003), it is possible that the sheath of the L3 larvae additionally contains some immunostimulatory compounds responsible for the greater IL-4 production noted in the antigen treated lambs in the present study although this needs further clarification. The increase in IL-4 on day 0 concurrent with the decrease in IFN-γ in NBDES suggests that the antigen treatment may have primed induction of tolerance. Tolerance is considered to be associated with an IL-4 based Th2 response (Strobel, 2001; Weiner, 1997) and IFN-γ cytokines secreted during an initial priming of Th1 immune responses which may occur before tolerance is established (Weiner, 1997). In contrast, while decreasing antibodies are typically associated with tolerance, the rapid initial decrease in serum IgA antibodies reported in the NBDES and NBCON groups on day -84 was likely attributed to the declining maternal antibodies rather than a sign of tolerance considering that both the antigen treated and the non-antigen treated lambs had similar antibody profiles. Additionally, the similar serum IgA profiles in the antigen treated lambs compared with untreated lambs post-infection and the decreasing FEC further suggest that treatment did not

successfully induce tolerance but rather skewed the immunological response towards immunity although this lack of difference between the groups in FEC suggests this was only partial.

The time from antigen treatment to the first nematode challenge may have been of importance for the outcome of the study. In the desensitising research by Greer et al. (2014) and the studies in Chapters 3 and 4, the first infection occurred a week following the last antigen injection while the NBDES in the present study were antigen treated at birth and infected four months later. It is possible that in the absence of a priming injection, infection may have occurred too late for the advantages of tolerance to be prominent and the immunological effect of the antigen had perished by the time of the first infection at 16 weeks of age, despite reports that tolerance can be maintained for several months following contact with an antigen (Strobel & Mowat, 1998). Furthermore, Strobel and Ferguson (1987) showed that the suppression of antibody responses associated with tolerance lasted up to six months after a single dose of ovalbumin. Potentially the lambs in the present study were too young at the time of treatment or first nematode challenge given there was no difference in performance between antigen treated and non-antigen treated lambs. Although the antigen treatment protocol by Greer et al. (2014) and from Chapters 3 and 4 was used in the present study these studies were conducted indoors with the antigen treatment carried out in four-and five-month-old lambs whereas the lambs in the present study were antigen treated either at birth or at weaning (three months of age) suggesting that the lambs in the present study might have been too young for the antigen treatment to have an effect, implying that the window for inducing mucosal tolerance or increasing protective immunity might have been missed since the immune system may have been unresponsive to the antigen at this time. Very young lambs are considered to lack the ability to acquire immunity to parasites (Dineen et al., 1978; Dineen & Wagland, 1966; Iposu et al., 2010; Kambara et al., 1993; Smith et al., 1985) and have a lower resistance to infectious diseases caused by an immunological hyporesponsiveness (Colditz et al., 1996). This is supported by Iposu et al. (2008) who found no effect of infection on performance or changes in serum antibodies in young lambs infected with different levels of *T. circumcincta*, suggesting that they were immunologically hyporesponsive and had not yet developed an immune response against the parasites. Moreover, Kambara et al. (1993), reported no difference in lymphocyte responsiveness between infected and uninfected lambs challenged with *T. colubriformis* L3 larvae at 8-20 weeks of age. However, the authors noted increased immunological response in older infected lambs compared with controls. Similarly, Vervelde et al. (2001) demonstrated different levels of protection in lambs of different age groups vaccinated against *H. contortus* with subcutaneous injections of ES products from the nematode. Reductions in worm burdens of 77% and 82% were seen in six-and nine-month-old lambs, respectively, however there was no reduction in three-month-old lambs. The older lambs also had greater antibody productions than the three-month-old group and the

authors suggested that the lower protection in youngest lambs could be due to suppressed immune responses. Furthermore, McClure (2009) observed a protective immunity following rectal administration of *T. colubriformis* L3 antigen placed onto the rectal mucosa in six-month-old lambs confirming that antigen treatment can be effective in older animals.

While previous desensitising research by Greer et al. (2014) showed a temporary increase in liveweight gain and feed intake during a period of six weeks in the antigen treated lambs, no effect on liveweight gain was seen in the present study. The generally greater live weight in the lambs treated with antigen at birth and their twins compared with the other four groups is likely a result of a higher feed allowance from birth until weaning when the NBDES and NBCON groups and their dams, for logistical reasons, were grazed separately from the other lambs with a greater quantity of feed being available in the paddocks where the NBDES and NBCON groups were reared. The antigen doses in the present study may have been inadequate to successfully induce tolerance and more adequate for establishing immunity, yet not sufficient to improve performance in the antigen treated lambs. This is supported by the lack of difference in serum IgA, liveweight gain, FEC and worm burden between the NBDES and NBCON lambs. Buchanan et al. (2013) showed that several low doses of oral ovalbumin antigen administered in new-born lambs proved to trigger immunity when subsequently challenged with ovalbumin and that a single high dose of oral ovalbumin antigen induced tolerance in new-borns with a negative impact on parental immunisation later in life. The authors suggested that whether lambs respond to an oral antigen with tolerance or immunity is dependent on dose or exposure. Moreover, Sun et al. (2006) demonstrated enhanced numbers of Foxp3+ Treg cells associated with mucosal tolerance in mice immunised with three low doses of 200 µg ovalbumin antigen and CTB adjuvant or one high dose of 20 mg ovalbumin administered orally. Similarly, Fujihashi et al. (1999) observed decreased IgG and IgE levels and reduced T cell responsiveness in mice immunised orally with 2.5 mg ovalbumin followed by subcutaneous injections with 100 µg ovalbumin/CFA or 100 µg HEL/CFA seven days later, indicating that mucosal tolerance was induced. The authors further reported hyporesponsiveness in mice given 25 mg ovalbumin orally and suggested that induction of systemic tolerance is possible with either high or low antigen doses.

In summary, the present study provides no evidence that the antigen treatment induced mucosal tolerance regardless whether it was administrated to a weaned lamb or neonate, although there were some immunological findings indicating that the antigen treatment regime induced a Th2 immune response with a greater response in the antigen treated lambs post-weaning compared with their non-antigen treated counterparts. However, it did not mitigate the negative effects of nematode infections on production in the lambs.

# Chapter 6

# General Discussion and Conclusions

## 6.1 General Discussion

The findings of this thesis demonstrate that repeated injections with a somatic *T. colubriformis* antigen were associated with Th2-based immunity and superior cytokine production in the antigen treated lambs with the most efficient antigen and delivery route being the antigen combination injected to the rectal submucosa. Nevertheless, it is clear that the current set of experiments did not successfully induce mucosal tolerance to any level that could mitigate the effects of infection and there was no evidence of the antigen treatment being effective in neonates.

Given the reductions in worm length in the COMB group (Chapter 3) and the RE group (Chapter 4) were comparable and greater than the worm length reductions in the remaining groups, this suggests that the combination antigen was more efficient than the individual larval antigens and the intra-rectal delivery route more effective than the subcutaneous route for delivery of the somatic nematode antigen. The reduction in FEC in the antigen treated animals in all three trials and the reduced worm burdens and shortened worm lengths in Chapters 3 and 4 are consistent with the increased serum IgA production. The declining serum IgA towards the end of these studies are in agreement with Sykes (2008) who described declining peripheral IgA levels related to establishment of immunity. Further indications of developing immunity were the increasing antibody levels and declining FEC that were evident in all three trials. These findings, except for the unexpected reduction in IgG towards the end of the study described in Chapter 4, are supported by Bisset and Morris (1996), Douch et al. (1994) and Shaw et al. (2012) who showed that elevated IgG1 and IgA were negatively correlated with FEC. It is unknown what caused this decline but it is possible that the poor health of the lambs on delivery has affected their immunological status and confounded the results. In contrast, serum IgA did not decline in Chapter 5.

Reduced FEC has been associated with increased IgE responses (Bendixsen et al., 2004; Gossner et al., 2013; Murphy et al., 2010; Shaw et al., 1999) and it has been found that IgE plays a significant role in parasite expulsion (Huntley et al., 2001; Miller, 1996). Although serum IgE was increased in the antigen treated animals in Chapters 3 and 4, the infected groups had similar serum IgE concentrations despite harbouring greater worm burdens. IgE also decreased unexpectedly towards the end of both studies. Since a major role of IL-4 cytokines is to induce IgE production by B cells (Abbas et al., 1996) it is

proposed that the elevated IL-4 in the antigen treated animals compared with the infected control groups may have been involved in the reduction of worm establishment that were evident in Chapters 3 and 4 despite inconsistent serum IgE and IL-4 concentrations.

Whereas IL-4 cytokines are secreted during a Th2 immune response it was surprising that the INF group in Chapter 3 had IL-4 concentrations comparable with the control group despite reduced FEC and increased antibody levels. In contrast to the indoor studies (Chapters 3 and 4) where the IL-4 concentrations increased post-infection in the antigen treated groups, there was an unexpected peak in IL-4 pre-infection in the antigen treated lambs in the grazing study. While it remains unclear what caused this rise, the subsequently low IL-4 concentrations further supports the suggestion that the worm reduction was associated with the production of IL-4 cytokines given there was no indication that the antigen treatment reduced worm establishment in the grazing study. Despite the rise in IFN-γ in Chapter 5 being consistent with the decrease in IL-4 and indicative of priming of tolerance, the elevated antibodies and FEC reduction are contradictive. Moreover, there were no indications of mucosal tolerance in the two indoor studies. While it has typically been considered that IL-4 is associated with immunity and IFN-γ with susceptibility to nematodes, challenge has been seen to upregulate the production of both Th1 and Th2 cytokines (Meeusen et al., 2005). Furthermore, Coltman et al. (2001) suggested that the expression of IFN-γ was linked to reductions in FEC and increased IgA production and can enhance Th2 immune responses.

Despite similar small intestinal worm burdens in Chapters 3 and 5 the lack of reduction in feed intake and growth in the infected lambs in Chapter 3, suggest a lower pathogenicity of the worms in this study since reduction in feed intake has been estimated to contribute up to 90% of production losses in lambs (Sykes, 1987; Vanhoutert et al., 1995) and is considered the greatest contributor to impaired performance as a consequence of gastrointestinal nematode infections. While the worm burden in the infected group in Chapter 4 was much greater than in the other two studies, 34,000 compared with 12,000 and 15,000 in Chapters 3 and 5, respectively, there was a lack of difference in performance between infected and uninfected lambs in the former study. Similarly, there was no difference in Chapter 3 and it is unknown what caused the lack of difference in these studies. The elevated Cu levels in the feed may have reduced the pathogenicity of the worms in Chapter 3. While Cu has proven less effective against intestinal nematodes than abomasal nematodes (Burke et al., 2004; Knox, 2002; Leal et al., 2014; Soli et al., 2010), some reduction on egg excretion in ewes treated with Cu oxide wire particles was observed by Burke et al. (2010) and it cannot be discarded that Cu has affected the pathogenicity. However, this does not explain the lack of effect on performance in Chapter 4. Given the carcass compositions in Chapters 3 and 4 indicated no reductions in tissue deposition nor energy

utilisation this suggests that the nematodes did not cause metabolic distress in the lambs. In contrast, Greer et al. (2014), reported improved performance in antigen treated lambs infected with 2,000 *T. colubriformis* day$^{-1}$ reflected in a 2.03 kg increase in carcass weight compared with infected controls and a 20% reduction in gross efficiency of use of metabolisable energy for net energy deposition in the infected lambs during the acquisition period but in those studies nematode infection was pathogenic. Potentially the control animals in Chapter 3 were not performing to their maximum production level. The poor condition of the lambs on delivery from the sheep milking facility has perhaps impacted on their growth. This suspicion was raised after the lack of difference in performance between infected and uninfected lambs was observed in Chapter 3. Therefore, the immunosuppressed animals were included in the following study (Chapter 4) as an additional positive control group. However, no significant difference in performance parameters were observed between them and the other groups. Despite evidence of negative impacts of the parasites, e.g. declining serum total protein and albumin concentrations suggesting greater pathogenicity of the worms in Chapter 4, it was not enough to reveal a difference in feed intake or carcass composition. Apart from low pathogenicity it is also possible that the INF lambs were quite resilient to infection thereby little noticeable difference on production. As such, showing any improvement in performance in antigen treated lambs is difficult when infection itself is not pathogenic. While the lambs treated with antigen at birth (Chapter 5) and their twins had a generally greater live weight compared with the other four groups, likely due to being grazed in paddocks with greater quantity of feed available, this did not appear to have impacted significantly on the result of the study as there was no differences in performance such as liveweight gain and carcass composition nor in immunological parameters. Although feed intake was not measured in Chapter 5, the lower liveweight gain in the infected lambs compared with the uninfected controls strongly indicates that feed intake was decreased and the larvae pathogenic enough to cause reduced growth. Kyriazakis, Oldham, Coop, and Jackson (1994b) recorded a depression in feed intake around 10% and 30% reduced liveweight gain in lambs infected with 2,500 *T. colubriformis,* a figure similar to that previously reported by Sykes and Coop (1976). While the lambs in Chapter 5 were exposed to larvae on pasture and additionally harboured an abomasal worm burden from grazing it is possible that this has had an impact on feed intake and performance. Likewise, Coop et al. (1982) and Greer et al. (2008) observed decreased feed intake and growth in lambs with similar worm burdens.

Unlike in Chapter 4, there was lack of evidence of direct pathological damage by the parasites in Chapter 3 reflected in the similar profiles in serum P and serum albumin between infected and control animals. The reason for the declining serum albumin concentrations in the control animals is unknown but is possibly a consequence of insufficient levels in the feed. Common features of gastrointestinal nematode infections are hypophosphataemia (Bown et al., 1989; Poppi et al., 1985; Wilson & Field,

1983) and hypoalbuminemia (Coop et al., 1976; Steel et al., 1980; Sykes & Coop, 1976) and while endogenous protein losses to the alimentary tract during nematode infections can reduce protein supply to muscle tissues (Bown, Poppi, & Sykes, 1984; Poppi et al., 1986), there was no indication of this in Chapter 3 confirmed by the similar carcass compositions and growth rates between infected and uninfected animals. In contrast, the reduction of serum albumin and serum total protein in INF in Chapter 4 indicate some leakage of plasma proteins to the gastrointestinal tract likely caused by the parasites. However, there was no reduction in growth to indicate replacement of endogenous plasma losses from muscle tissue. The slightly greater serum urea concentrations in the immunosuppressed lambs were concomitant with the lower lean tissue deposition on day 45 which presumably reflects availability of amino acids through catabolic effects of the corticosteroids (Turini et al., 2003). This in conjunction with the absent reduction in serum total protein and serum albumin in the immunosuppressed and antigen treated animals suggests that the corticosteroids and antigen treatment enhanced the lambs´ ability to replace protein losses into the alimentary tract as a consequence of infection.

Since it was hypothesised that the antigen treatment would induce tolerance the antigen was administered without an adjuvant given most adjuvants have immunostimulatory properties (Guy, 2007). However it appears that the antigen treatment boosted immunity rather than tolerance, thus it is proposed that the addition of an adjuvant could possibly have enhanced the immune response although this was not the intention. Previous immunising studies have shown promising results using aluminium hydroxide and beryllium hydroxide adjuvants with reductions in FEC and worm burdens (Jacobs et al., 1999; Wedrychowicz et al., 1995). Apart from the immunological findings and reductions in worm burdens in the present studies, suggesting a vaccinating response following antigen injections, there was some advantage from treatment on a production level in the RE group in Chapter 4 compared with the untreated infected group. Although there was a reduction in worm burden and worm length in the SC group in Chapter 4 which indicates some effect of the antigen when administered subcutaneously, there was no increased performance in this group. While there was an improved growth in the intra-rectally injected lambs during the last 21 days of the study (Chapter 4), the lack of effect on performance in the SC group, despite similar immunological responses, suggest that the addition of an adjuvant may further improve performance of immunised lambs. Anticipated obstacles to overcome in regards to mucosal immunisation are accessing the mucosal tissue for repeated immunisation and delivery of sufficient antigen over the mucosal barrier with the use of sufficient adjuvants (Sedgmen et al., 2004).

## 6.2 Conclusions

The studies reported here demonstrated that the antigen treatment induced immunity of the lambs with the combination antigen being more potent than the individual larval antigens (Chapter 3) and the intra-rectal administration route being more efficient than the subcutaneous route (Chapter 4) causing greater decreases in worm numbers and worm length and that neonate exposure does not provide extended protection (Chapter 5). There was no effect of antigen treatment in the grazing study in either of the groups treated at birth or at weaning suggesting that the lambs may have been unresponsive to the antigen or the dosage inappropriate. Overall, the lack of ability to provide reproducible desensitisation severely questions the validity of this approach to mitigate the impacts of gastrointestinal parasites.

## 6.3 Future Research

In order to better understand the nature of antigen treatment in lambs more work needs to be done. Elementary questions regarding the mucosal immune system in sheep needs to be investigated and it is suggested future research is carried out with focus on the immunological mechanisms involved in the immune response to mucosal antigen treatment to gain a better understanding of the complexity around mucosal immunity and tolerance in this species. Other relevant areas to investigate would be different dose rates and frequency of dosing. While the systemic route of immunisation has some practical benefits for administrating antigens under farm conditions, different routes of antigen delivery in various age groups would be worth exploring further, including fetal administration of nematode antigens. In conclusion, further research to identify optimal delivery routes and dose rates may provide novel approaches to induction of mucosal tolerance in sheep and potentially lead to successful development of nematode antigens as part of a successful integrated parasite control strategy.

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