THE EFFECT OF DIETARY PROTEIN AND SOME AMINO ACIDS ON IMMUNITY IN PARASITISED LAMBS

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Two experiments were carried out to investigate the effect of dietary protein and methionine and lysine on the immune system of lambs infected with gastrointestinal parasites. Both experiments were run concurrently.

In experiment I, the effect of dietary protein on the immune system of lambs was investigated. Thirty two, 8 week old lambs were individually penned, divided into two groups (n=16) and each group was fed either a low or high level of dietary protein [low protein group (79g MP/kg DM) and high protein group (157g MP/kg DM)]. These two groups of lambs were further divided in two groups (n=8), one acting as an unsensitized control, the other being sensitized with parasite larvae. Infective larvae (L3) of *Trichostrongylus colubriformis* (95 %) and *Ostertagia circumcincta* (5 %) were administered as a trickle infection at rates of 600, 1200, 1800 and 3000 L3/week for the first 4, second 4, third 3 and last 4 weeks, respectively. All lambs including unsensitized control animals were challenged with 30,000 L3 of *T. colubriformis* 17 days after the end of the trickle infection and lambs were slaughtered 11 days after this challenge . In experiment II, the effect of methionine and lysine (ML) in the diet on development of immunity in lambs was investigated. The same low protein (LP) diet used in experiment I was supplemented with ML that had been protected from rumen degradation.

Neither diet significantly increased live weight gain (LWG) in the 8-27 week old lambs over the whole period of the trial. However, there was a strong trend ($p<0.09$) for an enhancement due to HP at a number of different time points during sensitization and ML supplementation tended to improve ($p=0.06$) growth rate 6-9 week into the trickle infection. The effect of the high dietary protein on established small intestinal and abomasal worm burdens was not statistically significant, but the HP group tended to have decreased survival of *T. colubriformis* after challenge ($p=0.091$). The HP diet had no effect on abomasal worm population and faecal egg numbers. However with ML supplementation there was a significant difference in the worm counts of *T. colubriformis* in the small intestine acquired during trickle infection and the worm count of the ML group was half that of the LP group. Abomasal worm populations of *O. circumcincta* were also similar after sensitization and challenge irrespective of ML intake. Faecal egg output was decreased during trickle infection and following challenge due to ML intake. In general, both protein and ML supplementation improved parasite (L3) specific and nonspecific (mitogen) lymphocyte proliferation *in vitro* during the early trickle period ($p<0.05$). All sensitized animals irrespective of diet had elevated blood eosinophils and higher proliferative responses to L3 antigen ($p<0.05$). Levels of eosinophils correlated negatively with challenge L3 survival but L3 proliferation correlated negatively and positively with HP and ML diet groups respectively.

Key words: Dietary protein, fish meal, amino acids, methionine, lysine, parasites, gastrointestinal nematodes, *T. colubriformis*, *O. circumcincta*, immunity, sheep, lambs, worm burden, faecal egg numbers, leukocyte, eosinophil, antibody, lymphocyte blastogenesis.

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CHAPTER 1

INTRODUCTION

Parasites are organisms that metabolically depend on other organisms as a source of food or habitat at some stage in their life cycle, and this dependence frequently leads to adverse effects of varying degrees on their hosts (Gibbs, 1985; Sykes, 1994). As a result, they can seriously affect ruminants causing direct and indirect production losses (Brunsdon, 1988). In some countries, total loss could be as high as 30 % of the market value of animals or animal production (Gibbs, 1985). Thus, many control methods such as the use of anthelmintics, pasture management and the selection of genetically resistant animals have been used in an attempt to minimise losses caused by parasites and to increase animal production. However, all these methods have some shortcomings or disadvantages (eg. resistance to anthelmintics, residue problems or lack of effectiveness). Therefore, alternative methods are being researched. The enhancement of immunity to parasites by vaccination or dietary improvement is one such approach.

To be able to control parasitic infections, it is necessary to know the parasite life cycle, how they produce their pathophysiological and metabolic effects on their host, and how and to what extent the host immune system produces a response to parasites. Therefore, in the literature review, these topics are discussed and recent research findings indicated. Lastly, factors such as improved feeding, age, gender and genetic constitution of animals which can affect the immune system of host are discussed. However, to discuss all those topics in detail is beyond the scope of this study.

There are a number of studies (Abbott *et al.*, 1985 and 1986b; Bown *et al.*, 1991; Dobson and Bawden, 1974; Donaldson *et al.*, 1997; Kambara *et al.*, 1993; Kambara *et al.*, 1997; Kyriazakis *et al.*, 1996 and Van Houtert *et al.*, 1995a and 1995b) where the effects of protein supplementation on immunity to parasites are apparent and one (Coop *et al.*, 1997) where the effects of some amino acids on immunity and resilience of animals to gastrointestinal parasites in sheep are described. The slow development of immunity in young lambs is important for two reasons. Firstly, in an intensive sheep farming operation producing young prime lambs, parasites constitute a major animal health penalty. Secondly, it is a major impediment to the development of effective vaccines for use in young lambs.

The experiments in this thesis were designed to test whether the development of immunity in young lambs can be enhanced, and the studies are described in two separate consecutive chapters (Chapter 3 and 4). More specifically, the aim of this study was to

test whether a diet supplemented with protected dietary protein (fish meal) or with protected methionine-lysine and fed to young lambs could affect immunity to *Trichostrongylus colubriformis* and *Ostertagia (Teladorsagia) circumcincta*, and characterise some of the mechanisms involved.

CHAPTER TWO
REVIEW OF THE LITERATURE

2.1. GASTROINTESTINAL PARASITES IN RUMINANTS

2.1.1. ECONOMIC IMPACTS AND CONTROL METHODS

Gastrointestinal parasites have been shown to seriously influence all types of animals, particularly small ruminants, including their productivity (Gibbs, 1985; Sykes *et al.*, 1992). The economic impact of nematode parasites in ruminants can be summarised as follows: firstly direct production losses, such as mortality and a decrease in meat, wool and milk production and fertility; secondly increased production costs, such as the cost of control strategies (anthelmintic, labour, drenching equipment) and other parasite-related penalties (delay in achieving target weights, increased food requirements to achieve target weights, predisposition to other diseases, cost of unnecessary or ineffective treatments), and lastly animal welfare concerns. However, it is difficult to detect the economic losses caused by parasites because of the nature of the disease itself that often is subclinical and insidious. Invisible losses are often much greater than visible ones in parasitic diseases, for instance it is not so easy to assess the loss of condition or prolonged unthriftiness in terms of decreased capital value caused by reduced marketability (Gibbs, 1985).

Total losses from internal parasites can be as high as 30 % of the market value of animals (or animal production) in herds according to estimates from Germany, France and Peru (Gibbs, 1985). In New Zealand, total loss caused by parasitic diseases is about 270 million dollars per year (Brunsdon, 1988; Familton, 1991). In Australia, nematode parasites are economically recognised as the cause of the most significant disease in the sheep industry (Barger, 1993). About 50 million cattle and buffaloes, and about 150 million sheep and goats in the world die per year due to diseases according to 1977-78 estimates. It is quite realistic to attribute at least 5-10 % of the mortality and 10-20 % of the morbidity losses to helminth diseases in addition to US $ 500 million in sales of anthelmintics (Gibbs, 1985). In New Zealand, sheep and cattle farmers spend NZ$ 59 million per year for anthelmintics (McKenna, 1997). Although total losses from livestock diseases range from 15 to 20 % of the total value of animal production in the countries where intensive veterinary care of animals has been established, losses in the countries with less intensive veterinary care could be between 30 and 40 % (Gibbs, 1985).

In conclusion, parasites cause large economic losses in animal production throughout the world, and limitation of these losses in farm animals is a continuing preoccupation of authorities (Familton 1991; Sykes 1994; Van Houtert and Sykes, 1996a). Therefore, it is crucial to control parasitic diseases to be able to increase animal production, and it is also necessary to have sound information about worm biology and their population dynamics for correct design of worm control programs (Vlassof and Bisset, 1991). In other words, the aim of control is to keep parasite populations at levels that are compatible with economic production, and this can be achieved by three interrelated approaches; by the use of anthelmintics, by grazing management and by the utilisation of immunity (Donald, 1982).

2.1.1.1. Anthelmintics

The control of helminth parasites of livestock by chemotherapy has particularly been developed in the last 50 years. In the 1930s, mixtures of arsenic, copper and nicotine - that are accepted as being of marginal efficacy by today's standards- were used, although these often killed as many animals as the parasites that they harboured. In the 1960s, a new era began with the use of thiabendazole, the first broad spectrum anthelmintic. Today, the drugs available are not regarded as expensive, and they have a wide spectrum of activity and a high level of safety (Waller, 1982). There are currently three groups of broad spectrum anthelmintics active against nematodes. They employ different mechanisms to kill parasites: group 1, benzimidazoles and probenzimidazoles (albendazole, febantel, fenbendazole...) affect tubulin polymerisation; group 2, levamisole and morantel influence acetylcholine receptors; and group 3, avermectins and milbemycins (ivermectin, doramectin and moxidectin) affect chloride ion movement in the gamma-aminobutyric acid receptors (Coles, 1994).

The use of anthelmintics is still the most important control technique that is presently available for the control of gastrointestinal nematodes of sheep, and many farmers depend on it alone (Donald, 1982). However, some factors, such as the alarming development of anthelmintic resistance among nematode parasites of sheep and goats, the effects of chemicals on non-target organisms in the environment and increasing concerns about drug residues in animal products, have affected and discouraged their usage (McKellar, 1997; Waller, 1982). There is clear evidence that gastrointestinal parasites of sheep and goats develop resistance to anthelmintics more commonly than other species (Donald and Waller, 1982). Anthelmintic resistance in gastrointestinal

nematodes has been reported in many countries, such as Australia (resistance in *Haemonchus contortus, Ostertagia* and *Trichostrongylus* spp to benzimidazoles, levamisoles and avermectins), New Zealand (to benzimidazoles, levamisole/morantel, and avermectins), USA (to phenothiazine and benzimidazoles), Britain, Switzerland, The Netherlands, France, South Africa (to benzimidazoles, and avermectins), Brasil (to benzimidazoles, and avermectins), and Argentina, Paraguay and Uruguay (to avermectins) (Waller, 1985; Waller, 1997).

However, the life expectancy of certain drench families can be increased by using specific narrow spectrum products such as salicylanilides for *H. contortus*, by using the knowledge of epidemiology of the parasites to decrease the number of anthelmintic treatments and/or by using a combination of anthelmintic with different modes of action (McKellar, 1997). To use controlled-release and long acting drugs can also increase the effective life and efficiency of anthelmintics. In the controlled-release device, anthelmintic can be released at a constant level for a period of 90-100 days, and long acting drugs such as pharmacological variants of the macrocyclic lactone class of anthelmintics have a longer biological half-life (Barger, 1997).

In conclusion, it seems difficult to control gastrointestinal parasites only with anthelmintics. Anthelmintics as an integral part of control programs, should be used cautiously in close coordination with other husbandry procedures (Benz, 1985; Familton, 1991; Herd, 1993). In addition, anthelmintic resistance to all broad-spectrum anthelmintics has encouraged people to find alternative methods to be able to reduce the requirement for frequent anthelmintic treatments (Echevarria *et al.*, 1993).

2.1.1.2. Pasture management

A second control method is avoidance by the host animal to exposure to the infective stages of parasites on the pasture by means of pasture management and the creation of "safe" pastures (Benz, 1985; Vlassof and Bisset, 1991). "Safe" pasture refers to areas where larval populations are low enough not to impair the production of susceptible animals grazing them (Nichol and Everest, 1997). This is very appropriate for some countries since under temperate weather conditions as in New Zealand, more than 90 % of the total worm population is present outside the host (refugia) at any time in the life cycle period (Familton and McAnulty, 1997).

The development of free living stages on pasture takes place within a certain range of temperatures with the presence of sufficient oxygen and moisture. Development time

from egg to the infective larval stages is 5 to 7 days under optimum conditions. However, under field conditions this development period is profoundly affected by the season and therefore, usually lasts 2-3 weeks or more. Under New Zealand conditions, development percentage to the infective larvae is low (ranging from <1 % in the dry mid-summer conditions to 13 % in moist warm autumn conditions). Infective larvae are quite tolerant to environmental conditions and may survive up to 2.5-3 months in the summer depending on the degree of exposure to sunlight or desiccation. Some larvae from the autumn peak can survive 3-6 months, with numbers of them for more than 12 months, and some *Nematodirus* larvae up to 18 months (Familton and McAnulty, 1997; Vlassof and Bisset, 1991).

Lambs acquire their initial infection from two sources. First, the larvae that have overwintered because infective larvae can withstand adverse conditions for several months, even under freezing conditions as stated above. The second source of pasture contamination is from adult sheep. Compared to younger sheep adults can be more harmful in view of pasture contamination because they pass a greater volume of faeces (Benz, 1985; Familton, 1991; Familton and McAnulty, 1997; Gibson and Everett, 1972). For example, an adult sheep can pass 1 million eggs with faeces in a day and if 6 % of these eggs develop to the L3, this equates to 60 000 L3 per day (Familton and McAnulty, 1997). In addition, infection derived from the post-parturient rise in faecal egg count can exaggerate the initial infection in lambs (Gibson and Everett, 1972).

Temporary pastures are very useful in being able to minimise the availability of infective larvae for the hosts. The larvae, when present on herbage, are usually plowed under or buried in the process of establishment and those left on the soil surface are subjected to maximum harmful drying conditions. The survivability of larvae under these conditions is limited. At the same time, if animals are treated with an anthelmintic immediately prior to being turned onto such pastures, contamination can be minimal. However, most pastures which are of natural grass or an improved perennial grass are permanent and therefore, conditions on these pastures are optimum for survivability of infective larvae (Benz, 1985; Euzeby, 1981).

There is disagreement about the rotation of permanent pastures as a control practice. According to this procedure, animals are allowed to graze a pasture for a period then later are removed for several weeks and herbage is allowed to grow before the area is grazed again. Therefore, the infectivity of the pasture decreases over time since larvae die exponentially. This procedure is very convenient for maximum forage production but

does nothing to reduce the level of pasture contamination if the rotation is short (Benz, 1985). However, in South Africa, removal of the flock from pasture for 30 days, resulted in little reinfection during this period and proved that this method can be very efficient on worm control in hot dry conditions (Reinecke, 1994). Likewise, when the time of turnout onto permanent pasture was postponed for a month (from mid-May to mid-June) fewer overwintered larvae were picked-up and pasture contamination was significantly decreased on cattle pastures in Denmark (Nansen *et al.*, 1987).

Alternate grazing by sheep, cattle and horses is another pasture management method which usually depends on the species or genus of parasites. For example, there is no transmission (except for *N. battus*) from sheep to cattle or *vice versa* for *Ostertagia, Nematodirus* and *Bunostomum*. Very few transmission possibilities are available for *Cooperia spp* and *Trichostrongylus spp* of the small intestine. However, the abomasal worms *T. axei* and *Haemonchus spp* may be crosstransmitted between sheep and cattle (Euzeby, 1981). When weaned lambs and cattle were alternately grazed, live weight gain (up by 5 to 6 kg) and wool production (up by 23 %) were increased when compared with weaned lambs not alternated with cattle. Moreover, lamb performance in alternating groups was equal to that of weaned lambs drenched monthly (Nichol and Thompson, 1986). In Europe, control programs, which integrate minimum anthelmintic treatments with the use of safe pasture which were contaminated with very low level of nematodes, have been applied. However, a recent study in Scotland indicated that cattle parasite larvae may survive over 12 months at high levels (Bairden *et al.*, 1995) which makes it very difficult to generate "safe" pastures under such conditions.

In conclusion, the number of infective larvae on pastures can be reduced using some pasture management techniques and by means of this, the effective life of anthelmintics can be increased which means greater success in parasite control and animal production (McAnulty *et al.*, 1982).

2.1.1.3. Immunogenetic control

It is clear that there are very substantial differences in worm and faecal egg counts of individual animals when exposed to field infection under the same environmental conditions and field challenge. These differences in faecal egg counts which reflect significant differences in worm burdens of individual animals imply that the genetic constitution of animals can be important for the control of parasites (Dargie, 1984; Dineen, 1984; Ogilvie and De Savigny, 1982). A considerable amount of variation exists

between breeds of sheep (eg. Red Maasai versus Dorper) with respect to resistance to gastrointestinal parasites (Baker et al, 1993). Genetic influences are also important within breeds in that some individuals acquire resistance faster, with earlier and/or greater protective responses than others (Emery *et al.*, 1993; Wedrychowicz and Bezubik, 1990).

Windon *et al.* (1988) established lines of sheep that were high or low responders to vaccination and challenge infection, through selection based on an age-dependent responsiveness, and observed that general immunological competence to *T. colubriformis* was increased in high responder (resistant) sheep. These high responder animals had better antigen recognition, with enhanced parasite-specific cellular and humoral responses, and effector responses involving mast cells, globule leucocytes, circulating eosinophils and mediator release (Windon, 1996). Similarly, Romney sheep have been selected for nematode resistance to field challenge in New Zealand on the basis of faecal egg counts, with a heritability of 0.32 (Douch *et al.*, 1996).

2.1.1.4. Vaccines

There have been considerable advances in the development of experimental vaccines including the isolation of secreted enzymes and several structural antigens from the sheep parasites *H. contortus*, *O. circumcincta* and *T. colubriformis*. Following the initial success of the cattle lung worm (*Dictyocaulus viviparus*) and the canine hookworm (*Ancylostoma caninum*) vaccine, γ-irradiated, attenuated L3 (reviewed by Emery *et al.*, 1993; McFarlane, 1997) were used to induce immunity in the first generation gastrointestinal nematode vaccines.

Adams *et al.* (1989) observed a 90 % reduction in the faecal egg outputs from sheep vaccinated and infected with *H. contortus* and a 78 % reduction in sheep vaccinated with *T. colubriformis* after having developed an acquired immunity with irradiated larvae. However, Angus (1979) did not observe any significant difference in susceptibility of lambs to *H. contortus*, after parenteral administration of larval antigen in adjuvant together with the two doses of irradiated larvae. Boisvenue *et al.* (1987) inoculated helminth-free lambs subcutaneously with a purified metabolite of exsheathed third stage *H.contortus* larvae but this vaccination did not inhibit the development of worm populations in the lambs although worm egg production appeared to be inhibited.

Munn *et al.* (1993) observed that lambs vaccinated with extracts of adult *H. contortus* enriched with H11, an integral membrane protein from parasites intestinal microvilli,

showed an 89 % reduction in parasite egg production and an 88 % reduction in total worm burden at post-mortem, 35 days post-challenge. One of the recent research challenges is the development of a process to produce protective antigens in large volume. To achieve this goal, research has centred on the production of recombinant antigen vaccines made up of parasite gut antigens (*H. contortus*) or excretory-secretory antigens of infective larvae (*T. colubriformis*). As a result, vaccines against nematodes could provide good protection even if their efficacies are below 100 % and could be an alternative control in the future (McFarlane, 1997).

2.1.1.5. Diet and parasite control

Another alternative in controlling parasites is to help animals (especially those under dietary stress such as young growing lambs, pregnant or lactating ewes) to develop stronger immune responses which provide functional protection against nematodes and reduce the debilitating effects of parasites. This may be provided by improved feeding (Bown *et al.*, 1991; Donaldson 1997; Kambara *et al.*, 1993; Van Houtert *et al.*, 1995a). In this regard, the effects of protein and amino acid nutrition will be discussed elsewhere in this chapter in detail.

In conclusion, all parasite control methods have some shortcomings, therefore, control programs should not depend on only one method, but involve an integrated approach, for instance using anthelmintics, improved feeding, pasture management and genetically resistant animals (Nichol and Everest, 1997; Windon 1996; Wakelin, 1989).

2.1.2. LIFE CYCLE OF GASTROINTESTINAL PARASITES IN RUMINANTS

The trichostrongyloid parasites are small, hair-like worms of the gastrointestinal tract of animals and birds. Structurally they have got few cuticular appendages and a vestigial buccal capsule. The males have a well develop bursa and two spicules that are important in species differentiation. The life cycle of these parasites is direct, often non-migratory and the L3 is the infective stage (Urquhart *et al.*, 1987).

In New Zealand, sheep and cattle harbour many different nematode species. In sheep, *Haemonchus*, *Ostertagia* in the abomasum and, *Trichostrongylus*, *Nematodirus* and *Cooperia* in the small intestine most commonly cause production losses and clinical disease. In cattle, the abomasal parasites *Ostertagia ostertagi* and *Trichostrongylus axei* are the most common with secondary contributors being the small intestinal parasites *Trichostrongylus spp.* and *Cooperia spp.* These parasite species are typical of cool

temperate climatic zones around the world, and a moist and moderate climate as in New Zealand is especially favourable for the development and survival of parasite larval stages (Charleston, 1986; Vlassoff and Bisset, 1991). These parasites are the causative agents of trichostrongylosis, a common term used to describe the diseases caused by parasites in the *Trichostrongyloidea* and *Strongyloidea* superfamilies (Benz, 1985).

The life cycle of gastrointestinal nematodes covers a complex interaction among the parasite, the infected host, and the physical conditions of the pasture environment and climate (Vlassoff, 1986).

In nematodes, the sexes are separate and the females, that lay eggs or larvae, are often bigger than males. There are four moults, the successive larval stages being designated L1, L2, L3, L4 and L5 in the complete life cycle (Urquhart *et al.*, 1987). In the life cycle of most trichostrongyle nematodes, the basic aspects are quite similar. Adult parasites inhabit the alimentary tract and each species occupies a particular location. Adult female nematodes produce eggs and these pass to the outside with the host faeces. The embryo develops into the 1st stage larva within the egg after a day on the ground under favourable conditions. The 1st stage larva after leaving the egg feeds on bacteria and moults to the 2nd stage, shedding its cuticle in the process. Then, the 2nd stage larva moults to the 3rd stage, which is infective to the host, retaining the 2nd stage cuticle. Under optimum environmental conditions, this process from ovum to 3rd stage larva lasts 7-10 days, however, under field conditions, this varies with the season and usually takes 2-3 weeks. In New Zealand, the percentage of eggs which develop to the infective stage ranges from <1 % in the dry summer conditions to 13 % in moist warm autumn conditions. Unlike eggs L1 and L2, infective larvae (L3) are very resilient to adverse conditions and can survive up to 12 months or more (Benz, 1985; Pomroy, 1997; Vlassoff and Bisset, 1991).

Third stage larvae infect the host following oral ingestion of contaminated feed or water. Then, larvae pass to their predilection site, shedding the 2nd stage cuticle (exsheathment) in the process and enter the mucosal folds and digestive glands where they moult to the 4th stage within 1 or 2 days. The larvae emerge to the mucosa surface and moult to the 5th stage or adult stage after about 10 days following ingestion. Most trichostrongyle parasites gain sexual maturity and begin producing eggs in about three weeks (Benz, 1985).

Arrested larval development (inhibited larval development, hypobiosis), means a temporary cessation of the development of a nematode in its development, and can be

seen in some strains of nematodes. As it is a facultative characteristic, a number of factors affect and initiate arrested larval development. Firstly, environmental conditions are a stimulus for the free living infective stage larvae before ingestion by the host. Secondly, acquired immunity of the host is another factor which may be involved in arrested development of the L3 and L4 stages. However, the proportions of larvae arrested are not usually so high as in hypobiosis. As a result, arrested larval development is epidemiologically very important since it ensures the survival of the nematode in adverse conditions and subsequent maturation of larvae increases the contamination of the environment (Pomroy, 1997; Urquhart *et al.*, 1987).

Another phenomenon that impacts on the normal parasitic life cycle and is of significant epidemiological importance is the periparturient rise in faecal egg counts. The periparturient rise (Spring rise) refers to an increase in the numbers of parasite eggs in the faeces of animals around parturition. In sheep, the cause of this phenomenon has not been clearly elucidated but may result from a temporary relaxation in immunity due to changes in the circulating levels of either prolactin, adrenal corticoid or adrenocorticotrophic hormones which seem to decrease parasite-specific immune responses. The role of prolactin is ambiguous (Coop *et al.*, 1990; Jeffcoate *et al.*, 1990). Donaldson *et al.*, (1997), observed that protein supplementation can also affect the parasite status of breeding ewes around parturition period when their nutrient requirements are high. The rise in faecal egg count around parturition can be due to maturation of arrested larvae, an increase in establishment of infections ingested from pasture and decreased turnover of existing adult parasites, and an increase in fecundity of existing adult worm populations. Epidemiologically, this rise endangers new-born animals in addition to ensuring the survival and propagation of parasites (Urquhart *et al.*, 1987).

2.1.3. PATHOPHYSIOLOGICAL EFFECTS OF PARASITES IN RUMINANTS

Parasites are adapted to live in a particular part of the digestive tract, and they have a very specific association with their hosts. For instance, in the abomasum, *Ostertagia spp.* fourth stage larvae live in the glands until returning to the lumen as an adult but *T. axei* have a more superficial relationship with the host. Likewise, adult *T. colubriformis* worms have an intraepithelial association in the small intestine with the host (Sykes, 1994).

In the abomasum, parasites that damage the parietal and chief cells cause a reduction in acid secretion and therefore, an increase occurs in abomasal pH from 2-3 to 5-6 (Coop *et al.*, 1979; Sykes and Poppi, 1986). Large amino acid chains are not hydrolysed as insufficient hydrochloride acid is secreted in order to activate the enzyme precursor to pepsin. If intact protein molecules are not first hydrolysed to peptones and polypeptides in the abomasum, then normal dipeptidase functions cannot take place in the small intestine (Benz, 1985). Parasites also affect hormone and enzyme secretion.

Histologically, infections with abomasum can have serious consequences. Abomasal folds are dramatically reduced in height and numbers following parasitism, and in affected areas, disruptions of the tight intercellular junctions take place that results in abnormal exchanges between interstitial fluid and the abomasal contents. Therefore, in affected ruminants, abnormally high amounts of pepsinogen and gastrin may be detected in the blood plasma. Concurrently, significant losses of various plasma proteins including serum antibodies and other ions such as Na^+ and Cl^- occur into the gastrointestinal tract. Anaemia also is common with severe, prolonged infections due to lack of normal protein digestion, loss of interstitial proteins (Benz, 1985) as well as iron loss particularly with blood sucking parasites.

In the small intestine, parasites can destroy villi in the first 5-7 m or cause villous atrophy while crypts become longer and straighter than normal (Coop *et al.*, 1979; Sykes and Poppi, 1986). In addition to villous atrophy, crypt cell hyperplasia and destruction or distortion of the lamina propria can also be seen due to the activities of the worms or the operation of a local cell mediated immunity, or most probably, a combination of both circumstances (Angus *et al.*, 1979; Lloyd and Soulsby, 1987). In ruminants, these pathological reactions are seen particularly in sheep infected *with T. colubriformis, N. battus,* and *T. vitrinus*. During primary infection, villous atrophy, crypt hyperplasia and inflammation are most severe at the location of the parasites, probably because of direct physical damage. However, partial villous atrophy, which appears to be immunologically mediated, is seen around infected areas and in lightly infected sheep (Lloyd and Soulsby, 1987).

In ruminants, most gastrointestinal nematode infections are chronic, and accumulation of mast cells and globule leukocytes occurs in the intestine. Substances released from these cells such as histamine and leukotrienes can cause the characteristic leakage of blood proteins into the lumen, hypoproteinemia and alterations in protein metabolism. There is also a deficiency in brush-border enzymes that influence nutrient absorption (Lloyd and

Soulsby, 1987). In heavily infected animals, diarrhoea and plasma protein losses occur, that cause weight loss (Benz, 1985; Urquhart *et al.*, 1987).

2.1.4 METABOLIC EFFECTS OF GASTROINTESTINAL PARASITES

2.1.4.1. Food intake

The most important single effect of gastrointestinal parasites on animal performance is the reduction in voluntary feed intake (Sykes, 1994). Many parasites cause a reduction in feed intake and this may range from a progressive reduction in chronic infections to almost complete anorexia in acute parasitism (Coop *et al.*, 1976, 1977; Coop, 1981; Holmes and Coop, 1994; Leng, 1981; Sykes, 1997). Challenges with *T. colubriformis* and *O. circumcincta* in lambs caused reductions of 22-66 % in feed intake during weeks two and three of infection for up to 3 months (Bown *et al.*, 1988; Coop, 1981). In naive animals, total inappetence can be seen during massive acute infection. Diet may influence this and, Bown *et al.* (1991) detected a decrease in dry matter (DM) intake of lambs infected with 3000 *T. colubriformis* larvae/day by approximately 22 % in the groups infused with sodium caseinate and glucose, and by 32 % in an unsupplemented group. Ewes subjected to the same modest infection showed a 25-30 % decline in food intake during lactation when they lost their immunity to larval challenge (Sykes, 1994).

Fox *et al.* (1989a and 1989b) showed that feed intake was dramatically decreased (up to 77 %) from day 37 post infection in calves infected with 10 000 *O. ostertagi* larvae/day. The decrease in appetite caused nearly 73 % of the difference in live weight gain between the infected and *ad libitum*-fed control groups.

The mechanism of depression in food intake is not clear. There are some studies that indicate the disruption of hormones such as somatostatin, gastrin and cholecystokinin (CCK) (Poppi *et al.*, 1990; Titchen, 1982) but their roles remain unclear (Reidelberger, 1994).

2.1.4.2. Feed digestion

Parasites can also decrease the efficiency of food utilisation (Coop *et al.*, 1982). The efficiency of metabolisable energy available for growth and maintenance can be reduced by up to 50 % even with subclinical abomasal or intestinal parasitism in lambs (Coop, 1981; Leng, 1981; Sykes and Poppi, 1986).

2.1.4.3. Protein metabolism

Bown *et al.* (1991) observed that parasites (*T. colubriformis*) caused an increase in irreversible loss of endogenous protein from the small intestine rather than catabolism of protein to supply energy or failure of protein absorption. Unresorbed residues pass the large intestine and they are excreted either in the faeces or via the urine as urea following ammonia release in the hind-gut (MacRae, 1993).

The concentration and synthesis of circulating albumin is affected by a deficiency of plasma protein. In one study, hypoalbuminaemia was seen from weeks 4 to 24 in lambs following *T. colubriformis* infection even though the concentration of plasma globulin (IgG, particularly IgG1) was increased because of the development of immunity. In calves, serum albumin concentration was influenced and decreased about 30 % during weeks 3 and 4 with *Oesophagostomum radiatum* infection (Coop *et al.*, 1976). Gastrointestinal nematodes suppress the rate of skeletal muscle protein synthesis, but in many cases, increase the rate of liver protein synthesis. There was a direct correlation between the enteric loss of endogenous proteins, albumin synthesis and the faster rate of liver protein synthesis in trichostrongylosis (Coop *et al.*, 1976; Symons, 1989). In summary, parasitic infection of the gastrointestinal tract causes a leakage of plasma protein, and brings about the extra mucus secretion and proliferation of cells (Sykes, 1994). This may be counteracted by the supplementation of dietary protein which appears to improve resilience or allow continued productivity. This has been demonstrated in lambs (Coop *et al.*, 1997; Kambara *et al.*, 1993; Van Houtert *et al.*, 1995a).

2.1.4.4. Mineral absorption

In sheep, another common feature of abomasum and small intestine parasites is reduced bone growth with decreased bone matrix deposition (Bown *et al.*, 1989; Coop *et al.*, 1981;) because of a reduction in the deposition of Ca and P in the body (Bown *et al.*, 1988). Infection of the small intestine can significantly influence P absorption and induces P deficiency (Coop, 1981; Poppi *et al.*, 1990; Sykes *et al.*, 1975; Wilson and Field, 1983). Direct tissue damage in the intestine or change in pH because of the abomasal damage may indirectly reduce the P plasma concentration due to increased endogenous Ca and P losses, together with a net reduction in absorption of the minerals (Bown *et al.*, 1988, 1989). However, plasma P concentration was less affected by infection in lambs offered a diet supplemented with methionine (Coop *et al.*, 1997).

Gastrointestinal nematodes may have a significant effect on copper metabolism in sheep because of an elevation in pH in abomasal and duodenal digesta which affects duodenal copper concentration and hepatic uptake of copper (Bang *et al.*, 1990; Sykes, 1997). Thus, parasites can aggravate an existing Cu deficiency in the same animals (Hucker and Yong, 1986)

As a result, abomasal and intestinal parasitism frequently reduces skeletal growth in young lambs. Common bone lesions with these infections result in a reduction in growth of external dimensions of bones, measured as tibial length or as volume of bone, a reduction in osteoblast activity and a reduction in the degree of mineralisation of bone matrix. (Sykes and Poppi, 1986).

2.2. IMMUNITY TO PARASITES

2.2.1 Introduction: Immune system and parasites

The immune system as a major physiological system serves to protect the body against infections caused by parasites, microbes and viruses. This highly complex system has constitutive and adaptive elements that have multiple components and mechanisms involved in their activities such as multiple cell to cell interactions, specific and non specific control signals, amplification capabilities, feedback loops and memory properties (Tizard, 1982).

In mammals, the immune system is not totally successful in producing absolute resistance to helminth infections. Immunity to parasites develops slowly and rarely results in total elimination. This is not surprising because these organisms have adapted to an obligatory parasitic existence, and presumably this adaptation includes either overcoming or evading the immune system. However, parasites are more susceptible to attack by the immune system during certain times of their life cycle, eg. during the larval stages (Nansen, 1985; Outteridge, 1985; Tizard, 1982).

In ruminants, immune responses to the trichostrongyle parasites are very complex, in part because their structure is very complex. For instance, the total surface antigens of an ingested infective larva are much more complex than somatic antigens in even the largest bacteria. In addition to this, two parasitic moults take place leading to increases in the size of the larvae and in the complexity of the surface antigens (Benz, 1985). Ruminants have several immune response mechanisms to gastrointestinal parasites that involve a cascade of events such as recognition of nematode antigen, stimulation of the immune system to elicit the appropriate cellular response at the gastrointestinal mucosa, and the

release of products that promote the elimination of the parasites (Douch, 1990). There are several complex factors or defence mechanisms that affect the course of helminth infections.

2.2.1.1. Innate defence mechanisms

These include both the influences of host and helminth derived factors within the same host. Both intraspecies and interspecies competition can be seen in the latter case, particularly with respect to cestode infection where the presence of adult worms in the intestine delays the further development of larval stages in the tissues. The factors of host origin are age, breed and sex that may influence helminth burden through innate or acquired immune responses. The effects of age and sex on helminths may be hormonal, and parasites tend to synchronise their reproductive cycle with that of the host whose sexual cycle is seasonal. For example, ewes show a 'spring rise' in faecal nematode ova output, that coincides with lambing and the onset of lactation. Resistance to some helminths may be linked to factors such as blood type eg. sheep with haemoglobin AA are more resistant to infestations with *H. contortus* and *O. circumcincta* than are sheep with haemoglobin BB (Tizard, 1982).

Young ruminants, under about 6 months of age, are more susceptible to parasitic infections than are older animals (Dobson et al, 1990; Gibson and Parfitt, 1972; Kambara *et al.*, 1993). These animals develop only limited protective immunity to gastrointestinal parasites which is likely to be due to impaired immune responses such as antibody production, lymphocyte responsiveness to parasite antigen and changes in the populations of globule leucocytes in the intestinal mucosa (Kambara *et al.*, 1993; Kambara and McFarlane, 1996, Kambara *et al.*, 1997).

2.2.1.2. Acquired immune mechanisms

The gastrointestinal tract is probably the major site of antigenic stimulation because the range of antigenic material experienced by the intestine is immense and diverse (Moqbel and MacDonald, 1990; Newby, 1984; Newby and Stokes, 1984) In the small intestine, the structure of the lymphoid compartment is unique to this organ and gut-associated lymphoid tissue (GALT) consists of three basic elements known as lymphoid aggregated follicles called Peyer's patches, diffused lymphoid cells present in large numbers within the lamina propria, and intraepithelial lymphocytes present between the villar columnar epithelial cells. These components are associated with the process of antigen recognition,

processing, presentation and subsequent effector immune mechanisms including the initiation of an inflammatory reaction (Moqbel and MacDonald, 1990). The mesenteric lymph nodes and gastrointestinal tract contain 43 % of the total lymphocytes present in the body. These large typical nodes are receiving stations for dividing cells from the Peyer's patches and are the store for many of the effector T and B blasts that are antigen-specific (Moqbel and MacDonald, 1990; Outteridge, 1985).

Acquired immune defence mechanisms can be divided into two groups; humoral and cell-mediated immunity.

2.2.1.2.1. Humoral mechanisms

Humoral immunity is dependent on the expansion of B lymphocyte clones which mature into plasma cells to secrete immunoglobulins (antibodies) that function directly against parasites. In primary infections, immunoglobulins of various classes develop early and are present for several weeks (Benz, 1985). Immunoglobulins such as IgG, IgM, IgA and IgE are the most important following repeated infections. IgG is the most prevalent class circulating in the bloodstream and it is also produced locally by plasma cells in the lamina propria (Crook, 1990; Wakelin, 1984). In one study, immune sheep rejected most of their infective larvae in the first day following challenge, and this rejection was associated with an increased concentration of *T. colubriformis*-specific IgG1 and IgG2 in the intestinal mucus and the local appearance of globule leucocytes (McClure *et al.*, 1992). A moderate correlation existed between IgG titre and resistance (measured by FEC output) in 6-8 month old Romney lambs fed on New Zealand pastures (Douch *et al.*, 1996). Newby and Bourne (1976) also observed that IgG1 was the major immunoglobulin in the small intestinal secretions of calves. Cripps and Rothwell (1978), by introducing *T. colubriformis* L4 larvae into Thiry-Vella loops in worm-free and parasite resistant adult sheep, observed that the amount of IgG1, IgG2, IgM and albumin discharged from loops was increased in worm free sheep following the infection. In resistant sheep, IgA secretion into the gut was three times higher than that in worm-free animals. They concluded that the primary infection with *T. colubriformis* seemed to increase the movement of protein into the intestinal lumen and multiple infections had stimulated a local IgA response which is accepted as important in preventing pathology within the gut. IgA can take part in local immune responses since it crosses epithelial surfaces of the gut, the mammary gland and the bronchi (Nansen, 1985; Ogilvie and De Savigny, 1982; Tizard, 1982; Wakelin, 1978; Warren, 1982;) and is the predominant

immunoglobulin in the secretions of mucosal surfaces (Lloyd, 1981). Large amounts of IgA also enter the intestine with bile, and remain intact and functional in the intestinal lumen because of a secretory component (Wakelin, 1984). As a result, following secondary challenge of lambs with *O. circumcincta*, the production of IgA is increased both within the abomasum and in the lymph draining the infected site of resistant lambs (Smith *et al.*, 1987).

Although IgG, IgM and IgA class antibodies are produced in response to helminth antigens, IgE is also a significant immunoglobulin class involved in resistance to parasites. In parasitised cattle with *O. ostertagi*, IgE levels are often increased and many helminth infestations are associated with the characteristic signs of type I hypersensitivity including eosinophilia, local oedema, asthma and urticarial dermatitis (Ogilvie and De Savigny, 1982; Tizard, 1982; Wakelin, 1978; Warren, 1982;). In addition to being potent stimulators of IgE production against themselves, helminth antigens can also act as adjuvants, specific for IgE production against other, nonhelminth antigens. A positive passive cutaneous anaphylaxis (PCA) reaction to the worm antigens may be seen during many parasite infections such as oesophagostomiasis, ancylostomiasis, strongyloidiasis, taeniasis and fascioliasis (Tizard, 1982), but it was not present following *T. colubriformis* infection in lambs (Kambara *et al.*, 1997).

Although there are some negative effects of IgE and allergies, they seem to be of considerable benefit in controlling worm burdens. For example, self cure reactions can be seen in sheep infected with gastrointestinal nematodes, especially *H. contortus*. These parasites embed in the abomasal mucosa and secrete antigens during their third ecdysis that act as allergens. The combination of parasite antigens with mast cell-bound IgE causes mast cell degranulation and the release of vasoactive amines that stimulate smooth muscle contraction and increase vascular permeability, a local acute type I hypersensitivity. As a result, violent contractions of the intestinal musculature and an increase in permeability may take place allowing an efflux of plasma into the intestinal lumen. This may cause expulsion and dislodgment of the major portion of the worm burden (Nansen, 1985; Tizard, 1982).

While the other immunoglobulin classes have also a protective role against helminths, the IgE-dependent eosinophil-mediated response may be the most important resistance mechanism against helminths. The other immunoglobulins have a protective role against parasites by antibody-mediated neutralisation of proteolytic enzymes used by larvae to penetrate tissues, blocking anal and oral pores of the larvae with immune complexes as

antibodies combine with their secretory and excretory products, and prevention of ecdysis and inhibition of larval development by antibodies directed against exsheathing antigens. Antibodies can also block enzyme pathways, therefore, female *O. ostertagi* worms cannot develop vulvar flaps when grown in immune calves (Tizard, 1982).

2.2.1.2.2. Cell-mediated immunity

Parasite recognition by antigen presenting cells is necessary for the development of specific acquired immunity that takes place in the peripheral lymphoid system, be it humoral or cell mediated (Miller, 1984). Simple neutralisation by antibody that can be effective against bacteria and especially viruses does not often by itself affect parasites (Nansen, 1985). However, the adoptive transfer of lymphocytes from resistant animals to their susceptible cotwin conferred protection (Smith *et al.*, 1984).

T lymphocytes are known to be important in controlling cells behind many immune responses with the ability to organize and stimulate other effector cells against nonself molecules and organisms (Crook, 1990). There are two common classes of peripheral T-cells; T helper (CD4+) and T suppressor/cytotoxic (CD8+) cells. Levels of CD4+ cells were associated with resistance to *T. colubriformis* (Kambara and McFarlane, 1996) and the depletion of CD4+ cells *in vivo* in sheep prevents resistance to *H. contortus* (Gill, 1993). Helper T cells (Th) can be divided into at least two subsets (Th1 and Th2) in human, mice and probably in sheep. In general, resistance to helminth infections in mice and man are usually associated with Th2 mediated immune responses. The Th1 subset of helper T cells produces IL-2 and interferon gamma (IFN-γ), and Th2 subset cells produce IL-4, IL-5 and IL-10. These cytokines from Th2 cells may regulate antibody (IgE, IgG) production and the development of eosinophilia. The *in vivo* depletion of IL-4 with neutralizing monoclonal antibody prevents the occurrence of the parasite-induced IgE response in mice infected with *Nippostrongylus brasiliensis*. While anti-IL-5 blocked a helminth-induced eosinophilia in peripheral blood and tissue , a combination of anti-IL-4 and IL-3 antibodies inhibited nematode-induced mucosal mastocytosis (Scott and Sher, 1993).

Increased resistance to nematodes is closely related to the number of globule leucocytes or mucosal mast cells which are involved in the production of vasoactive amines from the mucosa of the gut. These substances suppress the mobility of parasite larvae (Douch *et al.*, 1996). Stankiewicz *et al.* (1993) demonstrated a correlation between numbers of globule leukocytes (GL) and larval migration inhibition activity in the mucus. In this

study, a significant positive correlation (r=0.92) was detected between numbers of GLs and larval migration inhibition, but a negative correlation existed between GLs, and the number of parasites in the intestine (r=-0.63), following *T. colubriformis* infection in sheep.

Buddle *et al.* (1992), Pernthaner *et al.* (1995), and Rothwell *et al.* (1993) showed that eosinophil numbers in tissues and blood had a relationship with immune responsiveness of sheep to *T. colubriformis* infection.

Immunity can influence gastrointestinal parasites in at least three characteristic situations: Firstly, the host can decrease the parasite population either by preventing their establishment or expulsion. The self cure reaction in sheep with haemonchosis is the best example of the expulsion phenomenon. This phenomenon is likely to be of a local immediate hypersensitivity reaction triggered by antigenic stimulation provided by the introduction of infective larvae. Vasoactive amines, such as histamine, 5-hydroxytryptamine, and leukotrienes appear to be the effector molecules responsible for the expulsion of parasites (Nansen, 1985; Outteridge, 1985). However, antagonists to leukotrienes and prostaglandins do not by themselves inhibit rejection even though corticosteroids do (McClure *et al.*, 1992). Secondly, some parasite species can become arrested during their larval development due to acquired immunity. The "Spring rise" seems to be closely connected with lambing and can be explained by a likely hormone-associated peri-parturient relaxation of immunity that results in increased susceptibility to establishment and resumption of the development of arrested larvae. Lastly, a reduction in size and egg-laying capacity of adult worms can be seen in chronic infections because of acquired immunity (Nansen, 1985).

2.3. NUTRITION AND IMMUNITY

2.3.1. Effects of nutrition on immunity

Nutrients can influence host response to diseases directly by acting on the immunocompetent cells or indirectly by changing metabolic, neurological, or endocrine parameters. Stability and integrity of cellular and subcellular membranes, and expression of cell surface receptors on immunocompetent cells, or changes in the circulating subsets of T cells can be affected by single nutrients. Throughout a variety of pathways, the ontogeny and maintenance of disease-resistance mechanisms of animals can also be regulated by nutritional factors (Reddy and Frey, 1990).

2.3.2.1. Energy and Immunity

Energy and protein malnutrition can sèriously affect immunity (Austic *et al.*, 1991; Chandra, 1991). Protein-energy malnutrition may influence the lymphoid tissues, particularly the thymus, and cause atrophy. The histomorphological effects on lymphoid tissues are caused by reduced cell proliferation because of decreased protein and DNA synthesis, and cytolysis because of increased levels of unbound glucocorticosteroids (Austic *et al.*, 1991; Chandra, 1991). However, energy was not a major limiting factor in efficient food utilisation of lambs with gastrointestinal nematode infections (Bown *et al.*, 1991). Similarly, Donaldson (1997) did not observed a significant difference in the resistance of ewes during the periparturient period given a diet containing different energy levels.

2.3.2.2. Minerals-trace elements and immunity

Iron deficiency can seriously influence cellular and humoral response - a deficiency seriously depressed circulating T cell levels and phytohemagglutinin-induced lymphocyte transformation in children (Gross and Newberne, 1980). Zinc deficiency significantly decreased thymus and spleen weights, peripheral blood lymphocyte counts, and serum γ-globulin levels in pigs, rats and mice. In rats, zinc deficiency seriously depressed the splenic response to phytohemagglutinin and concanavalin A (T-cell proliferative response) and pokeweed mitogen (T- and B-cell cooperation) (Beisel, 1982; Gross and Newberne, 1980). Copper deficiency decreased splenic lymphocyte proliferation in response to a variety of both T and B cell antigens. (Gross and Newberne, 1980; Reddy and Frey, 1990). Selenium deficiency decreased antibody production, but supplementation increased antibody titres in cattle and sheep and increased lymphocyte blastogenesis (Reddy and Frey, 1990). Calcium and phosphorus deficient chicks were much more susceptible to *Ascaridia galli* infestation and the parasites were more numerous and larger (Gross and Newberne, 1980).

2.3.2.3. Vitamins and immunity

Vitamins as coenzymes are essential for metabolism. Therefore, the immune response may seriously be affected by disruption of metabolism caused by vitamin deficiency which may increase morbidity and mortality from infectious diseases (Gross and Newberne, 1980).

Vitamin A is required to maintain epithelial tissue and prevent infection. Deficiency causes increased host susceptibility to infections, lymphoid tissue atrophy and affected cell morphologic features, blast cell formation, graft rejection and a changed primary-secondary humoral response (Beisel, 1982; Latshaw, 1991; Reddy and Frey, 1990; Sheffy and Williams, 1982). Pyridoxine (B6) deficiency caused a reduction in plaque-forming cell responses to sheep red blood cells, in delayed cutaneous hypersensitivity to purified protein derivative of tuberculin in thoracic duct T cells, in mixed leucocyte reactivity and in graft-versus-host reactivity in humans, monkeys, rats and mice (Beisel, 1982; Gross and Newberne, 1980; Sheffy and Williams, 1982). After immunisation with sheep red blood cells, thiamine-deficient rats showed an inhibition in splenic plaque-forming cell formation. Riboflavin deficiency usually results in a lesser primary response of antibody production after immunisation (Beisel, 1982; Gross and Newberne, 1980). Vitamin C deficiency usually increases host susceptibility to infections. It causes a reduction in size and motility and an increase in fragility of macrophages (Beisel, 1982; Gross and Newberne, 1980).

2.3.2.4. Effect of dietary protein on immunity

Investigations with animal models have indicated that simple dietary protein deficiency seriously influences immune function. Deficiency of dietary protein in animals reduced antibody affinity, reduced lymphocyte populations and response of lymphocytes to mitogenic stimulation, and decreased complement levels. These changes probably increase the susceptibility of host animals to infections (Austic *et al.*, 1991; Chandra, 1991, 1992).

Protein level of diet can also affect innate immunity of sheep. Dobson and Bawden (1974) observed that the increased susceptibility of protein deprived sheep {20-26 week-old Merino (25 %) x Border Leicester (75 %) lambs} to *Oesophagostomum columbianum* was associated with malfunctions in the innate immunity of the alimentary tract.

Protein supplementation increases the rate of acquisition of immunity to gastrointestinal parasite infections and increases the animal resistance to reinfections (Coop *et al.*, 1997). Abbott *et al.* (1985), demonstrated that a high protein diet (170 g CP/kg DM) did not affect the establishment or pathogenicity of infection with 125 *H. contortus* larvae/kg body weight (BW) in Scottish Blackface lambs. In contrast, the diet had some effects on early establishment in Finn Dorset lambs according to total faecal egg output. The same

authors (1986a, and 1986b) observed that a high protein diet (169, and 170 g CP/kg DM) in lambs increased live weight gain (resilience) with a single dose *H. contortus* (350 larvae/kg BW) infection although this diet did not affect the establishment of parasites. Lambs (3 months of age) offered a low protein diet (88 g CP/kg DM) were less able to withstand the pathogenic effects of parasites, and exhibited more severe clinical manifestations, such as inappetence, weight loss, oedema, anaemia, hypoproteinemia and hypoalbunemia.

Bown *et al.* (1991) investigated the effect of post-ruminal infusion of protein (a continuous infusion of 50.4 g/day CP as sodium caseinate) or energy (glucose) on the pathophysiology of *T. colubriformis* infection and body composition in Dorset Down x Coopworth wether lambs. They observed that increasing duodenal protein supply can significantly decrease the debilitating effects of internal parasites. Moreover, the protein supply, but not the energy had a positive effect on the ability of lambs to limit either parasite establishment or maintenance with *T. colubriformis*.

In an attempt to understand the immunological basis of such findings, Kambara *et al.* (1993) assessed the development of resistance to *T. colubriformis* in sheep, trickle infected either during 8-26 or 33-51 weeks of age and given two levels of dietary protein (11 and 20 % CP/kg DM) based on meat meal. They showed that the young animals offered a high protein diet developed better resistance as gauged by establishment of worm burden, faecal egg count and eggs *in utero* in the parasites. The more resistant lambs on the high protein diet had no evidence of improved acquired immunity such as elevated antibody titre, mucosal mast cell numbers or activity, or lymphocyte proliferation to parasite antigen following parasitism. In the older lambs only the higher level of dietary protein increased the *in vitro* T lymphocyte response to parasite antigen and mitogens, and the percentage of CD4+ cells was considerably increased in both the peripheral blood and mesenteric lymph in immunised animals (Kambara and McFarlane, 1996). In summary, immediate-type hypersensitivity reactions and antibody responses were dependent on the level of dietary protein and age of the sheep, and influenced the course of the disease.

Van Houtert *et al.* (1995a) offered fish meal as a source of protected protein at 0, 50 and 100 g per day to 3-month-old Merino wether lambs, and observed that lymphocyte stimulation *in vitro* in response to *T. colubriformis* L_3 antigen was significantly increased in infected animals at 8 months of age when given 100 g fish meal, but no effects were seen on the level of parasite-specific and non-specific circulating antibodies. Animals

offered high dietary protein were better able to reject established worms of the parasite and had decreased production losses (Van Houtert et al., 1995a, 1995b and 1996b). However, data by Kyriazakis *et al.* (1996) did not support the idea that protected protein supplements fed to sheep could affect the pathophysiology of long term subclinical intestinal parasitism (*T. colubriformis*), and the expression of acquired immunity in lambs. Lastly, Donaldson (1997) observed that protein supplementation with fish meal had a major effect on the establishment and possible rejection of *O. circumcincta* in periparturient ewes.

2.3.2.5. Effect of amino acids on immunity

There is little information about the effect of individual amino acids on immunity. Deficiencies of specific essential amino acids may primarily influence humoral response, except for methionine which predominantly affects cellular immunity when its function as a lipotrope is limiting. This amino acid works in concert with other lipotropes, such as folic acid and vitamin B_{12} which are vital to cellular nucleic acid synthesis and cell proliferation due to their role in single carbon (methyl group) metabolism (Sheffy and Williams, 1982).

Methionine and cysteine-cystine deficiency resulted in significant deleterious effects on the thymus, lymph nodes and spleen (Beisel, 1982). Methionine and cystine supplementation improved cell mediated phytohemagglutinin-P (PHA-P) responses and T cell dependent IgG responses when these amino acids were given at low levels to broiler chicks. In contrast, high supplemental methionine resulted in significant depression in both responses (Tsiagbe *et al.*, 1987a, 1987b). Marginal methionine-cysteine depressed humoral immune response in mice although there was no effect on resistance to *Salmonella gallinarum* infection in chickens with methionine deficiency. Likewise, a marginal methionine-choline diet increased susceptibility to infection, and decreased cell mediated immunity and lymphoid organ development in rats during gestation and weaning (Wattenberg, 1983). Finally, supplementation with S-containing amino acids can increase the resilience to *T. colubriformis* infection in growing lambs when they receive a low protein diet (Coop *et al.*, 1997).

2.3.2.6. Conclusion

Protein deficiency may significantly affect the ultimate immune response by affecting various facets of the response, such as nonspecific immunity, lymphocyte distribution and

function, cellular cytotoxicity, cytotoxic antibody, and the overall response depends on the interaction between these factors. In addition to these effects, dietary protein has significant effects on the tolerance and resistance of animals to gastrointestinal parasites, on the live weight gain, milk and wool production of animals, and on the elimination and egg production of parasites (Beisel, 1982; Wakelin, 1989). It is not known which of the individual amino acids, if any, are important limiting factors for immune function and resistance to gastrointestinal parasitism.

CHAPTER THREE

THE EFFECT OF DIETARY PROTEIN ON THE IMMUNE SYSTEM IN LAMBS INFECTED WITH *TRICHSTRONGYLUS COLUBRIFORMIS* AND *OSTERTAGIA CIRCUMCINCTA.*

3.1. INTRODUCTION

There are several factors that play a role in the development of immunity to parasitic infections in animals such as age, gender, genetic constitution of animals and feeding. The age of an animal affects the development of immunity to parasite infections (Dobson *et al.*, 1990; McFarlane, 1997). Young ruminants, under about 6 months of age, are more susceptible to parasitic infections compared with older animals (Gibson and Parfitt, 1972; Kambara *et al.*, 1993; Watson *et al.*, 1994). These animals develop only limited protective immunity to gastrointestinal parasites which is likely to be due to impaired immune responses such as low antibody production, less lymphocyte responsiveness to parasite antigen and low populations of globule leucocytes in the intestinal mucosa (Kambara *et al.*, 1993; Kambara and McFarlane, 1996). As a result, the acquisition of immunity by these animals to parasitic infections may be delayed until 8-24 months of age although this process is affected by several factors (Dobson *et al.* 1990; Lloyd and Soulsby, 1987; McFarlane, 1997; Van Houtert *et al.*, 1995a).

Animal gender can affect the susceptibility of the host to gastrointestinal parasites. Within genetic lines, ewe lambs were more resistant to *T. colubriformis* infection compared to rams and wethers (Windon *et al.*, 1988). Male sheep seemed also to be particularly susceptible to experimental infections with *T. colubriformis* and *H. contortus* when challenged around or after puberty (Barger, 1993). Likewise, Bawden (1969) observed that male lambs were more susceptible to infection with *O. columbianum* and they generally harboured more adult nematodes, compared with females. However, Knight *et al.* (1972), did not observe consistent effects on susceptibility to mixed worm species (*T. axei* and other 9 nematode species) due to sex. They only detected a significant difference in the numbers of *Strongyloides papillosus* resident in rams and ewes.

Genetic constitution of animals has a very important role in individual resistance to helminth infections (Windon and Dineen, 1981; Windon, 1996). There are very substantial differences in faecal worm egg counts of individual animals exposed to field

infection under the same environmental conditions and this trait is moderately heritable in some studies (Dargie, 1984; Dineen, 1984; Ogilvie and De Savigny, 1982). Windon *et al.* (1988) demonstrated that sheep could be divided into two groups termed high or low responders following vaccination with irradiated infective larvae and challenge infection, through selection based on age dependent responsiveness. High responder (resistant) sheep have higher immunological competence to *T. colubriformis* such as better parasite-specific cellular and humoral responses, and enhanced effector responses involving mast cells, globule leucocytes, circulating eosinophils and mediator release (Jones *et al.*, 1990; Windon, 1996). Gill (1994) detected an enhanced ability to mount parasite-specific cell-mediated immune response against *H. contortus* infection in resistant lambs.

In sheep, there are a number of studies that address the effect of protein intake on immunity and resilience of animals to gastrointestinal parasites. For example, an increased susceptibility in protein-deprived sheep to *O. columbianum* was associated with impaired function of the innate immunity of the gut (decreased peristalsis and failure of the mucin cell response) and with reduction of the adaptive immune response (Dobson and Bawden, 1974). Lambs given a high protein diet showed more resilience to *H. contortus* infection with less pathophysiological consequences (Abbott *et al.*, 1986b). Bown *et al.*, (1991) compared the effect of post-ruminal infusion of protein and energy on the pathophysiology of *T. colubriformis* infection in wether lambs. Although there was no significant effect of energy, protein infusion decreased faecal egg output and mean total parasite counts. Increased duodenal protein supply significantly decreased the debilitating effects of internal parasites. Kambara *et al.* (1993) observed that young male lambs (8-26 week-old) given a high protein diet (meat meal) developed better resistance to *T. colubriformis* establishment with improved growth rate. Van Houtert *et al.* (1995a and 1995b) reported that supplementary feeding with fish meal enhanced the expulsion of *T. colubriformis* burden in sheep and reduced the production losses caused by the parasite. Protein supplementation can also decrease the periparturient breakdown in resistance to gastrointestinal parasites, particularly due to *O. circumcincta* (Donaldson *et al.* 1997). However, dietary protein did not influence the establishment of *H. contortus* (Abbott *et al.*, 1985 and 1986a). Kyriazakis *et al.* (1996) observed that the use of voluntary selection of protein supplementation by sheep following infection with *T. colubriformis* infection did not significantly affect the pathophysiology of long term subclinical intestinal parasitism and the expression of acquired immunity.

The mechanisms of immunity to gastrointestinal parasites in sheep have been variously described in older sheep following protein supplementation (Kambara *et al.*,1997; Van Houtert, 1995a). However, the enhanced resistance to *T. colubriformis* infection found in lambs younger than 6 months when fed dietary protein supplements has not been defined, except that acquired immune mechanisms such as lymphocyte proliferation (Kambara *et al.*, 1993), specific antibody production, eosinophil production and gut leukotriene release do not seem to be involved (Kambara *et al.*, 1997), although local T19 (gamma-delta) cells may have a role (Kambara and McFarlane, 1996). Therefore, the aim of this study was to test whether dietary supplementation with fish meal could improve immunity to *T. colubriformis* infection in young lambs, and characterise some of the mechanisms involved.

3.2. MATERIALS AND METHODS

3.2.1. Animals

Thirty two Coopworth male lambs were maintained parasite free after birth and weaned at 3 weeks of age onto a standard moderate protein diet. At 8 weeks of age, they were individually penned, and randomly allocated into 2 groups to be given either a high protein (HP) or a low protein (LP) diet.

3.2.2. Diet

Animals were given 300 g of the diet per day at the beginning of experiment which increased to 870 g per day at the end. The amount of food offered was based on AFRC recommendations to give an average body weight increase of approximately 200 g/day in the HP group animals[‡] . The same amount of LP diet was offered to the LP group animals giving approximately equal amounts of energy, calcium and phosphorus as compared to the HP treatment groups. The addition of sodium bicarbonate and ammonium chloride was to aid in the prevention of urolithiasis, a potential problem in housed entire male sheep.

[‡] Calculated using RuMEMP (version 1.12) software developed by van Houtert MFJ and Zyskowski R, Lincoln University.

High protein diet (12.4 ME and 157 MPg/kg)

Barley grain	47.3 %
Lucerne hay	25.0 %
Fish meal	20.0 %
Molasses	5.0 %
Soya bean oil	1.0 %
Sodium bicarbonate	1.0 %
Ammonium chloride	0.7 %

Low protein diet (12.2 ME and 79 MPg/kg)

Barley grain	79.0 %
Lucerne hay	7.8 %
Molasses	5.0 %
Soya bean oil	1.0 %
Dicalcium phosphate	3.7 %
Lime ($CaCO_3$)	1.8 %
Sodium bicarbonate	1.0 %
Ammonium chloride	0.7 %

3.2.3. Parasite sensitization and challenge

Infective L_3 of *T. colubriformis* (strain LIU\89-6) and *O. circumcincta* (strain LIU\85-10) were separately cultured from the faeces of sheep that had been infected with a monoculture and were used for sensitization (vaccination) and challenge after being mixed 95 and 5 %, respectively.

Lambs (n = 16) given a high protein diet (HP) were randomly allocated into 2 equal groups and either sensitized (HP1) by oral infection with L_3 stages of *T. colubriformis* and *O. circumcincta,* or not (HP2) as controls. This "trickle" sensitization was carried out from the age of 8 until 23 weeks with 600, 1200, 1800 and 3000 L_3 per week for the first 4, second 4, third 3 and last 4 weeks respectively (given as 3 split doses in a week). After 15 weeks of trickle infection, sensitization was stopped and 17 days later (at 25-26 weeks of age), all animals (including controls) were orally challenged with 30,000 L_3 of the same parasite mix. Animals were slaughtered 11 days later. By the end of trial, lambs had been orally given 23,370 L_3 of *T. colubriformis* and 1,230 L3 of *O. circumcincta* as a trickle infection, and 28,500 L_3 of *T. colubriformis* and 1500 *O. circumcinta* as

challenge infection, respectively. Lambs (n = 16) offered a low protein diet (LP) were also divided into 2 groups and were either sensitized (LP1) or acted as a control group (LP2).The same procedure as described for the HP was followed for trickle and challenge infection.

3.2.4. Experimental procedure

3.2.4.1. Body and organ weights

Animals were weighed each week from birth to slaughter and their live weights (LW) and LW gains (LWG) were recorded. Carcass weights were measured 24 hours after slaughter. The thymus of each animal was dissected out at slaughter and immediately weighed.

3.2.4.2. Worm burdens

At necropsy, each abomasum, and small intestine was removed separately for all sheep the contents individually collected and assayed.

Worm burdens in abomasum: The abomasum was cut open over a container in which the contents were caught. The abomasal tissue was washed with a stream of water and carefully rubbed with fingers to remove any worms adhered. The contents and washings were then transferred to a beaker and made up to a volume of 2 litres. The contents of the beaker were thoroughly mixed and four 50 ml sub samples withdrawn and pooled in a labelled container to which 20 ml of concentrated formalin (37 % formaldehyde) was added. Four 5 ml abomasal aliquots were examined, this represented an one hundredth and eleventh of the original sample, therefore each worm counted represented 110 worms in the final abomasum count (McAnulty, 1997).

After washing the abomasum, the tissue was cut up into pieces and subjected to peptic digestion for 16-20 hours. After digestion, all material was passed through a 45 μ sieve and washed with a jet of water. The material collected on the sieve was fixed in 100 ml of 5 % formalin, and stored, pending examination. A one tenth aliquot of the sample was examined to give a total number of worms in the abomasal digest (McAnulty, 1997).

Worm burdens in small intestine: The same general procedure to the above was carried out in the proximal 6 metres.

3.2.4.3. Faecal egg counts

After collecting faecal samples from the rectum of each lamb, 1.7 g of faeces were placed in a jar with 5 ml of water and soaked overnight to soften. The next day, 46 ml of saturated NaCl was added and the sample mixed for 25 seconds, either by hand or with an electrical stirrer, until the faecal pellet was completely broken up. Following filtration a pasteur pipette was used to fill both chambers of a moistened McMaster slide with the faecal suspension. Any eggs present on the slide floated to the surface of the salt solution and stuck to the cover glass, where they could be counted. The number of eggs present in both chambers of the slide were counted under a microscope, totalled, and multiplied by 100 to give the number of eggs per gram (epg) for that sample. Thus, the method described had a sensitivity of one egg counted representing one hundred eggs per gram in the sample (McAnulty, 1997).

3.2.4.4. Blood collection

Blood samples were collected from the jugular vein into 10 ml vacutainers containing Na heparin. Within two hours, they were centrifuged at 2650 rpm for 20 minutes at room temperature. The buffy coat was used as a source of lymphocytes (see section 3.2.4.8) and plasma samples were collected and stored at -70 °C.

3.2.4.5. Leukocyte numbers

Blood leucocyte numbers were manually counted using an improved Neubauer haemocytometer. Whole blood was mixed 1:40 with 0.01 % Gentian violet and 1.5 % acetic acid (Kambara *et al.*, 1993).

3.2.4.6. Eosinophil numbers

Eosinophil numbers were manually counted after selective staining with eosin. Whole blood was mixed 1:5 with 0.05 % eosin and 5 % acetone. After 5 minutes, eosinophil numbers were counted (Kambara *et al.*, 1997).

3.2.4.7. Plasma antibody

Antibodies against *T. colubriformis* in plasma were measured by indirect ELISA using purified parasite antigen (see section 3.2.4.8). Flat-bottom plates (Falcon 3912, Becton Dickinson and Co.) were coated with 100 µg/ml *T. colubriformis* excretory-secretory antigen (ESA) in phosphate buffered saline (PBS), drained and stored at -70 °C.

Blocking buffer (200 µl 0.5 % bovine serum albumin - BSA - in PBS) was added and incubated at room temperature for 1-2 hr. Then, 100 µl of plasma (diluted 1/5 in PBS) was added after the plates were tapped dry. Plate wells were washed 6 times with washing buffer (0.05 v/v Tween 20/PBS), and after being tapped dry, 100 µl rabbit anti-sheep polyclonal antibody (1/5000) conjugated to peroxidase (DAKO) was added for an hour incubation which was removed by washing six times. Finally, 100 µl of substrate (50 ml substrate buffer + 20 mg OPD + 30 µl H_2O_2) was added and when colour developed (10-60 minutes), a stop solution (100 µl, 1.25 M H_2SO_4) was added, and optical density measured (OD_{492}) using an EIA reader (Bio-Rad, Model 2550).

3.2.4.8. Lymphocyte blastogenesis test (LBT)

Lymphocytes were prepared from the buffy coat of peripheral blood and the mesenteric lymph node that drained the duodenum (aseptically dissected at slaughter). Lymphocytes were separated by density-gradient centrifugation in Lymphoprep™ (Pharmacia). After washing 4 times in Dulbecco's Modified Eagle Media (DMEM)+EDTA (0.075 %), the cell suspension was adjusted to 2.3 x 10^6 lymphocytes per ml in DMEM containing 10 % fetal calf serum (FCS) with benzylpenicillin, and streptomycin sulphate. Two hundred µl of cell suspension was cultured in "U"-bottom microplates (Nunc) in triplicate with either 25 µl of buffer as a control, or Conconavalin A (ConA), or homogenate (L3AG) or excretory-secretory antigen (ESA) from *T. colubriformis* infective larvae. Plates were incubated for 48 h, and then for a further 20 h, after the addition of ^{3}H-thymidine (1µCi per well). Following incubation, the lymphocytes were harvested onto filter paper discs (Glass Fiber Filter Strips 240-1 for PHD cell harvester) using a cell harvester (PHD cell harvester. Cambridge Tech., Inc.) and ^{3}H-thymidine incorporation was measured in a liquid scintillation counter (Wallac 1409). The result was expressed as a stimulation index: mean counts per minute (c.p.m.) of stimulated cells divided by mean c.p.m. of cells cultured without mitogen or antigen (Kambara *et al.*, 1993).

Preparation of L3 antigen: Larvae homogenate was prepared from infective larvae of *T. colubriformis*. A suspension of L3 in phosphate buffered saline was frozen in liquid nitrogen, and disrupted by crushing and sonic vibration. The suspension was allowed to stand overnight at 4 ° C, then centrifuged at 30,000 g for 30 minutes at 4 °C. The

supernatant was collected, filtrated through a 0.2 μm filter and kept at -20 °C until further use.

Preparation of excretory-secretory antigen from the infective larvae (L3): This procedure was adapted from O'Donnell *et al.*(1989). There was a modification with the gas mixture used (about 60 to 70 % N_2) which was bubbled through the L3 suspended in 0.05 M succinic acid and 0.02 M sodium tetraborate at pH 2.8, for 2 to 10 hours.The exsheathment process varied from 40 to 70 % which was inversely proportional to the larval concentration of the suspension. The suspension was centrifuged at 1500 rpm for 5 minutes, the supernatant was collected and stored at -70 °C until further use.

3.2.4.9. Statistical analysis

To all results an analysis of variance was carried out using Systat (univariate and multivariate repeated measures analysis where appropriate) (Version 5, SYSTAT Inc., USA). Variables were diet (LP and HP) and parasite infection (sensitized or not). The Wilcoxon signed rank test (non-parametric) was used for comparing small intestine and abomasum worm burdens as the distribution of the data was skewed with or without transformation (Jacobs *et al.*, 1994; Kambara *et al.*, 1993). Faecal egg counts were transformed {log 10 (count+1)} to normalise data. Likewise, lymphocyte stimulation indexes were transformed by natural logarithims {$\log_n$ (count+1)} (Kambara *et al.*, 1993). Pearson correlations between certain measurements were carried out using SYSTAT.

3.3. RESULTS

3.3.1. Live weight gains

Over the period of the trial, the animals fed the high protein concentrate (HP1 and HP2) tended to have higher live weights and live weight gains (Table 3.1), compared to LP1 and LP2. This was more apparent during the last 5 weeks of the trial where the differences between pooled dietary groups had a p value of 0.081 when repeated measures analysis was used. The mean LW of groups HP1, HP2, LP1 and LP2 at the beginning of the trickle infection (8 weeks of age) were 11.08, 13.56, 13.38 and 11.58 kg, respectively. By 16 weeks of age, the LWs had increased almost 2-fold to 21.91, 23.2, 22.03 and 20.39 kg for HP1, HP2, LP1 and LP2 and at the end of the trial (27 weeks of age), the animals' LWs had reached 33.03, 35.29, 30.68 and 32.11 kg. Although, the differences between infected (HP1-LP1 = 2.35 kg) and control groups

(HP2-LP2 = 3.18 kg) due to diet effects were statistically not significant overall (p=0.153), during the first 4 weeks of infection the HP1 animals grew faster than the LP1 group (p values varied between 0.011 and 0.09 for diet*infection interactions). Over the period of the trial differences in LWGs were not significant due to infection (p>0.05)and diet infection interaction (p>0.05).

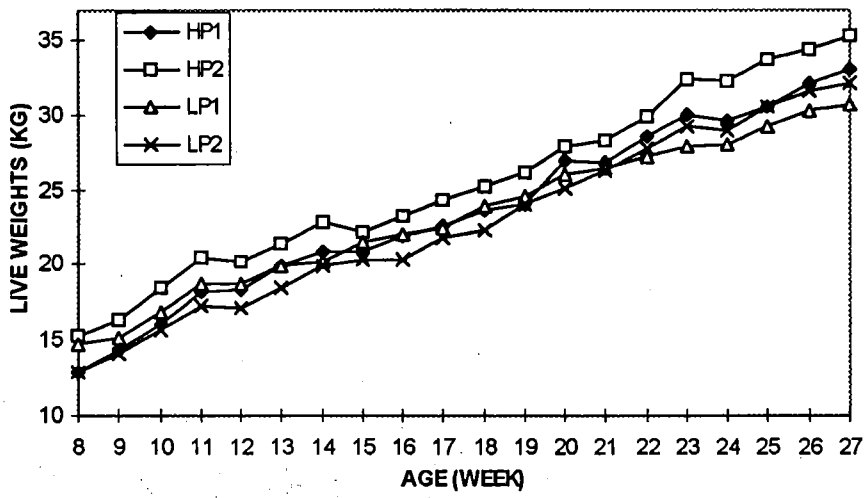

Figure 3.1: The effect of dietary protein and parasitism on body weights (LW). Changes in mean LWs of HP and LP group animals from the beginning of trickle infection (8 weeks of age) to end of trial (27 weeks of age). Trickle infection was stopped at 23 weeks of age and animals were challenged at 25-26 weeks of age.

Table 3.1: Mean live weight gains (LWG) of treatment groups.

Diet groups	**Live weight gains (g/day)**			
	Week 8-13	Week 14-20	Week 21-27	Week 8-27
HP1	193_a	145_a	127_a	155_a
HP2	193_a	131_a	147_a	157_a
LP1	152_a	123_a	96_a	124_a
LP2	163_a	132_a	141_a	145_a

Different letter subscripts in the same column indicate a level of significance (p<0.05).

3.3.2. Carcass weights

There were no significant differences in the carcass weights of animals due to diet (p=0.327) or sensitization (p=0.130) even though the control groups' (HP2 and LP2) carcass weights were slightly higher (15.39 and 14.51 kg) than that of sensitized groups (HP1 and LP1; 14.11 and 13.56 kg).

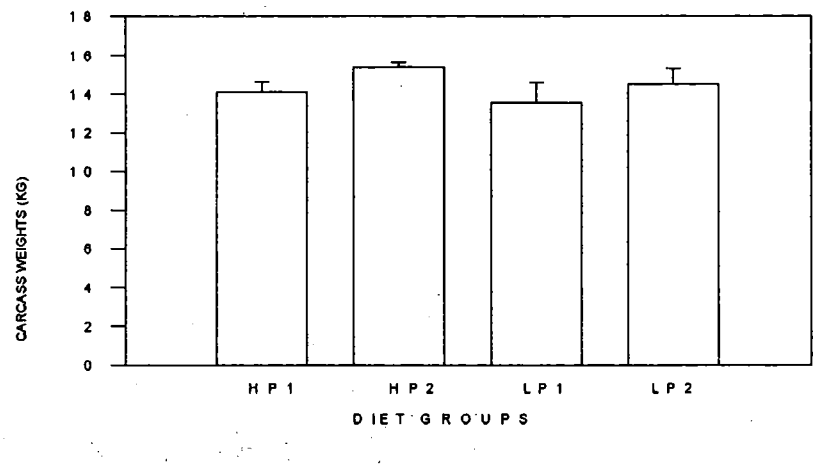

Figure 3.2: Mean carcass weights of animals according to diet.

3.3.3. Thymus weights

There were no significant differences in thymus weights due to diet (p=0.5) or sensitization (p=0.145). However, the mean thymus weights of the control groups (HP2 and LP2) were slightly higher (13.1 and 12.1 g) than that of sensitized groups (HP1 and LP1; 10.9 and 9.9 g).

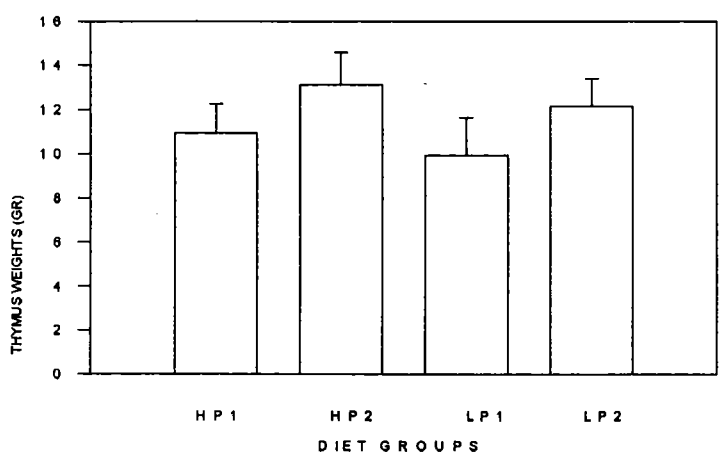

Figure 3.3 : Mean thymus weights of lambs.

3.3.4. Total worm burdens

As the distribution of worm burdens were not normal before or after log transformation and were heavily skewed, they were analysed by Wilcoxon signed rank test (non-parametric analysis).

Small intestinal worm burdens: Animals from the sensitized groups (HP1 and LP1) were given L3 larvae from 8 weeks of age until 23 weeks of age as a trickle infection, and all group (HP1, HP2, LP1 and LP2) animals were challenged at 25-26 weeks of age . The differences due to diet on the trickle worm population of *T. colubriformis* in the small intestine were not significant ($p>0.05$). There was a negative trend in sensitized groups between protein supplementation and worm numbers, that is the high protein diet (HP1) group had considerably decreased worm numbers (Table 3.2) compared with LP1 in the trickle worm population, but this was statistically not significant ($p=0.263$). L3 larvae were not given to the control groups (HP2 and LP2) during sensitization and these group animals remained worm free.

While, there was no significant ($p<0.05$) diet effect on the challenge worm population, sensitized groups (HP1 and LP1) had significantly lower worm populations ($p<0.05$) than that of control groups (HP2 and LP2). HP1 also tended to have lower number of challenge L_3 compared with LP1 ($p=0.091$).

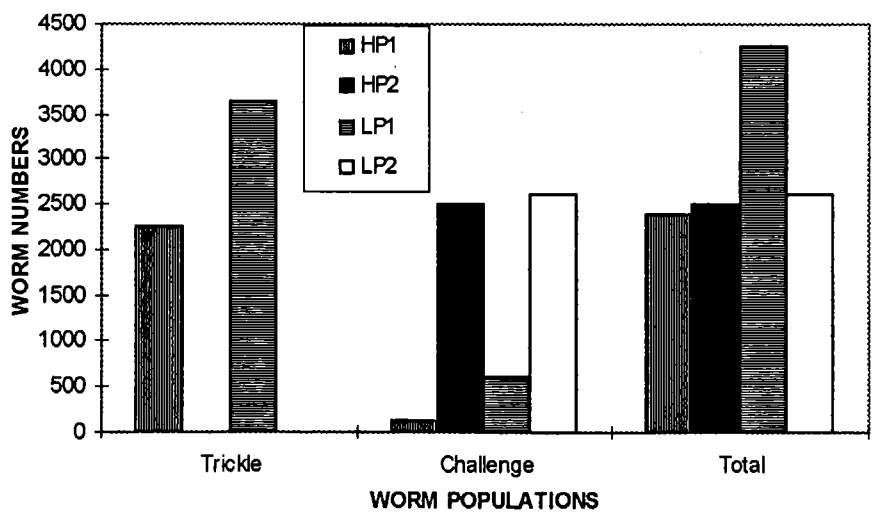

Figure 3.4: Arithmetic means of small intestinal worm burdens. Changes in mean worm numbers of trickle, challenge and total worm populations of *T. colubriformis* counted at slaughter. Trickle infection was continued for 15 weeks and all animals were challenged 11 days prior to slaughter.

Table 3.2: Arithmetic means of small intestinal worm populations of *T. colubriformis.*

Diet groups	Trickle	Challenge	Total
HP1	2267.5_a	121.25_a	2388.75_a
HP2	0_b	2505_b	2505_a
LP1	3653.75_a	605_a	4258.75_a
LP2	0_b	2616.25_b	2616.25_a

Columns with different subscripts are significantly ($p<0.05$) different.

Abomasal worm burdens: There were no significant differences ($p>0.05$) shown in trickle, challenge and total worm burdens from abomasum due to diet with *O. circumcincta* infection in sensitized groups (HP1 and LP1). Differences due to sensitization were significantly higher in sensitized groups ($p=0.000$) and no worms were detected in the control animals after challenge. This latter finding is likely to have been due to the fact that the challenge larvae were from a different passage history and did not contain *O. circumcincta.*

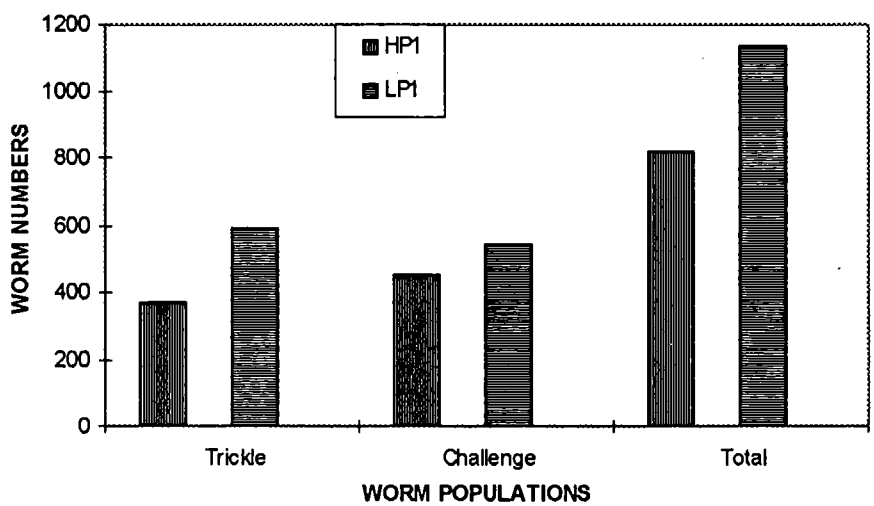

Figure 3.5: Arithmetic means of abomasal worm populations of *O. circumcincta.*

Table 3.3: Arithmetic means of abomasal worm burdens of *O. circumcincta.*

Diet groups	Trickle	Challenge	Total
HP1	367.5_a	452.5_a	820_a
HP2	0_b	0_b	0_b
LP1	588.75_a	543.75_a	1132.5_a
LP2	0_b	0_b	0_b

Columns with different subscripts are significantly ($p<0.05$) different.

3.3.5. Faecal egg counts

Faecal egg data was log transformed which tended to normalise data. When animals were trickle infected with L3 larvae of *T. colubriformis* and *O. circumcincta,* from 8 weeks of age to 23 weeks of age, faecal egg numbers followed a similar pattern in both sensitized groups (HP1 and LP1) irrespective of diet. At 9 weeks of age, egg count geometric means per gram of faeces were 108.14, 0, 193.09, and 0 in HP1, HP2, LP1 and LP2, respectively with much variability, geometric means were 138.96, 0, 32.65, and 0.93 at the end of trial. Differences in egg counts due to diet were not statistically significant ($p=0.739$). After challenge at 25-26 weeks of age, egg numbers in LP1 increased compared to HP1 ($p=0.093$).

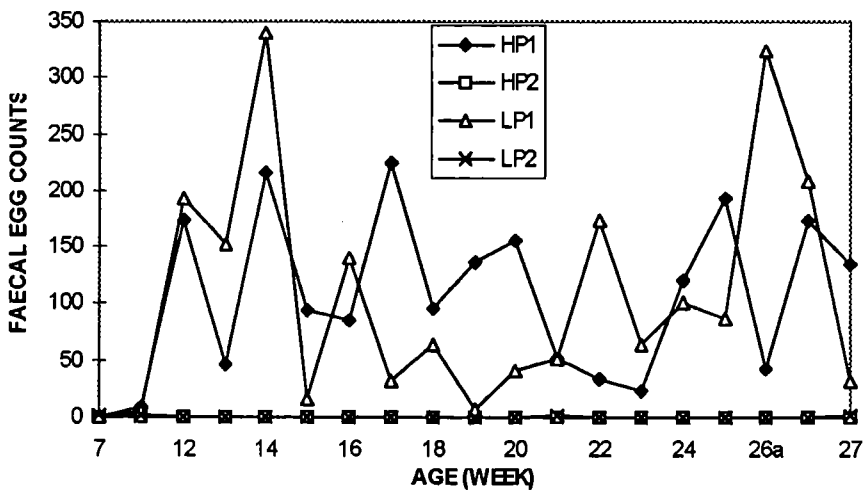

Figure 3.6: Geometric means of nematode egg count per gram of faeces in HP and LP diet groups.

A significant ($p<0.02$) positive correlation ($r>+0.4$) existed between worm burdens from trickle infection, and measured FEC, from 4 weeks into the trial until the end, in both HP and LP groups. However, a significant negative correlation ($r<-0.5$) existed between resultant challenge infection and trickle FEC after 4 weeks of trickle infection, in both HP and LP groups.

3.3.6. Blood leukocyte numbers

During the trial, leukocyte numbers from whole blood were counted and compared between dietary groups. Differences between dietary groups were statistically significant using a repeated measured analysis ($p=0.039$) being higher in HP groups over the whole trial. Differences at 8 ($p=0.030$), 12 ($p=0.038$) and 16 ($p=0.064$) weeks of age were significant. Leukocyte numbers were lowest in the LP2 group. Infection had no significant effect on leukocyte numbers.

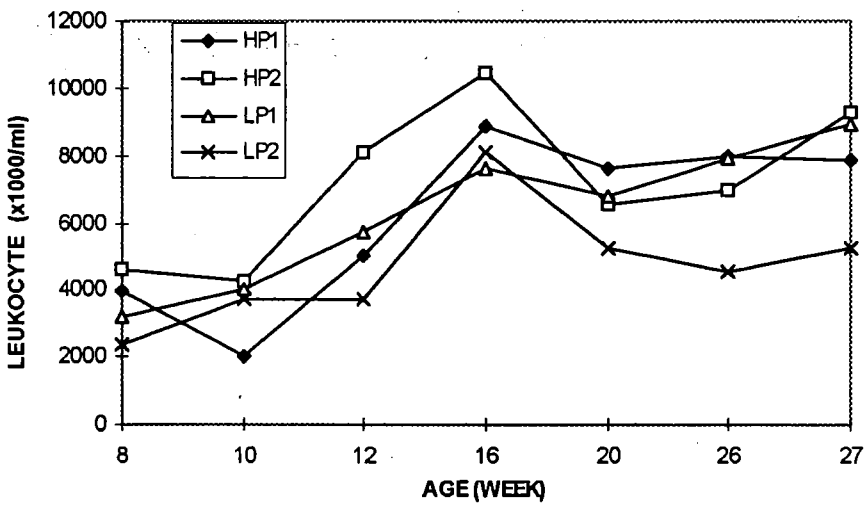

Figure 3.7: Mean numbers of leukocytes from blood.

3.3.7. Blood eosinophil numbers

Diet did not have a significant overall effect on eosinophil numbers in lambs during the trial ($p=0.115$). However, there was a significant difference between sensitized and control animals at 20 ($p=0.010$) and 26 ($p=0.007$) weeks of age, that is during the latter part of the sensitization and post challenge in the previously sensitized groups.

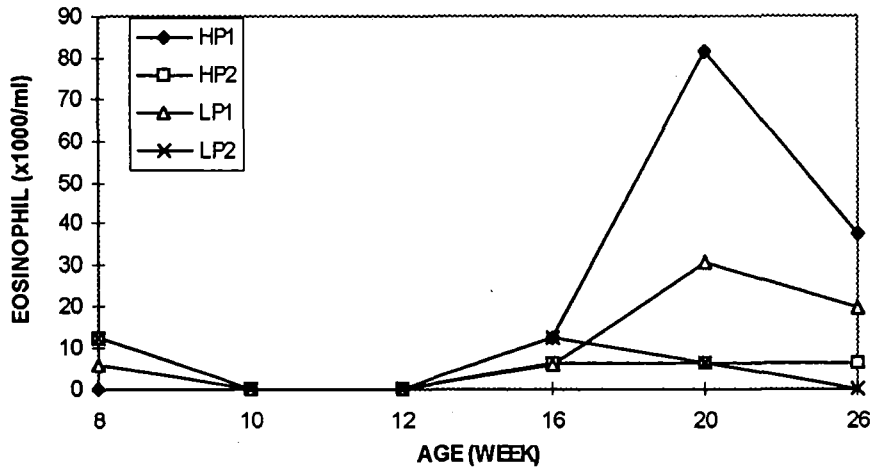

Figure 3.8: Mean eosinophil numbers in the blood.

While there was no significant correlation between trickle worm burdens and blood eosinophil numbers, there was a significant ($p<0.02$) trickle negative correlation ($r = -0.42$), between challenge L_3 numbers and eosinophil numbers at weeks 20 and 26 of trickle.

3.3.8. Plasma antibody

Antibody levels (against excretory secretory antigen of *T. colubriformis*) of plasma samples from lambs collected at 3 separate time points were assayed and were expressed as optical density at 492 nm after correction for interplate variation. There were no significant differences between groups due to diet ($p=0.904$) and sensitization ($p=0.493$). There was a slight increase in levels over the 19 weeks of the trial such that an increase in antibodies due to infection occurred by 27 weeks of age ($p=0.071$).

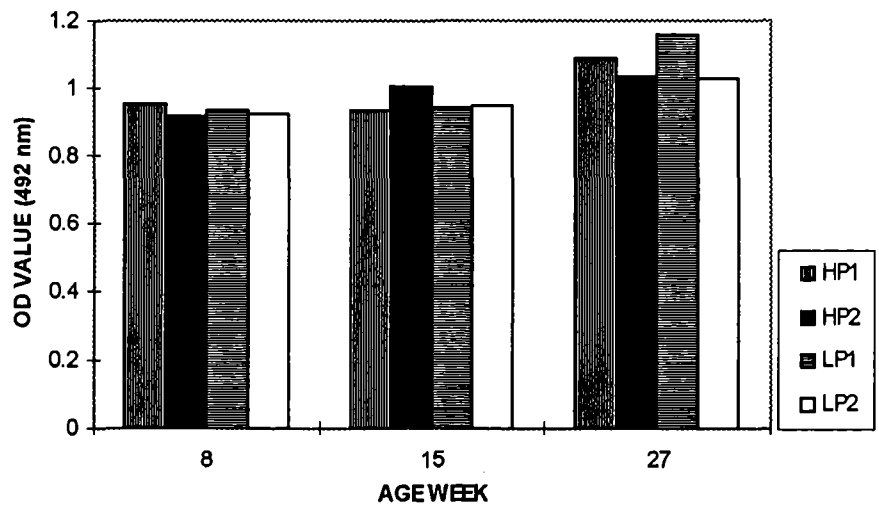

Figure 3.9: Mean OD values of plasma samples from lambs.

3.3.9. Lymphocyte blastogenesis test (LBT)

The lymphocytes that had been extracted from the peripheral blood or mesenteric lymph nodes of lambs were cultured separately with Concanavalin A (ConA), excretory-secretory L3 antigen (ESA) and whole antigen from the parasite infective larvae (L3Ag). The stimulation index (SI) following ConA was not significantly affected by dietary protein ($p=0.112$) over all the trial time period. The SIs of the HP groups were high at 8 weeks of age ($p=0.000$) which was due in part to contamination. The SIs of both HP groups increased from 12 weeks of age and maintained a high level to dramatically drop after 16 weeks of age. This elevation between 12-16 weeks of age was significantly different from the LP SIs ($p<0.05$). However, at both 18 and 24 weeks of age HP SIs were significantly lower ($p=0.000$), and at 27 weeks of age there was no diet effect ($p>0.05$).

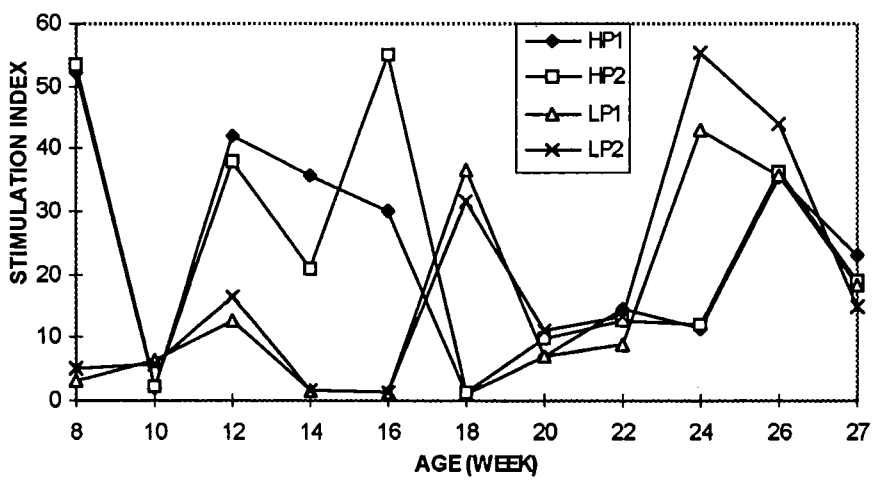

Figure 3.10: Arithmetic mean of SIs of lymphocytes stimulated *in vitro* with ConA.

When parasite antigen (ESA) was used to stimulate lymphocytes *in vitro,* there were no significant differences between the diet groups' SIs ($p=0.178$) and that from the infection groups ($p=0.183$) throughout the trial period. However, at 8, 14 and 16 weeks of age, the HP diet groups' SIs were significantly higher ($p<0.050$) than that of LP groups, with no significant differences due to sensitization. At 18, 20 and 24 weeks of age, LP SIs were significantly higher but at 27 weeks of age there was no significant diet effect. After challenge the sensitized groups' (HP1 and LP1) SIs increased for the last week of the trial, but this increase was not statistically significant ($p=0.124$).

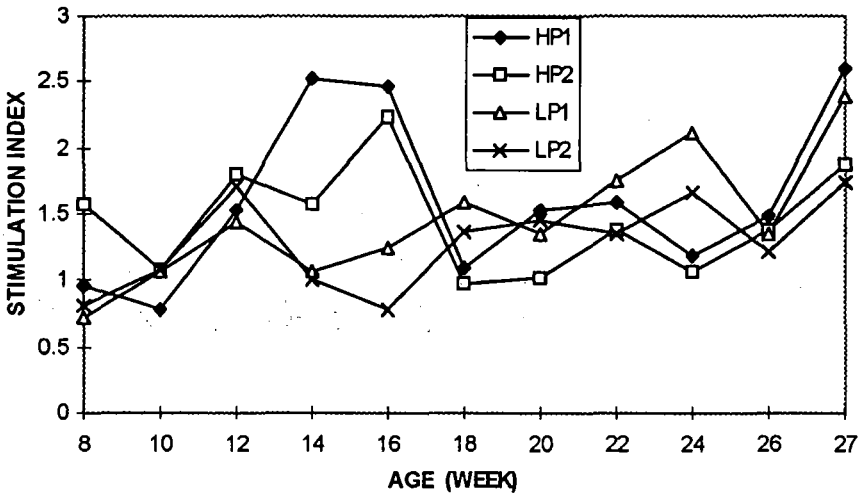

Figure 3.11: Arithmetic mean SIs of blood lymphocytes stimulated *in vitro* with ESA.

Over the whole trial, there was a dietary trend with the SIs of lymphocytes from lambs cultured with L3Ag ($p=0.071$). In particular, there was a significant difference between HP and LP SIs at 8, 14, and 16 weeks of age with the HP SIs being significantly higher ($p<0.05$). There was also a significant difference between the SIs of sensitized and unsensitized groups over the whole trial ($p=0.005$) but especially, from 20 weeks to 27 weeks of age.

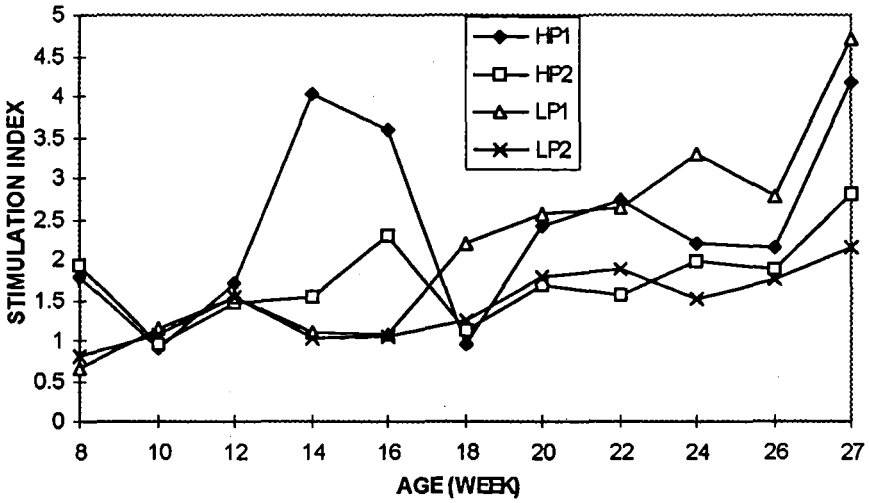

Figure 3.12: Mean SIs of blood lymphocytes stimulated *in vitro* with L3Ag.

There was no significant correlation ($p<0.05$), between L_3 Ag SI (log transformed) and FEC except momentarily after challenge (week 26) with a negative correlation of r=-0.39.

3.3.10. Lymph node lymphocyte blastogenesis test

Lymphocytes were collected aseptically from mesenteric lymph nodes draining the duodenum following slaughter of animals. These lymphocytes were cultured *in vitro* with the mitogen ConA, and the parasite antigens ESA and L3Ag, and their stimulation indexes were calculated. Differences between SIs were not significant, due to diet (p=0.718 for ConA, p=0.321 for ESA and p=0.122 for L3Ag) or sensitization (p=0.941 for ConA, p=0.141 for ESA and p=0.143 for L3Ag).

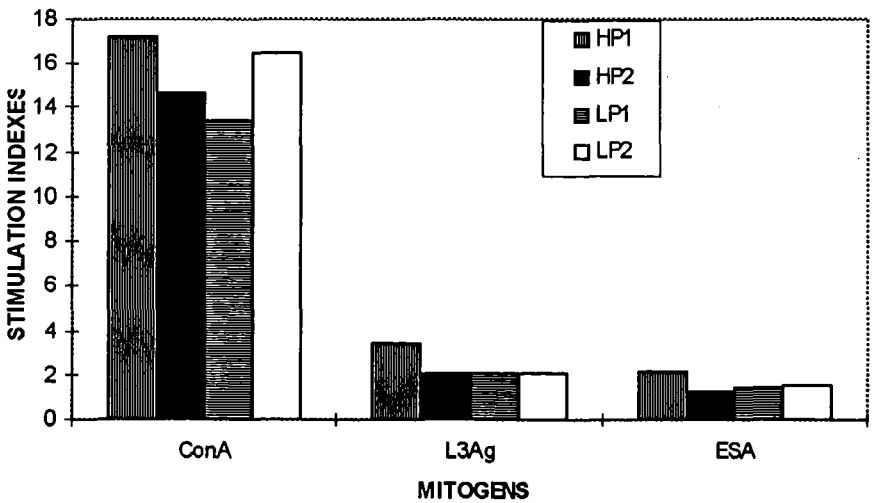

Figure 3.13: Mean SIs of lymphocytes from the mesenteric lymph node of lambs.

3.4. DISCUSSION

This study indicated that the high protein diet did not significantly increase live weight gain in 8-27 week old lambs over the period of the trial. However, there was a strong trend ($p<0.09$) over a number of different time points post infection. As the mean dry matter intake of all groups were similar by design, no effect of infection on feed intake was measurable. At the end of the trial, HP groups, particularly HP2 were heavier than LP groups but LWG differences were not significant. The mean body weight increase of all treatment groups followed almost the same pattern during the experiment. The growth rate of the parasitised animals over the first 4 weeks of infection was improved with extra dietary protein. At the end of the trial, extra protein enhanced growth rate irrespective of parasite infection. There were no significant differences in the carcass weights of animals because of diet or infection. These results were similar to those of Bown *et al.* (1991) where mean live weights of lambs given post ruminal protein supplementation and control animals were similar when infected with *T. colubriformis*. They differ in magnitude from those of Kambara *et al.* (1993) who showed that meat meal supplementation to lambs less than 19 weeks of age significantly improved body weight gains throughout an identical infection regime as this trial. These researchers showed that this was not the case in older (36-44 weeks of age) lambs which confirmed Bown *et al.* (1991) findings in older lambs. The parasite burden in this trial did not cause any clinical signs typical of gastrointestinal parasitism. This could be because the number of L3 given to animals as a trickle infection were too low to produce a pathophysiological effects and clinical signs. However, earlier experiments had indicated that this infection regime was "adequate" in producing an effect on body weight gain. An explanation could be that the infective larvae were "aged" and their that viability was low resulting in lower establishment rates. Alternatively, the offered food ration may have been inappropriate. At various (four) time points the food ration was adjusted to be substantially above the predicted ARC feeding requirements. In retrospect it may have still been inadequate for maximal divergence between HP and LP body weight increases.

These results indicate that an increase in protected dietary protein (fish meal) does not automatically reduce the production losses due to *T. colubriformis* and *O. circumcincta* in young lambs, but the latter is rather a product of parasite loading and dietary intake.

The thymus is a significant part of the immune system and pre-T lymphocytes migrate through the circulation from bone morrow to thymus. T-cell differentiation and antigen receptor selection occur in this organ (Crook, 1990). Protein deficiency has caused thymus atrophy in some species such as rats (Gross and Newberne, 1980). However, there was no significant difference in thymus weights due to diet or sensitization in this trial.

Establishment rates for *T. colubriformis* in the small intestine were 10 and 16 % for HP1 and LP1 during the sensitization period, and control groups remained worm free. Following challenge of all groups, establishment rates were much lower in sensitized groups (1 % in HP1 and 2 % in LP1) than those of control groups (9 % in HP2 and LP2). This probably reflects acquired immunity due to trickle infection but also indicates the poor viability of the *T. colubriformis* larval culture used for the challenge infection. However, establishment rates of *O. circumcincta* in the abomasum were much higher (30 for HP1 and 48 % for LP1) during the sensitization period. The HP1 group had a similar establishment rate to the LP1 group after challenge (30 to 36 %). No abomasal L_3 were detected in control groups following challenge which confirms a suspicion that the challenge dose had few if any *O. circumcincta* present. Results in the present study are intermediate to the results of Van Houtert *et al.* (1995a) and Kambara *et* al., (1993), who showed the absence and a marked effect of high protein diet on establishment rates of *T. colubriformis* respectively. This suggests that the 12 week sensitization period using low/moderate trickle levels used in the trial by Kambara *et al.*, (1993) may have been a major reason for the lack of acquired immunity, rather than age *per se*.

Overall, the effect of the high protein diet on small intestinal and abomasal worm burdens was not statistically significant ($p>0.5$). However, during sensitization there was a trend for the high protein group to have decreased worm numbers of *T. colubriformis* in the small intestine, which became particularly marked after challenge ($p=0.091$). As expected sensitized animals had significantly lower worm counts in the challenge population of the small intestine ($p<0.05$) than that of non-sensitized animals. Kambara *et al.* (1993) observed that while sensitized older animals had smaller worm burdens compared to unsensitized control animals following a standard challenge (30,000 L_3), this was not the case with young animals trickle infected at low levels for 12 weeks and fed a low protein diet. This variance in resistance found in low protein lambs could be explained by the

extra time for sensitization in this trial (15 versus 12 weeks). In addition, this trend (p=0.091) for protein supplementation to enhance resistance to challenge *T. colubriformis* L3 (ie establishment) although contrary to the findings of Van Houtert *et al.* (1995a) and Kyriazakis *et al.* (1996) supports the findings of Kambara *et al.*, (1993). The HP diet had no effect on abomasal worm populations and worm counts in HP and LP trickle infected groups were similar. No *O. circumcincta* worms were detected in the abomasum of control animals after challenge which probably reflects the low levels of those L3 in that particular preparation relative to *T. colubriformis* numbers (confirmed by light microscopy).

No statistical difference was observed between faecal egg numbers in the dietary groups (HP and LP). Egg counts were higher in the LP1 group at 12, 13, 14, 16, 22 26a and b weeks of age but egg counts were higher in HP1 at 15, 17, 18, 19, 20, 24 and 25 weeks of age. In general, there was a trend for the excretion of faecal eggs to be lower during the establishment of L_3 and after challenge in the high protein diet group. Results in the present study were in agreement with the results of Van Houtert *et al.* (1995a) who did not observe significant differences between faecal egg numbers from dietary groups given different amounts of fish meal (non, lower or higher) under trickle infections overall. However, Kambara *et al.* (1993) observed a significant difference between HP and LP groups when they measured the eggs excreted from a single secondary challenge infection given high levels of L3 (30,000).

During this trial, there was a significant diet effect on leukocyte numbers that were higher in the HP groups compared with LP groups until 14 weeks of age when they followed a similar pattern. There was a significant sensitization effect on leukocyte numbers at 26 week of age (p=0.004), but in general trickle infection had no effect on leucocyte counts.

There were no significant differences in eosinophil numbers due to diet. However, sensitization affected the number of eosinophils and there are significant differences between groups at 20 (p=0.010) and particularly at 26 weeks of age (p=0.007) - immediately after challenge. The reason for an eosinophilia being present in this study is likely to be due to the longer sensitization period (15 weeks) compared to a lack of eosophilia in the 12 week trial of Kambara *et al.* (1997). Van Houtert *et al.* (1995a)

observed significant difference in eosinophil counts in sheep due to diet which correlated with the rate of expulsion of *T. colubriformis*. In this experiment, the eosinophil numbers at ages 20 and 26 weeks (12 weeks post trickle and post challenge) correlated (r=-0.41) with the level of challenge burden, but not the long term established population.

There was no diet effect on antibody levels in lambs (p=0.904) but overall, there were some effects of sensitization, particularly at 24-27 weeks of age (p=0.070), being higher in sensitized groups. While this may indicate the beginnings of a response, in general the findings support the findings of Kambara *et al.*, (1997) and Van Houtert *et al.*, (1995a) who showed no increase in parasitic-specific antibodies at this age.

The lymphocyte blastogenesis test was carried out to measure the *in vitro* responses to specific parasite antigens (ESA and L3Ag) and non-specific mitogen (ConA). The SIs of lymphocytes cultured with ConA from the HP groups were significantly higher for the first 8 weeks but they decreased over the following 16 weeks of age. Although LP SIs were higher for the second ten weeks, there was not a significant difference (p=0.112) between the HP and LP groups over the entire trial period. These results were similar to Kambara *et al.* (1993) where results for ConA stimulation in young lambs (8-24 week old) did not indicate a significant difference between the dietary groups. SIs of lymphocytes cultured with ESA were similar in HP and LP groups. SIs were significantly higher in the HP groups at 8, 14 and 16 weeks of age, but then followed the same patterns in all diet groups, except at 24 weeks of age when LP SIs were significantly higher. No significant effect of sensitization on SIs was observed with ESA stimulation. In the present study, similar results were observed for L3Ag stimulation and differences in SIs were not significantly different in the groups due to diet, but there was a strong trend (p=0.070) which was higher in the HP groups. SIs of lymphocytes from sensitized groups were significantly higher and this was more apparent after challenge which shows the effect of sensitization on L3Ag stimulation. Although Kambara *et al.* (1993) observed a significant sensitization effect only in older lambs (33-51 week-old), there was a suggestion of a developing response to L3Ag in lymphocytes taken from lambs 10 and 11 weeks post sensitization. In the present study, the L3Ag response occurred from 12 weeks after the beginning of sensitization (20 weeks of age), until the end of the trial (27 weeks of age). This trial has highlighted the importance of sensitization period length

- which had been predicted by Dobson *et al.*, (1990) - particularly in young lambs with low trickle infection. That is, after 17-19 weeks of trickle infection immune responses such as lymphocyte proliferation, a blood eosinophilia and the production of antiparasite antibody had commenced. There was no significant correlation between proliferative response to L3 Ag and protection as measured by FEC changes. This finding may be an indication of the limitations of an assay such as lymphocyte proliferation. As it is believed that protection against helminths may be reflective of a TH_2 response (Scott and Sher, 1993), the production of IL-2 present in the expansion of lymphocyte populations may not directly indicate resistance.

CHAPTER FOUR

THE EFFECT OF METHIONINE AND LYSINE ON THE IMMUNE SYSTEM IN LAMBS INFECTED WITH *T. COLUBRIFORMIS* AND *O. CIRCUMCINCTA*

4.1. INTRODUCTION

Many factors such as age, gender, genetic constitution, physiological status of animals, and diet have effects on host immunity to gastrointestinal parasites (McFarlane, 1997). Diet, particularly protein intake has a role in the immunity and resilience of animals to gastrointestinal parasites. In an early study, Dobson and Bawden, (1974) observed an increased susceptibility in protein deprived sheep to *O. columbianum* and this was associated with impaired function of the innate immunity of the gut with reduction of the adaptive immune response. Lambs given a high protein diet had better resilience to *H. contortus* infection with less pathophysiological consequences (Abbott *et al.*, 1986b). Bown *et al.*, (1991) observed that although there was no significant effect of energy, protein infusion (casein) decreased faecal egg output and mean total parasite counts, and decreased the debilitating effects of internal parasites. Kambara *et al.* (1993) also observed that young male lambs given a meat meal-based high protein diet developed better resistance to the establishment of *T. colubriformis* infection with an improved growth rate. Van Houtert *et al.* (1995a and 1995b) reported that supplementary feeding with fish meal enhanced the expulsion of an existing *T. colubriformis* burden in sheep and reduced the production losses caused by the parasite.

However, very few studies have shown the effect of individual amino acids on immunity to infectious diseases in livestock. Tsiagbe *et al.* (1987a and 1987b) described the effects of supplements of methionine (0.063-1.45 %) and cysteine (0.203 %) on the growth and immune responses of broiler chicks. Methionine and cystine supplementation improved cell mediated phytohemagglutinin-P (PHA-P) responses and T cell dependent IgG, but not IgM, responses when these amino acids were given at these levels.

Ahmed and Qadri (1985) offered 35-40 day old rats different diets: high protein (18 %), low protein (7 %) diet, low protein supplemented with lysine (0.68 %), low protein supplemented with Lys (0.68 %) + methionine (0.26 %), and low protein supplemented with Lys (0.68 %) + Tryptophane (0.12). They obtained highest antibody titres (from 1:256 to 1:512) to tetanus toxoid intramuscularly injected in the thigh, in the animals

given the high protein diet, with the second highest value (from 1:128 to 1:256) in animals given low protein diet supplemented with lysine and methionine. These results indicate that dietary protein and methionine-lysine can have effects on antibody production.

Coop *et al.* (1997) offered an *ad libitum* diet which was either a low protein, a low protein diet supplemented with protected methionine (2 %) or a high protein diet, to five month-old Suffolk cross lambs infected for 10 weeks by giving 3000 L3 larva of *T. colubriformis* per day, followed by a challenge infection. The addition of methionine to the diet increased both the efficiency of feed utilisation in the infected lambs, and increased animal resilience, but did not affect resistance.

The aim of this study was to investigate the effect of protected methionine-lysine on immunity to *T. colubriformis* infection in young lambs and to characterise some of the mechanisms involved.

4.2. MATERIALS AND METHODS

4.2.1. Animals

Thirty two Coopworth male lambs were maintained parasite-free after birth, weaned at 3 weeks of age and given a standard diet (CP = 15 %) until they reached 8 weeks old and weighed approximately 14 kg. They were then randomly allocated into 2 groups, individually penned and given either a low protein diet supplemented with methionine-lysine (ML) or a low dietary protein (LP) for 19 weeks.

4.2.2. Diet

Animals were given 300 g of their respective diets per day at the beginning of experiment and 870 g per day at the end of trial. The amount of food offered was based on AFRC recommendations to give an average body weight increase of approximately 50 g daily in the LP group, and the same amount of LP ration plus methionine-lysine was given to the ML group[‡]. Thus equal amounts of energy, calcium and phosphorus were offered to the two dietary treatment groups relative to stage of the trial. The addition of sodium bicarbonate and ammonium chloride was to aid in the prevention of urolithiasis, a potential problem in housed entire male sheep.

[‡] Calculated using RuMEMP (version 1.12) software developed by van Houtert MFJ and Zyskowski R, Lincoln University

Low protein diet (12.2 ME and 79 MPg/kg)

Barley grain	79.0 %
Lucerne hay	7.8 %
Molasses	5.0 %
Soya bean oil	1.0 %
Dicalcium phosphate	3.7 %
Lime ($CaCO_3$)	1.8 %
Sodium bicarbonate	1.0 %
Ammonium chloride	0.7 %

The LP group (n=16) were fed solely this ration but in the ML group (n=16), this diet was supplemented with 70.5 mg methionine and 188 mg lysine per kg live weight (LW) of lamb. This was based on previous estimations (Barry, 1981; Fraser *et al.*, 1990; Van Houtert, *pers comm.* Smartamine™-ML, (Rhone Poulenc) that contained 15 % methionine and 50 % lysine monohydrochloride and was protected from ruminal degradation, was fed for supplementation at a rate of 470 mg per kg LW, that would supply 70.5 mg methionine and 188 mg lysine per kg LW.

4.2.3. Parasite sensitization and challenge

Infective L_3 of *T. colubriformis* (strain LIU\89-6) and *O. circumcincta* (strain LIU\85-10) were separately cultured from the faeces of sheep that had been infected with a monoculture and were used for sensitization (vaccination) and challenge after being mixed together 95 % and 5 % respectively.

Each of the diet treatment groups ML and LP were randomly allocated into 2 groups (n=8). The animals within one of the groups (ML1) were sensitized and the other animals remained uninfected as a control group (ML2). The animals in ML1 were sensitized by oral infection (manual dosing) with *T. colubriformis* and *O. circumcincta* L_3. This "trickle" sensitization was carried out from the age of 8 to 23 weeks with 600, 1200, 1800 and 3000 L_3 given for the first 4, second 4, third 3 and last 4 weeks respectively (given as 3 split doses in a week). After 15 weeks of trickle infection, sensitization was stopped and 17 days later, all animals (including controls) were orally challenged with 30,000 L_3 of the same parasite mix. Animals were slaughtered 11 days later.

4.2.4. Experimental design and procedure

Measurements of live weight, carcass weight, thymus weight, total worm burden, faecal egg count, lymphocyte and eosinophil numbers, plasma antibody titres, and lymphocyte blastogenesis (LBT) were made and compared between treatments (diet and sensitization), following the same experimental procedures described in Chapter 3.

4.2.5. Statistical analysis

To all results an analysis of variance was carried out using Systat (univariate and multivariate repeated measures analysis where indicated) (Version 5, SYSTAT Inc., USA). Variables were diet (LP and ML) and parasite infection (sensitized or not). The Wilcoxon signed rank test (non-parametric) was used for comparing small intestine and abomasum worm burdens as the distribution of the data was skewed with or without transformation (Jacobs *et al.*, 1994; Kambara *et al.*, 1993). Faecal egg counts were transformed {log 10 (count+1)} to normalise data. Likewise, lymphocyte stimulation indexes were transformed by natural logaritims {$\log_n$ (count+1)} (Kambara *et al.*, 1993). Pearson correlations between certain measurements were carried out using SYSTAT.

4.3. RESULTS

4.3.1. Live weights

The animals supplemented with ML, particularly the ML1 group showed higher live weight gains compared with the LP group animals (see Figure 4.1 and Table 4.1). At the beginning of the trickle infection (7-8 weeks of age), animals LWs were 13.71, 11.86, 13.83, and 12.84 kg for LP1, LP2, ML1 and ML2 groups, respectively and by 16 weeks of age, LWs were almost doubled at 22.03, 20.39, 23.63 and 21.26 kg. In the ML2 group, two animals were deleted from the trial; one of them died due to acidosis, and the other one did not start eating pellet food. In the same group, another animal died at the end of 23 weeks of age due to uraemia following bladder rupture. Thus, there were 5 animals in this group at the end. At the end of 27 weeks of age, there was marked difference between the LWs of LP1 and ML1 (ML1-LP1 = 4.35 kg) and slight difference between LP2 and ML2 (LP2-ML2 = 0.61 kg). Over of the period of the trial, differences due to diet ($p=0.592$) and sensitization ($p=0.404$) were not statistically

significant. However, during the period of 6 to 9 weeks after the beginning of trickle infection an improvement in LWG with ML supplementation was apparent (p=0.06).

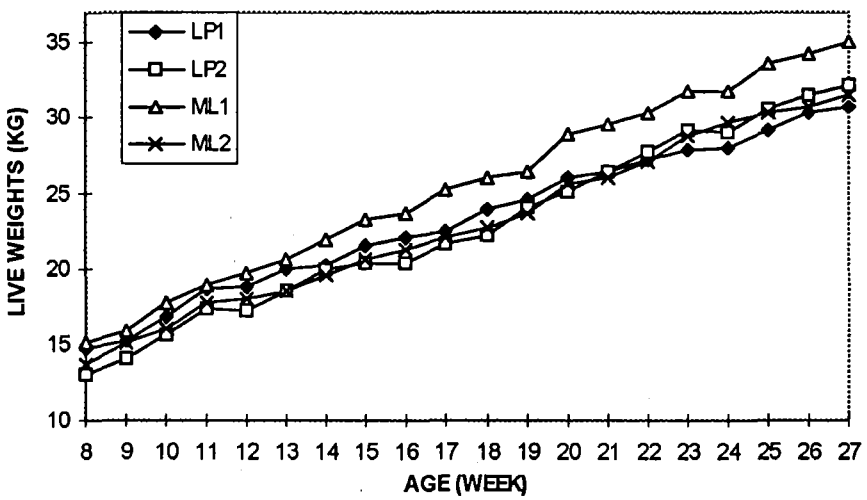

Figure 4.1: The effect of methionine-lysine supplementation on LWG. Mean changes in the LWs of LP and ML group animals.

Table 4.1: Mean live weight gains of treatment groups.

Diet groups	**Live weight gains (g/day)**			
	Weeks			
	8-13	14-20	21-27	Total
LP1	152_a	123_a	96_a	124_a
LP2	163_a	132_a	141_a	145_a
ML1	153_a	168_a	125_a	149_a
ML2	120_a	143_a	127_a	130_a

Columns with dissimilar subscripts are significantly different ($p<0.05$).

4.3.2. Carcass weights

When animal carcasses were weighed after slaughter, there were no statistically significant differences between animals due to diet (p=0.549) or sensitization (p=0.882).

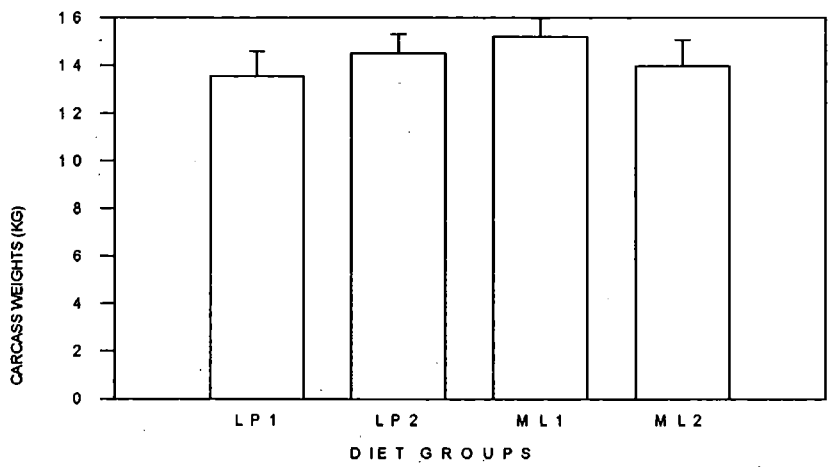

Figure 4.2: Mean carcass weights of animals according to diet.

4.3.3. Thymus weights

Following slaughter when the thymus of each animal was collected and weighed, no differences were observed due to diet or infection (p>0.05).

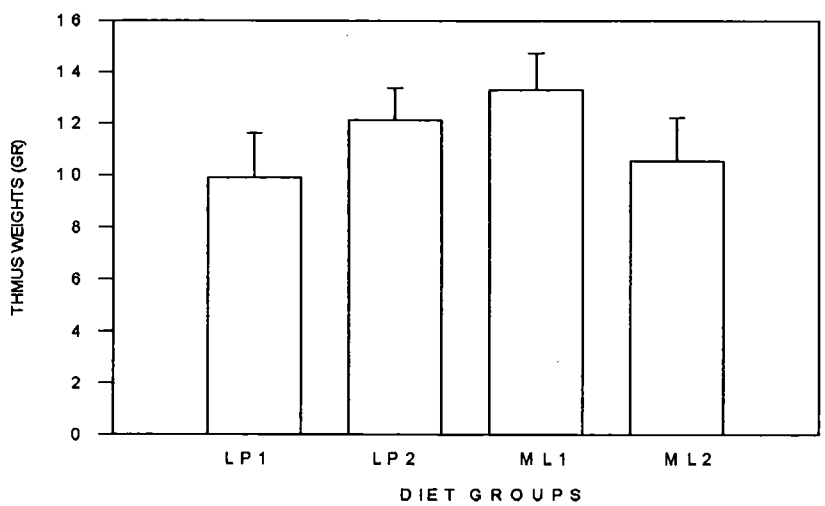

Figure 4.3: Thymus weights of animals.

4.3.4. Total worm burdens

As the distribution of worm burdens were not normally distributed before or after transformation they were analysed by Wilcoxon signed rank test (non-parametric analyses).

Small intestinal worm burdens: Differences in the trickle worm population of *T. colubriformis* due to diet were statistically significant in sensitized groups ($p<0.017$) with ML1 having significantly lower worm counts than LP1. As differences in challenge worm counts due to diet were not statistically significant ($p=0.263$) the overall difference in total worm counts between LP1 and ML1 groups was also significant ($p=0.05$). Differences in challenge worm counts due to infection was significant ($p<0.05$) with sensitized groups (LP1 and ML1) having lower worm counts.

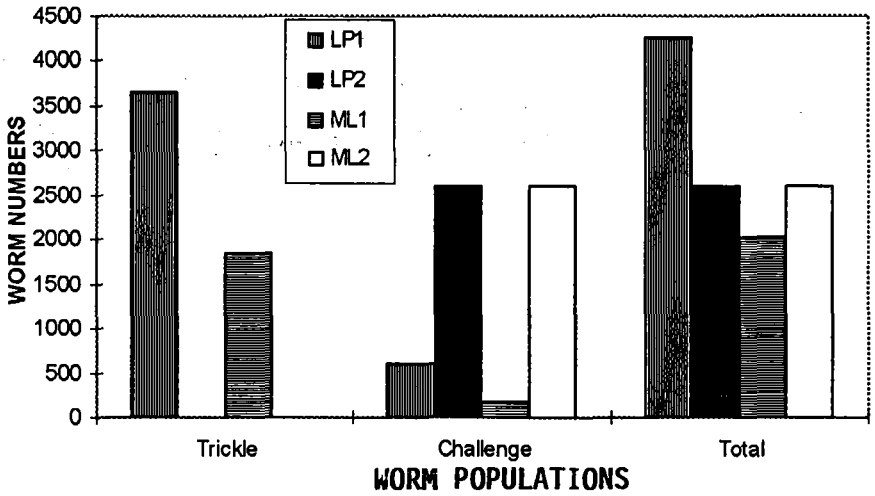

Figure 4.4: Arithmetic means of small intestinal worm populations. Differences in mean worm numbers of trickle ($p<0.05$) and total worm populations of *T. colubriformis* between LP1 and ML1 ($p=0.05$) due to diet were statistically significant.

Table 4.2: Arithmetic means of small intestinal worm populations of *T. colubriformis*.

Diet groups	Trickle	Challenge	Total
LP1	3653.75_a	605_a	4258.75_a
LP2	0_b	2616.25_b	2616.25_b
ML1	1845_c	186.25_a	2031.25_b
ML2	0_b	2603.75_b	2603.25_b

Columns with dissimilar subscripts are significantly different ($p<0.05$).

Abomasal worm burdens: Trickle, challenge and total *O. circumcincta* burdens were detected after animals were slaughtered. There were no significant differences in the worm counts of trickle and challenge populations due to diet, between LP and ML groups and p values for differences between LP1 and ML1 due to diet were 0.674. As expected there was a highly significant difference between the established worm population in the parasitised group and the naive controls ($p<0.001$). However, the unexpected finding, that infective larvae were not found in the challenge controls reflects the fact that a different passage was used which did not contain *O.* circumcincta.

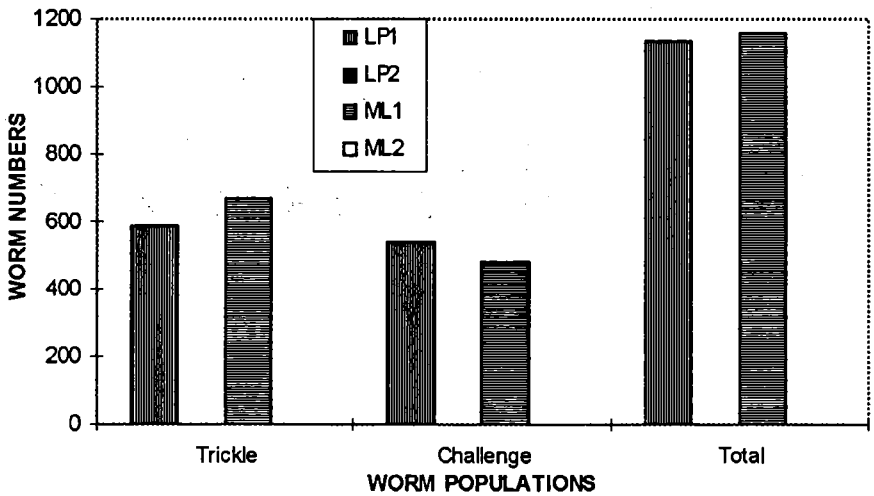

Figure 4.5: Arithmetic means of abomasal worm populations of *O. circumcincta.*

Table 4.3: Arithmetic means of abomasal worm populations of *O. circumcincta.*

Diet groups	Trickle	Challenge	Total
LP1	588.75_a	543.75_a	1132.5_a
LP2	0_b	0_b	0_b
ML1	672.5_a	485_a	1157.5_a
ML2	0_b	0_b	0_b

Columns with dissimilar subscripts are significantly different ($p<0.05$).

4.3.5. Faecal egg counts

Animals were trickle infected with L3 larvae of *T. colubriformis* and *O. circumcincta* from 8 weeks to 23 weeks of age. Trickle infection was stopped at 23 weeks of age and 17 days later, all animals were challenged. During the trial period, faecal egg counts were detected weekly. After log transformation differences between sensitized groups (LP1 and ML1) due to diet were not significant over the period of the trial (p=0.673). However, differences in egg counts were significant at 12 (p=0.029) and 14 (p=0.030) weeks of age (4 and 6 weeks after trickle started) when the ML1 group had significantly lower numbers of eggs than that of LP1. Following 14 weeks of age, egg counts dramatically decreased in LP1 and stayed lower until 21 weeks of age. On the other hand, egg counts increased in ML1 from 15 weeks of age and maintained its higher level until 19 weeks of age before dropping to low level (epg<150) at the end of the trial. After challenge, egg counts significantly increased in LP1 (p=0.027) and there was a strong trend (p=0.07) for the ML supplemented and trickle infected group, to have less faecal eggs compared to the LP trickle infected group.

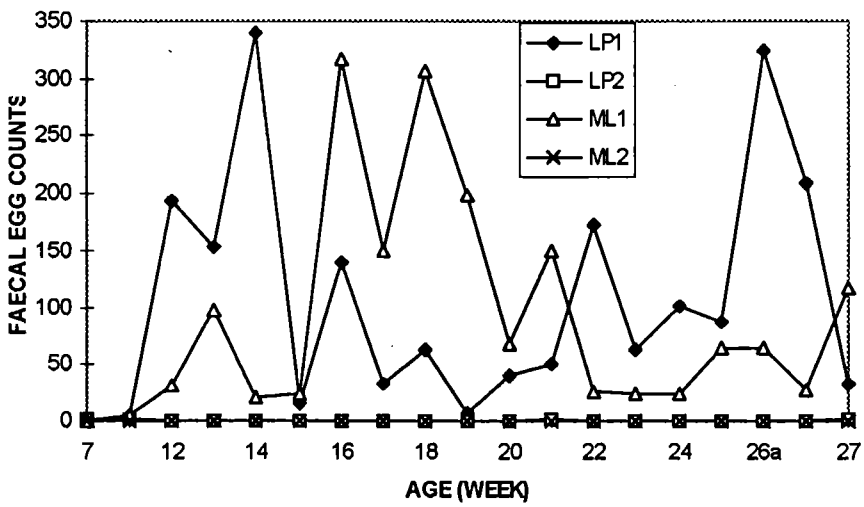

Figure 4.6 Geometric means of faecal egg counts per gram of faeces.

In general, there was a strong positive correlation (r= 0.5) between trickle worm burdens at slaughter and weekly FEC after 3 weeks of trickle. Conversely, there was a negative correlation (r=-0.45) between the challenge infection and weekly FEC after 3 weeks of trickle infection.

4.3.6. Blood leukocyte numbers

There were no significant differences in leukocyte numbers between dietary groups (p=0.427) but a significant difference (p=0.038) existed between trickle infected and non-sensitized animals.

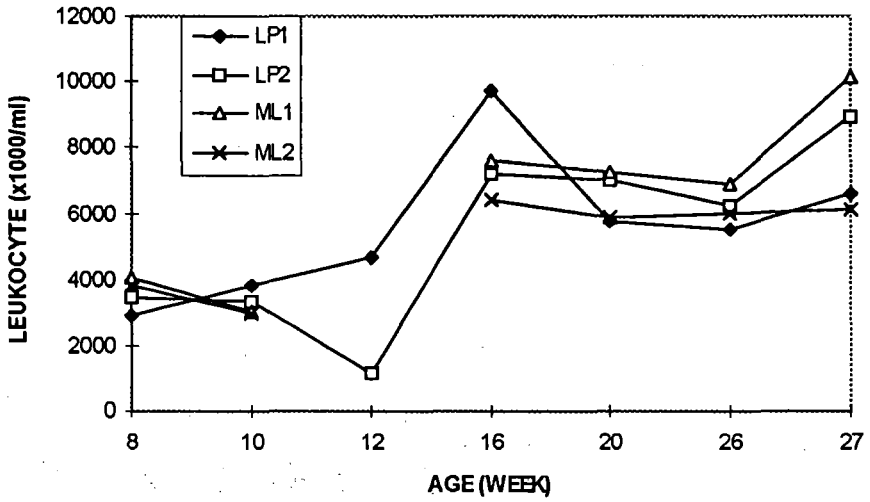

Figure 4.7: Blood leukocyte numbers of lambs.

4.3.7. Blood eosinophil numbers

There were no statistically significant differences in eosinophil numbers between diet groups throughout the trial. However, eosinophil numbers of the LP groups were significantly higher (p=0.053) at 26 weeks of age.

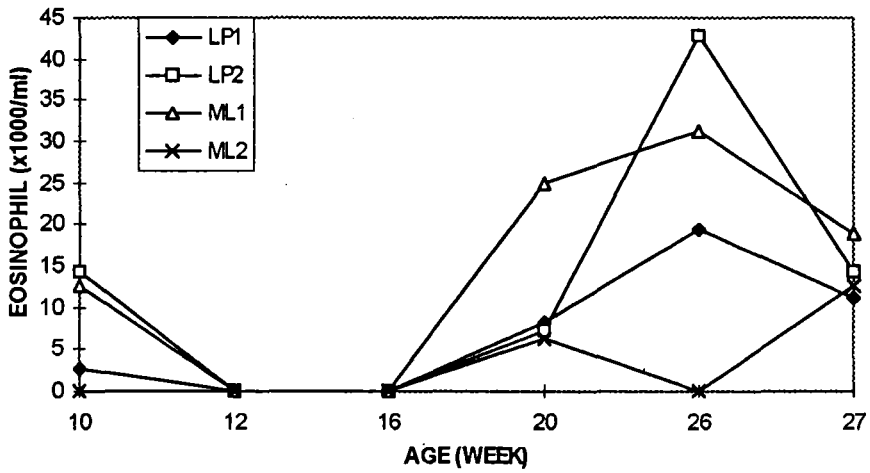

Figure 4.8: Blood eosinophil numbers of lambs.

There was a moderate negative correlation (r=-0.3) between levels of blood eosinophils between weeks 20-26 and challenge L_3 worm burden but no significant correlation existed with the established trickle infection.

4.3.8. Plasma antibody

Antibody levels (to excretory-secretory antigen of *T. colubriformis*) of plasma samples from lambs taken at 3 separate time points were measured and these were expressed as optical density at 492 nm after correction for interplate variation. There were no significant differences between groups due to diet (p=0.094) and sensitization (p=0.712). There was a slight increase in levels at 24-27 weeks of age, but this was much less than the response normally described following infection in older animals.

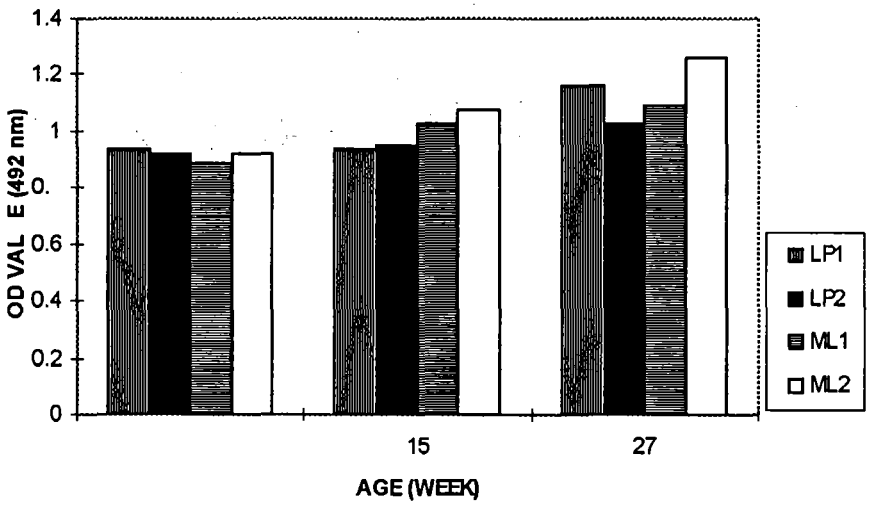

Figure 4.9: Mean OD values of plasma samples from lambs.

4.3.9. Lymphocyte blastogenesis test (LBT)

Lymphocytes that had been extracted from the peripheral blood or mesenteric lymph nodes of lambs were cultured *in vitro* with Concanavalin A (ConA), excretory-secretory antigen (ESA) and whole antigen of *T. colubriformis* infective larvae (L3Ag).

The differences in SIs of lymphocytes cultured with ConA were significantly different due to diet, being higher in the ML groups (p =0.022) when repeated measures analyses (MANOVA) were applied. However, SI was not consistently higher in the ML groups throughout the trial and it dramatically increased from 10 weeks to 14 weeks of age, then equally dramatically dropped to the same level as that of low protein animals (LP). At the trial end, the SIs were the same in all groups. No significant differences were observed due to sensitization (p=0.400).

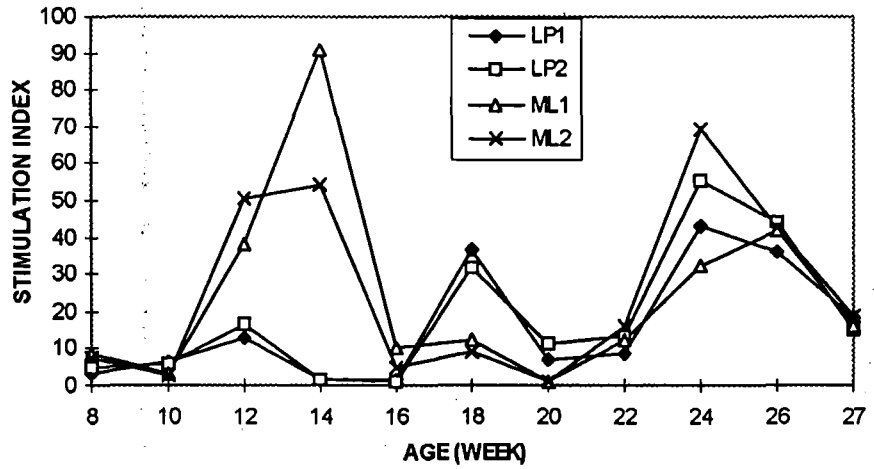

Figure 4.10: Mean SIs of lymphocytes from lambs cultured with ConA. SIs were significantly higher in ML groups (p=0.022).

The SIs of lymphocytes cultured with ESA were not significantly different either because of diet (p=0.178) or sensitization (p=0.183). There was a significant increase in SIs at one time point (16 weeks), but it rapidly decreased to the same level as the LP group at 18 weeks of age, and differences at 14 and 16 weeks of age were significant ($p<0.05$). After 18 weeks of age, the SIs followed almost similar patterns until the end of the trial.

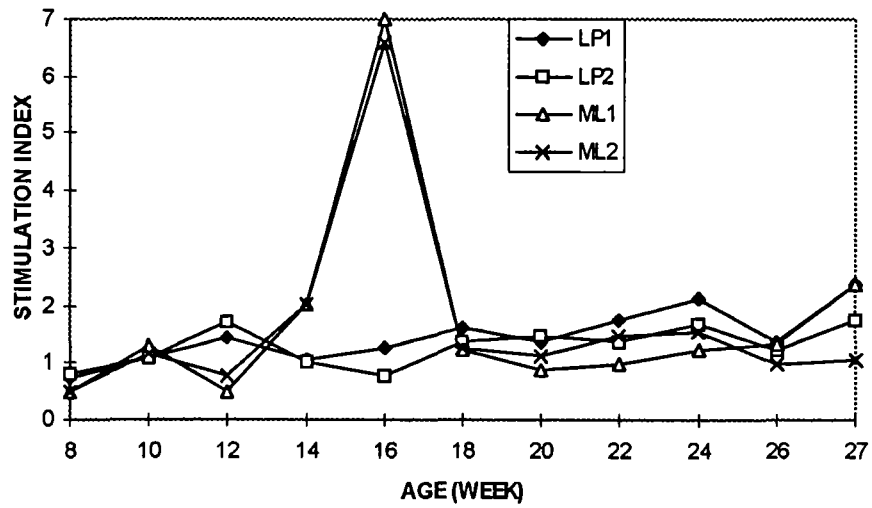

Figure 4.11: Mean SIs of blood lymphocytes from lambs cultured with ESA.

The transformed SIs of lymphocytes from lambs cultured with L3Ag were significantly higher in the sensitized groups (p = 0.001) compared to that of control groups. Within sensitized groups, the mean SI of LP1 animals was higher than the SI of ML1 animals.

Likewise, in the control group, the mean SI of LP2 was higher when compared with ML2. The SIs of lymphocytes from LP1 animals were also significantly higher (p=0.015) due to diet than that of lymphocytes from ML1 animals. After challenge, the SIs in infected groups significantly increased, but SIs in control groups did not increase. As a result, the SIs of lymphocytes from LP groups were significantly higher than that of ML groups over the period of the trial due to diet (p=0.001) and sensitization (p=0.007).

From week 24 of age (16 weeks of trickle) there was a moderate positive correlation (r>0.35) between FEC (log) and proliferative response to L_3 antigen.

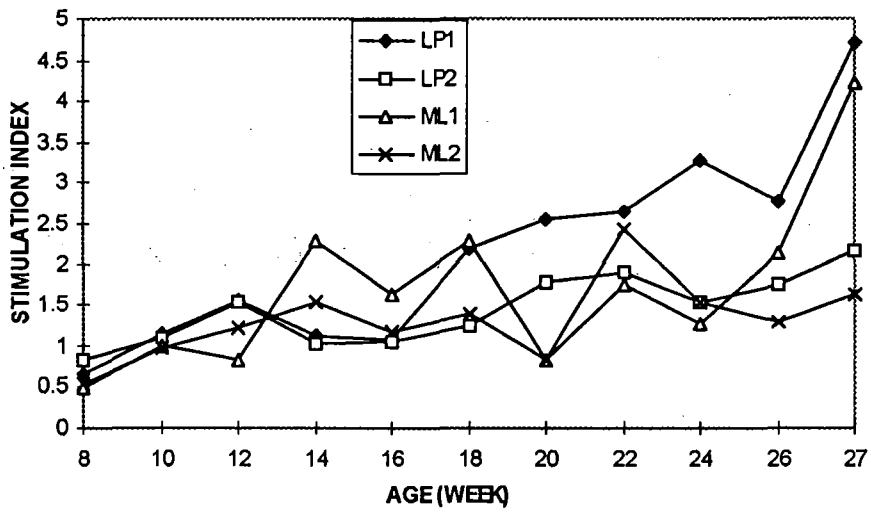

Figure 4.12: Mean SIs of blood lymphocytes from lambs cultured with L3Ag.

4.3.10. Lymph node lymphocyte blastogenesis test

Following slaughter of animals, lymphocytes were collected from the mesenteric lymph node and were cultured *in vitro T. colubriformis* infective larvae antigen (ESA and L3Ag) and non-specific (ConA) mitogens. The SIs of these lymphocytes were almost the same and there were no significant differences due to diet or infection.

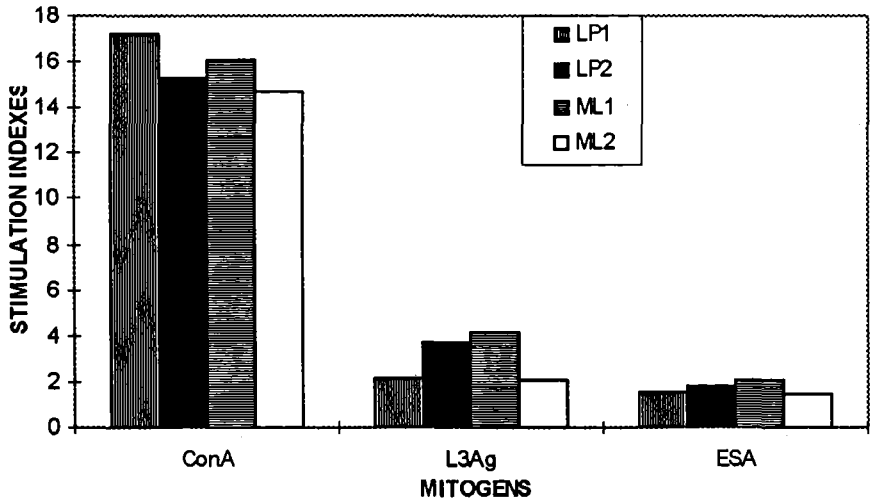

Figure 4.13: Mean SIs of the lymphocytes from mesenteric lymph nodes.

4.4. DISCUSSION

Over the period of the trial, the supplementation of the LP diet with methionine-lysine had no statistically significant effect on LWG. However, the mean BW of animals in LP and ML groups increased through the trial period and the ML1 group mean BW was 4.35 kg higher than that of the LP1 group mean at the end of trial. During the period 6-9 weeks after the start of the trickle (14-17 weeks of age), ML supplementation helped LWG. This is the time when the effects of infection typically become pronounced, and this is supported by the FEC data which had peaked approximately 2 weeks earlier. The food offered to animals in the 2 dietary groups was restricted but did increase during the period of the trial, therefore it was not possible to measure potential food intake in this experiment. In retrospect, the minimal effect on live weight gain may have been due to the impaired viability and subsequent establishment of the sensitizing infective larvae, and this constrasts with the findings of Kambara *et al.*, (1993) using protein (meat meal) supplements. Methionine and lysine supplementation did not significantly affect the carcass or thymus weights of animals even though they were highest in the ML1 group.

In the small intestine, establishment rates of the sensitizing populations of *T. colubriformis* were lower in the ML1 group (8 %) compared to the LP1 group (16 %). During sensitization, methionine-lysine supplementation significantly decreased the establishment rates of *T. colubriformis*. Following challenge, establishment rates were significantly lower in sensitized groups (0.7 for ML1 and 2 for LP1) than that of control groups (9 % for both ML2 and LP2). This low level of establishment in the control

unsensitized animals probably reflects the low viability of the infective larvae in the challenge innoculum. However, in the abomasum, the establishment rate of trickle *O. circumcincta* in the ML1 group (54 %) was slightly higher than the rate for the LP1 group (48 %). Challenge population establishment rates of L_3 were similar (32 % for ML1 and 36 % for LP1).

There was a significant difference ($p=0.017$) between the trickle worm population of the sensitized groups (LP and ML) in the small intestine. The worm count of the ML1 group was half that of the LP1 group. However, differences in challenge worm counts in the small intestine of previously sensitized animals were statistically not significant ($p>0.05$) due to ML supplementation. These results indicate that animals supplemented with dietary ML may have an improved resistance to incoming larvae but particularly have an improved ability to expel mature worms or inhibit larval development. Clearly this ML effect is an acquired immune characteristic. Thus our findings are in contrast to the findings of Coop *et al.* (1997) where they showed an improvement in resilience but not in resistance to established *T. colubriformis* populations with ML or HP supplementation. Abomasum worm populations of *O. circumcincta* were also similar after sensitization and challenge irrespective of diet (LP1 or ML1). In the control groups, no worms were detected after challenge which reflected the likelihood that *O. circumcincta* L3 larvae given orally were nonviable.

In the sensitized groups, the levels of faecal egg counts (FEC) were not consistent but in general they were higher in the LP1 group 6 to 8 weeks post infection ($p<0.5$). Then FEC increased in the ML1 animals and remained higher from 8-13 weeks post infection. The faecal egg counts of the LP1 group increased dramatically following challenge infection. Because of these oscillatory decreases and increases, differences in egg counts were not significantly affected by diet over the duration of the trial. These findings are in agreement with the findings of Coop *et al.* (1997) who showed statistically insignificant differences in faecal egg counts due to ML supplements in the diet.

Supplementation with ML or sensitization had no significant effect ($p=0.427$) on leukocyte numbers. No significant differences were detected in eosinophil numbers of lambs due to diet ($p=0.115$) and sensitization ($p=0.115$). However, there was a significant increase due to sensitization at 20 and at 26 weeks (following challenge).

There was a trend for ML supplementation to enhance the level of parasitic-specific antibody ($p=0.094$). The effect of diet was significant at 15 weeks of age ($p=0.024$) but not at 27 weeks of age ($p=0.113$). Sensitization had no effect on antibody level ($p=0.712$). The overall levels are low compared to background and compared to levels in older immuno-competent sheep and therefore are regarded as unimportant in terms of protection. This finding is supportive of previous work showing a lack of appearance of parasite antibody in this age of lamb following a similar sensitization (Kambara *et al.*, 1997; van Houtert *et al.*, 1995).

Supplementation with ML had a significant effect on the SIs of lymphocytes cultured *in vitro* with ConA ($p=0.022$). The SIs of lymphocytes from ML supplemented lambs were much higher between 12 to 16 weeks of age. The SIs of the LP groups subsequently increased and all groups were the same by the last week of trial. Supplementation with ML irrespective of trickle infection significantly increased the SIs due to ESA stimulation at 14, 16 and 18 weeks of age($p<0.05$). However, SIs of LP groups were significantly higher than that of ML groups at 20, 22 and 24 weeks of age ($p<0.05$) leading to overall lack of significance due to ML supplementation ($p=0.178$). There were highly significant effects of diet ($p=0.001$) and infection ($p=0.007$) on the SIs of lymphocytes cultured with L3Ag throughout the trial. In similar fashion, ML supplementation enhanced the response from 14-18 weeks of age superceeded by higher levels in the LP group. SIs were significantly higher in trickle infected groups.

The results of the lymphocyte stimulation data taken together show that dietary supplementation with ML has a non specific effect *in vitro*, 4 to 8 weeks after the start of the trickle infection and provision of the supplement. The fact that this effect is apparent with non specific mitogens (Con A) and with parasite Ag approximately 2 weeks later indicate that the improvement is not likely to be related to antigen presentation but rather something "downstream" from the moment of T cell activation. This time period corresponds to the reduction in faecal egg excretion rate during the early period of parasite establishment when ML supplements are offered. However, given the moderate positive correlation between FEC and L_3 proliferative response it is difficult to assign any of this *in vitro* activity to effective immune response *in vivo*. On the other hand, a moderate negative correlation existed between blood eosinophil levels late in the

sensitization period and challenge L_3 numbers suggesting that the former may have a role in preventing establishment of new infections.

In conclusion, results obtained from this study showed that dietary supplementation with protected ML significantly decreased the worm burdens of *T. colubriformis* following trickle infection and this is associated with certain immune cells.

CHAPTER FIVE

GENERAL DISCUSSION

Gastrointestinal parasites seriously affect all types of animals, particularly small ruminants and cause significant production losses. Many control methods such as the use of anthelmintics, pasture management and the selection of genetically resistant animals have been used to limit these losses and to increase animal production. However, all these methods have some shortcomings or disadvantages such as the development of resistance to anthelmintics, residue problems or lack of effectiveness. Therefore, alternative methods are required. The enhancement of immunity to parasites by dietary improvement is one such approach. There are a number of studies which have shown that protein (Abbott *et al.*, 1985, 1986a and 1986b; Abbott and Holmes, 1990; Dobson and Bawden, 1974; Donaldson *et al.*, 1997; Kambara *et al.*, 1993; Kambara *et al.*, 1996; Kyriazakis *et al.*, 1996 and Van Houtert *et al.*, 1995a and 1995b) and amino acid (methionine) supplementation (Coop *et al.*, 1997) have a role in the development of immunity and resilience to gastrointestinal parasites in sheep. The aim of this study was to test if a diet supplemented with protected dietary protein (fish meal) or methionine-lysine, fed to young lambs could affect immunity to *T. colubriformis* and *O. circumcincta*, and characterise some of the mechanisms involved.

The mechanisms of immunity to gastrointestinal parasites in sheep have been described in older sheep following protein supplementation (Kambara *et al.*, 1997, van Houtert *et al.*, 1995a). However, the enhanced resistance to *T. colubriformis* infection found in lambs younger than 6 months when trickle infected for 12 weeks and given dietary protein supplements has not been defined, except that acquired immune mechanisms such as lymphocyte proliferation (Kambara *et al.*, 1993), specific antibody production, eosinophil production and gut leukotriene release do not seem to be involved (Kambara *et al.*, 1997), although local T19 (gamma-delta) cells may have a role (Kambara and McFarlane, 1996).

Lambs given a high protein diet showed more resilience to *H. contortus* infection with less pathophysiological consequences (Abbott *et al.*, 1986b). Bown *et al.*, (1991) observed that post-ruminal infusion of protein (casein) had a lessening effect on the

pathophysiology of *T. colubriformis* infection in wether lambs, and this was confirmed by Kambara *et al.*, (1993) supplementing with meat meal and van Houtert *et al.*, (1995a) using fish meal. In this study, the addition of an HP supplement to the diet did not influence live weight gain in a major way; only during the first 4 weeks when the trickle infection typically began to influence productivity and during the last few weeks. The addition of protected methionine-lysine to the diet had a more pronounced effect than the HP diets at improving live weight gain particularly at certain periods such as at mid trickle. The balance between infection level, and diet (intake and quality) is clearly important and the outcome on productivity is difficult to predict accurately as indicated by other workers (Kyriazakis *et al.*,1996*)*. Gross measurements of the thymus were either too insensitive to detect differences found in rodents due to dietary protein and amino acids or the differences between dietary groups may have been inadequate. Alternatively, protein deficiency effects on thymic development may only be apparent in the prenatal foetus - in some ways the equivalent of the neonatal mouse - with respect to ontogeny of lymphocyte populations.

Previous work has indicated that protein supplementation of the diet for growing lambs improves resistance to parasite by expulsion of established *T. colubriformis* populations (Kyriazakis *et al.*, 1996, van Houtert *et al.*, 1995a) as well as preventing the establishment of new infections (Kambara *et al.*, 1993). However, dietary protein did not influence the establishment of *H. contortus* (Abbott *et al.*, 1985 and 1986a). As predicted by models (Dobson *et al.*, 1990), the duration of exposure to low continuous levels of infection appears to be critical and greater than 3 months of trickle infection appears to be necessary, at least for Coopworth lambs, to developed signs of acquired immune response to *T. colubriformis,* when low levels of infective larvae are ingested. The addition of either a high protein or a protected methionine-lysine mix affected worm burdens of *T. colubriformis.* In the case of the fish meal based diet, the established (due to trickle) worm burdens were not affected, but there was a trend for incoming L_3 challenge numbers to be decreased. When supplemented with ML the numbers of incoming L_3 were affected less than with HP supplementation. However, the expulsion of the established worm (trickle) population was most obvious with ML supplementation. It would appear from this experiment that the supply of both supplements interfere with resident worms and also the survival of incoming larvae but to

varying degrees of significance supporting both van Houtert *et al* (1995a) and Kambara *et al* (1997).

The production of faecal eggs was affected by both supplementation regimes at the beginning of the primary infection and after challenge, but was most marked with specific ML treatment. The fact that established populations of *O. circumcincta* or incoming infective larvae were not influenced by supplementation with either protein or ML may indicate that the mechanism for rejection for these abomasal species are different, or rather that the level of infection was low and no acquired immune response had developed. Previous research has shown that the acquisition of resistance to *H. contortus* (another abomasal worm) was influenced by dietary protein (Abbott and Holmes, 1990).

The blood eosinophil numbers became elevated during the latter part of the trickle infection and after challenge in all groups, but diet did not affect this result. The levels of eosinophils at weeks 20 and 26 correlated negatively with a subsequent challenge with *T. colubriformis* i.e. helped resist incoming larvae, but did not affect an existing worm burden. Numerous articles have indicated association between blood eosinophils and resistance (Buddle *et al.*, 1992) and the mechanism of eosinophil action has been implicated with expulsion of an established population of *T. colubriformis* (van Houtert *et al.*, 1997) and establishment of incoming L_3 (Kambara *et al* 1997). This experiment supports the latter, but not former experiment, irrespective of diet.

In general, the supplementation of diet with protein or ML improved the lymphocyte proliferative response to non specific mitogens and specific parasite antigen over an early part of the trickle infection for 3 weeks. A slight delay with parasite Ag stimulation may reflect the extra amount of antigenic processing time not needed by the mitogen. Thus, the acquisition of cell mediated immunity may be dependent on adequate protein and/or amino acids. However, there was only a parasite-specific association measured late in the trickle infections of groups with HP and LP diets and this correlated negatively with worm faecal egg output that indicated a role by lymphocytes affecting the resident worm population in the small intestine, particularly in terms of their fecundity. Interestingly, the sensitized groups supplemented with ML had a lower proliferation response. However, this does not necessarily mean that lymphocytes have no role in protection in

this instance. So called TH_2 cells which may release IL-5 necessary for differentiation of eosinophils may not exhibit a marked proliferative response. Note that there was a negative correlation between blood eosinophils and challenge L_3 burdens.

In summary, the provision of extra protein and specific amino acids have an effect on the establishment and maintenance of *T. colubriformis* infections. The mechanisms as to how these factors influence immunity have still to be clarified but appear to involve both common and unique pathways.

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APPENDICES - SUMMARY OF EXPERIMENTAL DATA

Mean live weights

Diet	Age (weeks)									
	8	9	10	11	12	13	14	15	16	17
HP1	12.95	14.3125	16.0375	18.2125	18.275	19.8875	20.85	20.9	21.9125	22.55
HP2	15.275	16.4125	18.45	20.4875	20.15	21.4375	22.7875	22.1875	23.2	24.275
LP1	14.7625	15.1875	16.8375	18.6875	18.8	19.975	20.2	21.5125	22.025	22.4375
LP2	12.975	14.1125	15.6875	17.275	17.1875	18.525	19.9	20.375	20.3875	21.725
ML1	14.725	15.725	17.95	19.0375	19.5375	21.15	22.475	23.6875	23.6875	25.4375
ML2	14.6625	16.0625	17.125	19.0375	19.35	20.2125	21.4625	22.5375	23.025	24.2

Diet	Age (weeks)									
	18	19	20	21	22	23	24	25	26	27
HP1	23.6125	23.975	26.9125	26.75	28.5	29.975	29.65	30.5625	32.0375	33.025
HP2	25.2125	26.1375	27.8625	28.2375	29.8	32.325	32.2	33.7	34.4125	35.2875
LP1	23.875	24.5875	26.0125	26.4	27.2	27.8875	28	29.175	30.3125	30.675
LP2	22.25	24.025	25.1	26.325	27.725	29.1625	28.975	30.55	31.525	32.1125
ML1	25.8125	27.225	28.8375	29.375	31	32.3	32.0125	34.3875	35.175	36.4375
ML2	24.9375	25.55	27.9125	28.7375	29.325	30.825	31.75	32.95714	33.51429	34.11429

Mean worm numbers

Diet	Trickle	Challenge	Total
HP1	2270	121.25	2393.75
HP2	0	2507.5	2507.5
LP1	3653.75	605	4258.75
LP2	0	2593.75	2593.75
ML1	1845	186.25	2031.25
ML2	0	2603.75	2603.75

Mean geometric egg numbers

Diet	Age (weeks)								
	7	11	12	13	14	15	16	17	18
HP1	0	9.46	173.18	46.24	214.78	94.41	84.33	224.39	95.5
HP2	0	0	0	0	0	0	0	0	0
LP1	0	5.15	193.09	152.8	340.2	15.26	138.96	32.27	63.12
LP2	0.94	0.93	0	0	0	0	0	0	0
ML1	0	4.66	30.84	98.31	20.18	24.24	316.69	148.97	307.32
ML2	0	0	0	0	0.78	0	0	0	0

Diet	Age (weeks)									
	19	20	21	22	23	24	25	26a	26b	27
HP1	136.46	154.88	50.52	34.44	22.54	120.5	192.75	42.66	173.78	134.9
HP2	0	0	0	0.78	0	0	0	0	0	0
LP1	7.55	40.21	50.52	173.18	62.97	100.63	86.3	323.34	209.38	32.65
LP2	0	0	0.94	0	0	0	0	0	0	0.93
ML1	198.1	67.71	149.7	26.93	24.1	24.6	64.01	64.01	28.1	116.7
ML2	0	0	0.78	0	0	0	0	0.78	0	0

Mean blood leukocyte numbers

Diet	Age (weeks)						
	8	10	12	16	20	26	27
HP1	3957.14	2000	5000	8857.14	7628.57	8000	7885.71
HP2	4622.22	4266.67	8088.89	10488.9	6577.78	6977.78	9266.67
LP1	3200	4000	5750	7600	6800	7900	8900
LP2	2350	3700	3700	8100	5250	4550	5250
ML1	2400	3550		8000	6775	5700	5700
ML2	3950	2850		6200	5550	6462.5	9850

Mean blood eosinophil numbers

Diet	Age (weeks)					
	8	10	12	16	20	26
HP1	0	0	0	12.5	81.25	37.5
HP2	12.5	0	0	6.25	6.25	6.25
LP1	5.55556	0	0	5.55556	30.5556	19.4444
LP2	12.5	0	0	12.5	6.25	0
ML1	12.5	0	0	6.25	37.5	6.25
ML2	12.5	0	0	25	0	12.5

Antibody mean OD values

Diet	Age (weeks)		
	8	15	27
HP1	0.95445	0.93620	1.08616
HP2	0.91685	1.00770	1.03757
LP1	0.9345	0.93854	1.15991
LP2	0.92238	0.94941	1.02654
ML1	0.887	1.02883	1.09229
ML2	0.92488	1.07938	1.259

Mean SIs of lymphocytes from blood cultured with ConA

Diet	Age (weeks)										
	8	10	12	14	16	18	20	22	24	26	27
HP1	52.1744	1.78375	41.9641	35.6463	29.8773	0.92513	6.96888	14.3958	11.5203	35.4953	22.9116
HP2	53.2836	2.09613	37.9858	20.95	54.8055	1.12913	9.63238	12.7659	12.1478	36.3516	19.0921
LP1	3.09625	6.34788	12.7186	1.59926	1.35488	36.4779	6.93738	8.85488	42.8085	35.8381	18.2869
LP2	4.93388	5.83838	16.4121	1.63838	1.19763	31.7365	11.0943	13.1985	55.3958	43.8459	14.9453
ML1	8.48375	3.0255	38.3159	91.027	10.0565	12.2068	1.08025	12.202	32.2488	42.1096	16.1064
ML2	7.66138	2.772	50.6628	54.4811	4.8485	9.0965	1.06875	16.3239	69.4606	42.5303	19.0841

Mean SIs of lymphocytes from blood cultured with ESA

Diet	Age (weeks)										
	8	10	12	14	16	18	20	22	24	26	27
HP1	0.96713	0.7815	1.534	2.524	2.46638	1.10088	1.53575	1.58663	1.1795	1.48463	2.58786
HP2	1.57613	1.07475	1.79475	1.57463	2.23338	0.96825	1.0165	1.38363	1.072	1.38663	1.87913
LP1	1.24913	1.12138	1.89775	1.2775	2.04025	1.15225	1.12188	1.67988	1.3	1.26113	1.79525
LP2	0.78538	1.1155	1.125	0.95513	0.83263	1.53063	1.296	1.20957	1.89588	1.21488	2.38438
ML1	0.477	1.273	0.47238	2.01475	6.98725	1.22675	0.85575	0.96138	1.21525	1.30663	2.368
ML2	0.484	1.15713	0.756	2.00975	6.57663	1.26888	1.11125	1.46938	1.524	0.98486	1.05271

Mean SIs of lymphocytes from blood cultured with L3Ag

Diet	Age (weeks)										
	8	10	12	14	16	18	20	22	24	26	27
HP1	1.78775	0.89163	1.71688	4.01488	3.58788	0.95963	2.40588	2.73313	2.18575	2.15263	4.17425
HP2	1.9285	0.96113	1.45863	1.53638	2.302	1.13075	1.67363	1.5675	1.96625	1.876	2.80013
LP1	0.66575	1.1345	1.54138	1.10663	1.06525	2.19175	2.55413	2.63657	3.28438	2.77513	4.71163
LP2	0.81625	1.08325	1.53025	1.01713	1.041	1.24463	1.77325	1.882	1.52138	1.75625	2.15688
ML1	0.49138	1.00438	0.8185	2.28713	1.62388	2.28188	0.8295	1.74813	1.25925	2.134	4.23075
ML2	0.53613	0.97375	1.20975	1.518	1.162	1.37263	0.83238	2.43138	1.53188	1.298	1.62186

Mean SIs of lymphocytes from mesenteric lymph nodes

	Antigen/mitogen		
Diet	ConA	L3Ag	ESA
HP1	17.23	3.45375	2.17375
HP2	14.6388	2.07125	1.28
LP1	13.4225	2.03125	1.41625
LP2	16.4263	2.06	1.49125
ML1	16.1063	4.185	2.03625
ML2	14.7171	2.08714	1.46571