IMMUNITY TO ABOMASAL PARASITES IN LAMBS

By

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DECLARATION

I declare that the work described in this thesis is my original work, any collaboration and assistance having been duly acknowledged.

Samuel A.J. Strain

Some of the work described in this thesis has been the subject of the following publications;

The genetic control of IgA activity against *Teladorsagia circumcincta* and its association with parasite resistance in naturally infected sheep. Strain, S.A.J., Bishop, S.C., Holmes, P.H., McKellar, Q.A., Mitchell, S., Stear, M.J. (In Press)

The influence of protein supplementation on the immune response to *Haemonchus contortus*. Strain,S.A.J., Stear, M.J.
Parasite Immunology (In Press)

The recognition of molecules from fourth-stage larvae of *Ostertagia circumcincta* by IgA from infected sheep. Strain,S.A.J., Stear, M.J.
Parasite Immunology 21 163-168

Resisting resistance. Stear, M.J., Strain, S.A.J.
The Veterinary Journal (Guest Editorial) 1999, 157, 111

Mechanisms underlying resistance to nematode infection. Stear,M.J.
Strain,S.A.J.
International Journal for Parasitology 1999, 29, 51-56

The relationship between the number and size of nematodes in the abomasum and the concentration of pepsinogen in ovine plasma. Research in Veterinary Science 1999 67, 89-92

The processes influencing the distribution of parasitic nematodes among naturally infected lambs. Stear,M.J., Bairden,K., Gettinby,G., McKellar,Q.A., Park,M., Strain,S., Wallace,D.S.

Parasitology 1998, 117, 165-171

The genetic basis of resistance to *Ostertagia circumcincta* in lambs. Stear,M.J., Bairden,K., Bishop,S.C., Buitkamp,J., Duncan,J.L., Gettinby,G., McKellar,Q.A., Park,M., Parkins,J.J., Reid,S.W.J., Strain,S., Murray,M.
The Veterinary Journal 1997, 154, 111-119

How hosts control worms. Stear,M.J., Bairden,K., Duncan.J.L., Holmes,P.H., McKellar,Q.A., Park,M., Strain,S., Murray,M., Bishop,S.C., Gettinby,G.
Nature 389, 27

and presentations;

How lambs control infection with *Ostertagia circumcincta.* (1998) Stear,M.J., Strain,S., Bishop,S.C.
5th International Veterinary Symposium.

The mechanism underlying genetic resistance to *Teladorsagia (Ostertagia) circumcincta.* (1998) Stear,M.J., Strain,S.
2nd International Conference on Novel Approaches to the Control of Helminth Parasites of Livestock.

IgA is the major mechanism controlling growth and fecundity of *Ostertagia circumcincta.* (1998) Strain,S.A.J., Stear,M.J.
2nd International Conference on Novel Approaches to the Control of Helminth Parasites of Livestock.

Protein Supplementation is associated with an increased IgA response against *Haemonchus contortus.* (1997) Strain,S.A.J., Stear,M.J.
The British Society for Parasitology.

Protein Supplementation is associated with an increased IgA response against *Haemonchus contortus.* (1997) Strain,S.A.J., Holmes,P.H., Stear,M.J.
The Association of Veterinary Teachers and Research Workers.

DEDICATION

To my parents who showed me the value of farming

To my wife

Soli Deo Gloria

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LIST OF ABBREVIATIONS

DMSO: Dimethylsulphoxide

EDTA: Ethylenediaminetetraacetic acid

HCl: Hydrogen Chloride

Ig: Immunoglobulin

IL: Interleukin

IFN: Interferon

MHC: Major Histocompatibility Complex

PBS: Phosphate Buffered Saline

RPMI: Roswell Park Memorial Institute

SDS: Sodium dodecyl sulphate

TEMED: Tetramethylethylene diamine

Zn: Zinc

PCV: Packed cell volume

Th: T helper cell

KDa: Kilodalton

CCK: Cholecystokinin

PMSF: Phenyl methyl sulphonyl fluoride

TPCK: N-tosylamide-L-phenylalanine chloromethyl ketone

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ABSTRACT

The parasitic nematodes *Teladorsagia (Ostertagia) circumcincta* and *Haemonchus contortus* are two of the most important pathogens of sheep and goats worldwide. Control has largely depended upon the use of anthelmintics. However with the widespread use of these compounds has come the emergence of parasite resistance. Other modalities of prevention and treatment are urgently needed. One of the most promising is the use of genetically resistant sheep. Although it is widely accepted that breeding for resistance to these parasites is possible, the mechanisms of resistance are unknown. The purpose of the work described in this thesis was to identify the mechanism of resistance to these parasites in young lambs.

Lambs infected with *T. circumcincta* are incapable of controlling their worm burdens. However, it appears that some are capable of controlling the growth and therefore the fecundity of adult female worms. Work described in chapter three shows that the most important mechanism controlling the growth and fecundity of this parasite is the local IgA response. 933 lambs were studied over 5 years. Faecal egg counts were performed on these lambs and 485 of these lambs were slaughtered and the average female worm lengths determined. Analysis showed a highly significant effect of parasite specific IgA on worm length. Those lambs with higher IgA responses to fourth-stage larvae had on average shorter worms. This response was heritable. Thus genetic resistance to *T. circumcincta* acts by reducing worm fecundity and works through a parasite-specific IgA response. In addition, this response is sex related with male lambs having the poorest responses and females the best.

Not only is the quantity of IgA important in determining host resistance, but also the specificity. Chapter four details work done in investigating the antigen specificity of the IgA response to *T. circumcincta.* Here, the recognition of antigens from fourth-stage *T. circumcincta* by plasma IgA was studied in a group of 30 ten-month-old Scottish Blackface sheep which had been naturally, then deliberately infected. There was a heterogeneous pattern of antigen recognition amongst the sheep. Two antigens with approximate molecular weights 87 000 Da and 129 000 Da were significantly associated with a reduction in mean adult worm lengths. The observed variation in recognition of these two antigens on fourth-stage preparations accounted, in a statistical sense, for nearly 40% of the total variation in worm length. In addition to the variation in antibody mediated recognition of these two parasite molecules, three other components have been implicated in regulating worm length. They are a 37 000 Da band from adult worms, the amount of fourth-stage larval specific IgA in the abomasal mucosa and the density-dependent influence of adult worm burden. Together these components and their interactions accounted for over 90% of the variation in worm length. These results indicate that the parasite-specific IgA response or something extremely closely associated with it, is the major immunological mechanism controlling worm length. For another mechanism to control worm length it would have to account for more of the variation. It is difficult to envisage such a mechanism existing.

Teladorsagia circumcincta is an extraordinarily successful parasite. One factor that undoubtedly contributes to its success is its ability to suspend development by going into inhibition at the early fourth-stage. Four mechanisms appear to underlie larval inhibition; genetic variation in the parasite, larval

response to seasonal changes, the host immune response, and density-dependent inhibition of larval development. To help define this further, the influence of immune responses and of the number of adult worms was studied in both naturally and deliberately infected sheep and the results are described in chapter five. Variation among sheep in the number of inhibited larvae was significantly associated with variation in the size of the IgA response to fourth-stage larvae, with the specificity of the antibody response to third, fourth and adult stages, and with the number of adult parasites present. These results indicate that the IgA response may influence larval development and that the density-dependent constraints on larval development do not work through the IgA response. The immunological and density-dependent associations were additive. Taken together, the IgA response, antibody specificity, and numbers of adult parasites accounted, in a statistical sense, for over 90% of the variation in numbers of fourth-stage larvae present.

In addition to *T. circumcincta* it was of interest to see if IgA may be involved in the development of immunity to another abomasal parasite *Haemonchus contortus.* Hampshire Down lambs were trickle infected with *H. contortus* over ten weeks. The lambs were offered one of two diets: a basal diet and a diet supplemented with additional protein. Plasma samples were taken for determination of IgA responses and the lambs necropsied and the adult female worms measured. Those lambs on the supplemented diet had shorter adult worms and produced significantly more anti-parasite IgA. There was a significant association between IgA against third-stage or fourth-stage larvae and adult worm length. Most of the difference between the two dietary groups could be accounted for by differences in IgA responses. Therefore, IgA may be a

major mechanism controlling fecundity of *H. contortus* and the magnitude of the IgA response is influenced by the quality of the diet.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 GASTROINTESTINAL PARASITISM IN SHEEP

1.1.1 General

Sheep production constitutes an important part of livestock farming in many parts of the world today. In many areas of the world they are the only livestock and therefore are the sole source of income to owners and landusers. Within the UK there are approximately 20 million ewes and 24 million lambs. The estimated sheepmeat value per annum is over £1 billion and wool approximately £30 million (Clarkson & Winter 1997).

Parasitism constitutes the largest single constraint on sheep production worldwide. The costs incurred by parasitism are both direct and indirect. There are the direct effects of sheep mortality and reduced productivity, and the indirect effects of increased production costs incurred through treatment, increased labour and enforced changes in pasture management.

Of the gastrointestinal parasites (table 1.1), *Haemonchus contortus* and *Teladorsagia circumcincta* are two of the most prevalent and economically important (Allonby & Urquhart 1975, Urquhart et al 1987). *H. contortus* is predominantly found in tropical/sub-tropical regions while *T. circumcincta* is the predominant species in temperate regions. Both are remarkably successful parasites of sheep and goats in their respective regions. Other parasite nematodes rarely achieve similar high levels of prevalence or intensity of

infection as measured either by faecal egg counts or worm burdens (Shaw & Dobson 1995). Farmed ruminants may harbour more parasites than free roaming populations because of the combination of high stocking densities and restricted pasture leading to high pasture contamination. In such an environment, those parasites with higher fecundity or better viability would be expected to predominate. *H. contortus* is a highly fecund parasite with even moderately infected sheep having very high faecal egg counts (Urquhart et al 1987). One estimate suggested that the daily egg output from one worm could be 9000 eggs (Cushnie & White 1947). However, *T. circumcincta* is considerably less fecund than *H. contortus* and does not appear to be significantly more fecund than other nematode parasites found in temperate regions although definitive data is lacking. Its success may be the result of a superior ability of larvae to survive both on pasture during adverse weather conditions, and within hosts in an inhibited state (Boag & Thomas 1977; Stear et al 1997a).

PARASITE	SITE	REGIONAL DISTRIBUTION
Haemonchus contortus	Abomasum	Tropical/sub-tropical
Teladorsagia circumcincta	Abomasum	Temperate/sub-tropical
O. trifurcata	"	"
O. leptospicularis	"	"
Trichostrongylus axei	Abomasum	Worldwide
T. colubriformis	Small intestine	"
T. vitrinus	"	"
Cooperia curticei	Small intestine	Worldwide
C. surnabada	"	"
Nematodirus battus	Small intestine	Temperate
N. filicollis	"	"
N. spathiger	"	"
Bunostomum trigonocephalum	Small intestine	Worldwide
Gaigeria pachyscelis	Small intestine	Tropical
Strongyloides papillosis	Small intestine	Worldwide
Trichuris ovis	Large intestine	Worldwide
Chabertia ovina	Large intestine	Worldwide
Oesophagostum columbianum	Large intestine	Tropical/Sub-tropical
O. asperum	"	"
O. verulosum	"	"

Table 1.1. Nematode parasites of sheep.

1.1.2 *Haemonchus contortus*

1.1.2.1 Description

H. contortus belongs to the superfamily Trichostrongyloidea of the order Strongyloidea and the class Nematoda. It is large compared to other trichostrongyloid parasites with adults measuring 2.0-3.0 cm. It is found in the abomasum of sheep, goats and cattle. Adults can be identified by their reddish-brown colour, a consequence of their blood-filled intestine. The ovaries of the female wind spirally around the intestine. This gives them their characteristic red and white barber's-pole appearance. The males have an asymetrical dorsal lobe and barbed spicules. Both have cervical papillae and a lancet within the buccal capsule that they use to obtain blood from mucosal vessels.

1.1.2.2 Life Cycle

H. contortus is a blood-feeding parasite of the abomasum. It has a direct life-cycle. The adult female parasite lays eggs in the abomasal lumen from where they are excreted in the faeces. On the herbage the parasite develops through three larval-stages L1, L2, and L3 over a minimum of five days. The L3 stage keeps the second stage cuticle which allows the parasite to resist desiccation. This allows them to survive desiccation for up to 3 months. The L3 are ingested by the sheep and exsheath in the rumen. In the abomasum they develop through the fourth larval stage (L4) stage within the gastric gland and subsequently into adults on the abomasal surface. The pre-patent period is three weeks.

1.1.2.3 Epidemiology

Tropical/sub-tropical regions

H. contortus develops optimally in warm climates. High humidity is necessary for larval development and survival and so the level of rainfall is a major constraint on its success. Due to the high fecundity of the female, pasture can become contaminated very quickly. In addition, immunity in adult sheep exposed to endemic infection is often not complete and can break down allowing for continual pasture contamination. In some areas of the world the parasite is able to arrest development (hypobiosis) at the beginning of dry periods when faecal eggs and larvae would be unable to survive. In other areas where there is a more frequent rainfall there appears to be no hypobiosis.

Temperate regions

Most commonly, there is a single annual life cycle. Larvae are ingested in the early summer and become arrested. They complete their development in the following spring that often coincides with lambing. Consequently, clinical haemonchosis is rarely a problem in these areas.

1.1.2.4 Pathogenesis and Clinical signs

The disease produced is a direct consequence of the adults feeding on host blood. This leads to a protein losing gastropathy, which is exacerbated by anaemia. Under prolonged exposure the anaemia can become non-regenerative due to inadequate replenishment of iron. The clinical signs are a corollary of hypoproteinaemia and anaemia and thus can include reduced packed cell volume (PCV), mucus membrane pallor, oedema (particularly sub-mandibular), ascites,

lethargy, melaena, progressive weight loss, weakness, tachypnoea, and tachycardia. Typical findings at necropsy include petechiation of the abomasal mucosa, evidence of bone marrow exhaustion, and dark brown abomasal contents due to haemorrhage, and carcase oedema.

The disease can be categorised into three syndromes depending on the degree of parasitism; acute, hyperacute, and chronic haemonchosis. In acute haemonchosis there is a dramatic fall in packed cell volume, which stabilises at a low level. However, as a result of iron loss a non-regenerative anaemia ensues which leads to death. In hyperacute haemonchosis sheep die suddenly as a result of a severe haemorraghic gastritis from a massive worm burden of up to 30,000. Chronic haemonchosis is seen where sheep are exposed to lower parasite burdens over a prolonged period. The chronic blood loss, often compounded by poor nutrition, leads to weight loss, weakness and decreased productivity.

1.1.3 *Teladorsagia (Ostertagia) circumcincta*

1.1.3.1 Description

T. circumcincta parasitises the abomasum of small ruminants and disease is associated with the emergence of the late L4 from the gastric gland and, the presence of adult parasites. Like *H. contortus, T. circumcincta* belongs to the superfamily Trichostrongyloidea of the order Strongyloidea and the class Nematoda. Adults appear light brown and measure 0.6-1.2 cm. Males have long slender spicules with three distal branches.

1.1.3.2 Life Cycle

T. circumcincta has a simple direct life cycle where eggs are laid in the lumen of the abomasum and are passed in the faeces. In the faecal pat the eggs hatch and develop through two stages to the infective third stage. In optimum conditions the development of eggs to larvae can occur within two weeks. When the faecal pat is moistened the larvae migrate onto the herbage. The L3 are ingested by the sheep and exsheath in the rumen. From there they pass into the abomasum and enter the gastric glands of the abomasum. In the abomasum they develop through a fourth-stage into the adult within the gastric gland and then emerge into the lumen 7-8 days after ingestion. Larvae can arrest their development at the early fourth-stage of development and resume development some time later (Armour et al 1966; Dunsmore 1960). The minimum prepatent period is 13 days.

1.1.3.3 Epidemiology

In temperate regions eggs are passed in the faeces of lambs during the spring reaching a peak in late summer (Boag & Thomas 1977). The source of infection for lambs is believed to come from overwintered larvae, eggs passed by ewes during the peri-parturient period, and from lambs developing patent infections (Boag & Thomas 1970). Thus pasture contamination builds up during the summer to give clinical teladorsagiasis. After October the majority of ingested larvae become arrested. These arrested larvae can subsequently develop to adults during the following spring. This can give rise to Type II teladorsagiasis although it is much less common in sheep than in cattle infected with *O. ostertagi* (Armour & Bruce 1974).

1.1.3.4 Pathogenesis and Clinical signs

Disease is thought to be a consequence of the presence of fourth-stage larvae within the gastric gland (Armour et al 1966). Infected gastric glands become stretched as the larvae grow and the surrounding epithelium becomes hyperplastic. Parietal cells are replaced by undifferentiated epithelial cells leading to a reduction of functional gastric gland mass. This de-differentiation occurs not only in infected glands but also in adjacent uninfected ones. The loss of parietal cells leads to an increase in abomasal pH and a failure to convert pepsinogen to pepsin and thus to a reduction in digestive efficiency. The actual effect of this on the host of this is uncertain as there is evidence that the small intestine can compensate for failure to digest abomasal protein (Parkins et al 1973). There is also evidence that infection stimulates zymogen cells to secrete pepsinogen. Elevated levels of plasma pepsinogen can be detected in infected animals. This may be due either to leakage across the damaged mucosa or direct secretion into the circulation (McKellar 1993). The elevation in pH can also leads to a failure of bacteriostasis.

The mucosa becomes oedemic and hyperaemic with occasional mucosal sloughing. At the cellular level, there is a leakage of plasma protein across the mucosal membrane, which may be due to a breakdown or incomplete formation of intracellular junctions. This is the main cause of the reduced nitrogen digestibility seen in infected animals (Parkins et al 1973). In addition, infection causes inappetance. The possible mechanisms of this are reviewed later.

The clinical signs are therefore a consequence of a relative protein deficiency. Unlike in cattle, teladorsagiasis in lambs rarely causes diarrhoea. The main clinical signs are a depression in feed intake and reduced liveweight

gain. Infection leads to reduced nitrogen digestibility, reduced calcium and phosphorous deposition, poor carcase conformation, and impaired wool growth (Sykes & Coop 1977; Symons et al 1981). Thus, the economic impact is through a depression in productivity. High levels of infection have been associated with population crashes in feral sheep on St. Kilda (Gulland 1992). Those animals that survived population crashes were less heavily parasitised than those that died. Whether these deaths can be directly attributed to parasitism is uncertain but even a moderate experimental infection can reduce growth rate by approximately one third (Coop et al 1985).

1.1.4 Present Control Strategies

Control at present is dependant the use of anthelmintics and management of grazing.

Most overwintered larvae die out during the spring (Boag & Thomas 1970). Therefore it is possible to alternate pastures grazed by sheep with those grazed by cattle annually. Similarly, crops can be alternated with grassland and if cattle are available there could be a rotation of cattle, sheep, and crops (Armour & Coop 1991). However, these strategies are only applicable to *T. circumcincta* as *H. contortus* can infect both cattle and sheep. Unfortunately, strategies dependent on management alone are seldom practical as 'clean' pasture is rarely available. In addition, they can occasionally lead to parasitic gastroenteritis from those parasites such as *Trichostrongylus colubriformis, Trichostrongylus axei* and *Nematodirus battus* that are capable of crossing the species barrier between cattle and sheep. Also, there is evidence that a small proportion of infective larvae can survive beyond 2 years on the herbage and

upper soil layers (Armour et al 1980). Therefore there is a risk that reliance on pasture management alone may fail. Pasture that has been free from sheep for 12 months can still harbour sufficient larvae to allow for sufficient generations to develop during the subsequent grazing year that clinical teladorsagiasis appears.

More frequently, anthelmintics are used in conjunction with management in a "dose and move" strategy where animals are treated and moved to pasture known to be free of infection. However 'clean' pasture is rarely available and most commercial situations call for the exclusive use of anthelmintics.

One of the most important sources of infection for lambs is the peri-parturient rise in faecal egg output in ewes during the last trimester of pregnancy and early lactation (Armour & Coop 1991). This appears to be due to a relaxation in the immune response to parasites resident within the abomasum and to freshly acquired larvae. Ewes are normally given an anthelmintic approximately one month before lambing and again shortly afterwards. Lambs are given anthelmintics at differing intervals dependent upon the preferences of the farmer.

The universal use of anthelmintics has led to the evolution of anthelmintic resistance in both parasites (Jackson 1993). Initially, resistance developed slowly against the less efficient early benzimidazoles, but with the introduction of more efficient anthelmintics, selection pressure for resistance has increased. Presently there is resistance to pharmaceuticals within each of the anthelmintic groups available for treatment in both parasite species. There has been no convincing evidence that if selection pressure is removed there is a reversion to susceptibility to anthelmintic (Jackson 1993). This is supported by findings that

parasites resistant to benzimidazoles are as fit as susceptible parasites from the same strain (Elard et al 1998).

For strategies aimed at delaying the onset of resistance to be successful they must use a minimum of chemoprophylaxis in order to minimise the number of parasite generations exposed to anthelmintic while maximising the efficacy of the drug in order to remove heterozygous resistant genotypes. An important concept when considering this is the relationship between the parasite population within an animal (the infrapopulation) and the one on the pasture (suprapopulation). If there is a large infrapopulation and a small suprapopulation and the host is wormed frequently then there will be a rapid increase in the number of resistance alleles within the total worm population. So, for example, a dose and move strategy where animals coming from a highly contaminated pasture are treated and then moved onto a 'clean' pasture is likely to select for parasite resistance.

One method to reduce selection for resistance is to rotate between groups of anthelmintic used. Resistance to one anthelmintic will confer cross-resistance to others within the same group and so rotation must be between anthelmintic groups. It has been shown that annual rotation is of more benefit than rotation after each treatment (Waller et al 1989).

Other strategies aimed at increasing the parasite kill are the use of splitting the dose of anthelmintic and combination therapy. Splitting the dose over two days increases the efficacy of benzimidazole drugs as it is related to the length of time the parasite is exposed to the drug (Sangster et al 1991). Combination therapies are based on the premise that it is highly unlikely that resistance alleles to two groups of anthelmintics will be found on one parasite. Such a strategy has

been shown to delay the emergence of resistance compared to strategies employing single drug therapies (Jackson 1993). However, simultaneous resistance to both classes may emerge at the same time with both strategies. Such a strategy employing two or more anthelmintics would add to the cost of production and may not be economically feasible. At best all of these strategies will only delay the emergence of resistance.

Perhaps one of the largest contributors to resistance emergence is the frequent underdosing of animals. Underdosing selects for heterozygous resistance thus increasing the number of resistance alleles within a population. The dose given should be that dose needed for the heaviest lamb but often a lower average dose is given thus underdosing the heaviest lambs.

1.1.5 Future control strategies

1.1.5.1 Vaccines

Vaccines have proven to be an extremely useful means of protection against a vast range of microorganisms. This success and the need for efficient strategies for the prevention of parasitic infections has led to a large volume of work directed towards the development of vaccines (Newton & Munn 1999). Two types of vaccines have been studied and used. The first type is based on antigens that are accessible to the host's immune system during natural infection. The advantage of such a vaccine is that continual natural exposure acts as a booster to the primary vaccine. However, many of the antigens that the host mounts an immune response to are non-protective and there is considerable variation in the identification of parasite molecules between animals (Haswell-Elkins et al 1989; McCririe et al 1997). The second method has been to look for

antigens that are hidden from the afferent immune system during normal infection (Munn 1997). The target antigens must be inaccessible to the host's immune system but must be accessible to antibodies or other immune components induced by the vaccination. The advantage of such vaccines is that the parasite will not have evolved mechanisms to evade immune responses to such antigens. However, the main disadvantage is that continual boosting of the immune response through natural exposure would not be expected to occur. Despite this there is evidence that vaccination with the hidden antigen H11 does confer a long period of immunity (Andrews et al 1997).

Currently there are successful vaccines against three parasites of veterinary importance; *D. viviparous, Taenia ovis,* and *Boophilus microplus*. The first two consist of natural antigens while the vaccine against the tick *B. microplus* consist of a protein based on the gut membrane protein Bm86 (Willadson 1997). The vaccine against *Dictyocaulus viviparous* (Dictol™) consists of X-irradiated third-stage larvae and is accepted as the best means of control of bovine lungworm (David 1999). However, the protective antigens in this vaccine remain unknown. In comparison several protective antigens have been identified from oncospheres of *T. ovis* for vaccination of sheep (the intermediate host) although a commercial vaccine is not currently available (Newton & Munn 1999).

Currently a number of antigens are being studied as nematode vaccine candidates and several have shown promise in vaccine trials. Most of the work on nematode vaccine candidates has focused on *H. contortus* (table 1.2). However there are reports of some work that has been done on analogues to some of the antigens on *H. contortus.* An equivalent to Hc-sL3 on *T. circumcincta* has been identified by both anti-Hc-sL3 antisera and by probes

using antibody secreting cells isolated from draining lymph of infected animals (Newton & Munn 1999). Similarly the homologues of H11, H-gal-GP and the 70kDa cysteine protease in *T. circumcincta* (O12 and O-gal-GP respectively) are reported to have been identified and characterised, although there are as yet no published details of their efficacy in vaccine trials.

There is as yet no certainty over the mechanisms of resistance induced by these vaccines. *In vitro* killing of third stage larvae in the presence of anti-Hc-sL3 is eosinophil dependent (Ashman et al 1995). Also immunity induced by this antigen appears to be IgE independent as animals vaccinated with pertussis toxin adjuvant which strongly induces an IgE response abrogated immunity (Jacobs et al 1995).

Molecule	Type of molecule	Molecular Weight (kDa)	Natural (N) or hidden (H) antigen	Reduction in FEC (%)	Reduction in worm burden (%)	Reference
Hc-sL3	Unknown	70-83	N	64-69	45-55	(Jacobs et al 1995)
Adult 15 & 24 kDa E/S antigens	Unknown	15 & 24	N	77	82	(Schallig et al 1997)
Contortin	Microvillar surface associated polymer	Complex mixture of gut associated proteins	H	-	78	(Newton & Munn 1999)
H11	Microsomal membrane aminopeptidase	Doublet with mean Mol weight 110	H	71-99	60-98	(Newton & Munn 1999)
H-gal-GP	Aspartyl protease, neutral endopeptidase,and has cysteine protease activity	Non-reducing conditions runs as five bands of 31,40,42,170 and 230 kDa	H	93-95	53-72	(Smith et al 1994)
P1	Unknown	Complex mixture including proteins of 45,49 and 53kDa	H	69	30 (Not Significant)	(Smith et al 1993)
P52/p46	Unknown	Mixture of 46, 52 and trace amounts of a 100kDa	Uncertain thought to be hidden but reports that traces could be detected in abomasal mucus	34	53	(Jasmer et al 1993) (Newton & Munn 1999)
35/55 kDa	Cysteine protease	35 & 55	H	93	87	(Boisvenue et al 1992)
70 kDa	Cysteine protease	70	H	76	46	(Newton & Munn 1999)

Table 1.2 Vaccine candidates for *H. contortus*.

1.1.5.2 Biological Control

Biological control is targeted at the free-living stages of the parasite. Two means of biological control that have provoked interest are the use of nematophagous fungi and species of grass used.

When the chlamydospores of nematophagous fungi are ingested by parasitised animals and excreted in the faeces they develop in the faecal pat and produce hyphae that trap and kill developing nematode larvae (Waller & Faedo 1996). *Duddingtonia flagrans* appears to survive passage through the ruminant gut more efficiently than other nematophagous fungi (Faedo et al 1998) and most recent work has focussed on this fungus. Clinical disease has been averted in trials where calves naturally infected with *O. ostertagi* were given *D. flagrans* (Nansen et al 1995). Feeding lambs infected with predominantly *T. circumcincta* with *D. flagrans* led to a reduction in newly acquired worm burdens of 62 % (Githigia et al 1997). Further work is necessary to determine the optimum dose of chlamydospores and the environmental impact of seeding pasture with nematode trapping fungi.

There is some evidence that changing the type of forage fed to lambs affects the worm burdens and faecal egg counts of lambs suffering from parasitic gastroenteritis. In one study feeding lambs sulla (*Hedysarum coronarium*) led to significant reductions in the worm burdens and faecal egg counts of lambs compared to others fed other types of forage (Niezen et al 1998a). It has been suggested that plants that contain condensed tannins reduce larval establishment and increase nematode mortality (Niezen et al 1998b). However, it remains unclear how certain forages might affect parasite nematodes. Condensed tannins reduce the amount of protein degradation in the rumen thus increasing rumen

bypass protein (Waghorn 1987). It could be that the effect of changing the forage is to improve the host's protein digestion, which in turn improves the host's immune response.

1.1.5.3 Genetic Resistance

The observation that different animals have differing susceptibilities to infectious disease is widely recognised. Several investigations have shown that sheep can be bred for resistance to gastrointestinal parasites and that individual breeds appear to be more resistant to infection than others (Gray & Woolaston 1991). This increased resistance appears to work through immune responses that are more effective. The genetic influence on the immune response is reviewed later.

Genetic resistance can be exploited in several ways. Breeds resistant to infection can be used in the place of those that are more susceptible. For example, the Red Massai breed is more resistant to *H. contortus* than other European breeds (Mugambi 1994). However, for breed substitution to be effective the new breed must be acceptably productive. Resistant breeds are often smaller and a common perception is that they are less productive than the larger, more susceptible breeds. Associated with this is the perception of farmers that the new breed is inferior to the larger breed even when evidence to the contrary is available. For breed substitution to be effective, the farmers need to be persuaded, which can be a difficult task to achieve.

Resistant breeds can also be exploited by the use of cross-breeding and the development of a composite population (Nicholas 1993). However, there is little

published work on the use of such techniques to develop resistance amongst sheep to nematode infections.

Most of the work to use genetic resistance to infection has been the selection from within populations of sheep for breeding programmes. The success of such programmes depends upon several factors. They include the heritability of resistance (generally approximately 0.3 (Stear & Wakelin 1998)), the intensity of selection (which must be offset by avoiding inbreeding), the genetic variation of the trait within the population, the accuracy of the selection process (for example using best linear unbiased prediction methods which uses information from relatives), the time from one generation to the next and the size of the population.

1.2 IMMUNITY TO GASTOINTESTINAL PARASITES

1.2.1 General

Current opinion generally regards the immunological mechanisms controlling gastrointestinal parasites as complex (Miller 1984). Much work has gone into developing laboratory models for studying parasite/host interactions.

The advantages of using models are clear. They are relatively cheap and allow extensive manipulation of host responses by comparing the various responses to infection of different strains of host, notably inbred, congenic, recombinant inbred, mutant and genetically modified. In addition, the extensive knowledge of rodent immune systems and the availability of reagents to investigate immune responses have allowed extensive work to be done in defining these immune responses.

However, despite the undoubted usefulness of rodent models there remain limitations to their usefulness. Clearly, models are only indicators of what may be happening in the definitive host/parasite. Any conclusions drawn must be confirmed in the species under investigation. In some cases the parasites under investigation are not natural parasites of the model host making the model yet more artificial.

It is however worthwhile to identify those major findings from models that may be pertinent in order to develop a context to look at immune responses to *T. circumcincta* and *H. contortus*.

1.2.2 Disease Models

1.2.2.1. *Heligmosomoides polygyrus*

Adults of *H. polygyrus* are found in the small intestine of the mouse but are not thought to be pathogenic. The females are larger than the males with tightly coiled bodies. Eggs are passed in the faeces and the larvae hatch and develop through 2 moults to infective third stage larvae within 4-6 days. After ingestion the larvae lose their sheath in the stomach and migrate to the small intestine where they burrow into the mucosa. The larvae moult to adults, 6-8 days after infection and can remain viable for several months.

Infection of mice with *H. polygyrus* is usually chronic (Behnke 1987). However, this does not appear to be due to any ability of the parasite to resist expulsion mechanisms. For example, *Trichinalla spiralis* evokes a rapid inflammatory response which is sufficient to cause the expulsion of third stage and adult *H. polygyrus* in concurrently infected mice (Behnke et al 1992). However, *H. polygyrus* is able to slow down the normal immune response to *T.*

spiralis and thus delay worm expulsion (Behnke et al 1993). This is associated with a delay in mastocytosis and a reduction in production by mesenteric lymphocytes under mitogen stimulation of IL-9 and IL-10 normally seen during *T. spiralis* expulsion (Behnke et al 1992).

It appears to be adult parasites that modulate the immune response (Kurtzhals et al 1998). If infection is stopped before adult parasites develop, immunity is developed that is sufficient to prevent the establishment of subsequent larval infections (Wahid & Behnke 1992). The mechanism for this immunosuppression remains unclear. It may be mediated through impaired antibody production (Pritchard et al 1994), suppression of mastocytosis (Dehlawi & Wakelin 1988), hypergammaglobulinaemia (Robinson & Gustad 1996), or non-specific binding of IgG1 by a superantigen (Robinson et al 1997a).

However, immunomodulation is not seen in all strains of mice. Adult worms were used to successfully immunise mice to larval challenge in NIH mice but increased susceptibility to infection in an outbred strain of mouse (Pleass & Bianco 1994). It would appear some strains of mice are able to overcome any immunomodulatory effects of adults.

Immunity to the parasite can be partially abrogated by a systemic infection with *Trypanosoma congolense* (Fakae et al 1997). Whether this is due to a redirecting of the immune response away from a mucosal response towards a systemic response or is a reflection of "stress" from concurrent infections remains unknown.

Lymphocytes derived from the thymus are known as T cells. They can be divided into two sub-populations according to the expression of the cell surface receptors CD4 and CD8. CD4+ cells can be further categorised by the cytokines

they produce into Th1 or Th2 cells (Mosmann et al 1986). Although concerns about the danger of adhering too rigidly to this model have been raised (Allen & Maizels 1997), it remains a most useful model in elucidating basic types of immunological response. Th1 responses are characterised by production of IFN-gamma, IL-2 and lymphotoxin while Th2 responses are characterised by production of IL-4, IL-5, IL-9 and IL-10. The Th2 response is generally associated with an effective immune response to parasitic infections as measured by the control of worm burden and worm fecundity.

CD4+ cells are essential to the development of effective immunity in mice infected with *H. polygyrus.* When mice are given anti-CD4 monoclonal antibody, host immunity is totally abrogated (Urban et al 1991a). In addition IL-4 appears critical to immunity. Treatment with anti IL-4 or with anti-IL-4 receptor monoclonal antibody increases both parasite survival and fecundity (Urban et al 1991b). Similarly treatment with IL-4 decreases fecundity and leads to worm expulsion (Urban et al 1995).

The passive transfer of IgG1 transfers immunity (Pritchard et al 1983). However, treatment with anti-IL-4 monoclonal antibody blocks immunity but does not block the polyclonal IgG1 response (Finkelman et al 1990). This could be because IL-4 is needed either to recruit an essential cell type or for the production of parasite-specific IgG1. The former possibility is supported by the observation that the effect on the immune response of passively transferring IgG1 is augmented by the concurrent adoptive transfer of immune mesenteric lymph node cells (Williams & Behnke 1983).

Identification of differences in worm burdens between and within outbred populations is often confounded by the large and over-dispersed variation in

worm burdens within host populations (Stear et al 1996b). This variation imposes on studies of immune responses the need to study large numbers of hosts to allow reliable conclusions to be drawn. To reduce this variation, inbred strains of mice are often used. However, in mice given primary infections of *H. polygyrus*, there was no difference in the variance of worm burdens between inbred and outbred strains of mice. This suggests no advantage in using inbred rather than outbred strains to control variance (Tanguay & Scott 1992). This is in contrast to work with *Trichuris muris* where outbred lines had a greater variance in worm burdens (Wakelin 1975). This may reflect a difference in immune induction in that *T. muris* is able to induce a rapid and effective response in the host whereas *H. polygyrus* does not, leading to chronic infections.

Resistant and susceptible mouse strains are identified through deliberate infections. However, doubt has been cast on how representative this is of natural exposure. In one study it was found that resistant and susceptible strains when allowed to acquire infection naturally harboured indistinguishable parasite burdens and had similar faecal egg counts (Scott 1991). No explanation was proposed for this observation.

Most studies involving the effect of diet on immunity have focused on the effects of protein and the mineral Zinc (Zn). Diets low in protein are associated with poor immune responses (Keymer & Tarlton 1991). However most of the studies have concentrated on the effect of severe protein restriction. The normal maintenance protein requirement for mice is regarded as 12-14% total diet (UFAW 1987). Diets of 3% protein have been associated with reduced ability to control worm burdens and fecundity (Boulay et al 1998) and diets of 2% increased larval establishment compared to mice on 16% protein diets (Slater

1988a). For immunisation with irradiated larvae to be successful mice had to be on diets of 8% protein or greater (Slater & Keymer 1988). Mice on diets lower than this remained unprotected and showed a significant delay in eosinophilia.

Zn deficiency is associated with increased adult worm burdens (Shi et al 1995). However typically diets deficient in Zn will contain only trace amounts of the mineral (0.75 mg/kg compared to the recommended 30mg/kg). Animals on such a diet had reduced IL4, IL10 and IFN gamma, and a reduced eosinophilia (Hai et al 1997).

1.2.2.2 *Trichinella spiralis*

Trichinella spiralis is a cosmopolitan parasite, able to infect many mammals. First-stage larvae are found encysted in muscle. After ingestion of infected muscle by a predator or scavenger, the larvae become free and moult through four stages within the small intestine to the adult within 4 days. After copulation the females burrow deeply into the mucosa via Lieberkühn's glands. The females are viviparous and larvae enter the lymph, and reach the blood stream via the thoracic duct. From there they are distributed throughout the body. Each larva then enters a striated muscle fibre cell that becomes de-differentiated to form a nurse cell. Within these cells, the larvae grow for a number of weeks until they measure approximately 1mm when they become encysted. Within these cysts the larvae can remain viable for many years until ingested by another mammal.

Resistance to infection is associated with a type 2 immune response. What is less clear is which aspects of the Th2 response are important. One of the limitations in understanding the immunological responses are the substantial

differences in response seen between mice and rats (Bell 1992). This is compounded by there being many more reagents to examine the responses in mice compared to rats. Therefore, caution must be exercised in drawing parallels between the two model hosts and with other hosts.

During infection in naïve mice there is an initial Th1-type immune response (Goyal et al 1994). This is characterised by production of IFN-gamma followed by a switch to Th2 related cytokines (IL-4 and IL-5) (Ishikawa et al 1998). The rapidity of this switch appears to be dependent upon the strain of parasite used, ranging from 2 to 8 days (Goyal et al 1994). The timing of the switch may reflect differences in antigenic profiles. In the closely related species *Trichinella pseudospiralis* this switch takes place much more rapidly (Wakelin et al 1994). This may reflect a form of immunomodulation, allowing the first-stage larvae to establish and develop quickly to adults in order to produce progeny while preventing further incoming larvae to establish and compete for this niche.

IL-4 appears to be involved in adult worm rejection and establishment of muscle larvae. Mice treated with anti-IL-4 receptor monoclonal antibody have a small increase in the number of adult parasites surviving and in the number of larvae establishing in muscle cells (Finkelman et al 1997).

IgE is important in parasite estblishment. Rats depleted of IgE had increased numbers of muscle larvae (Dessein et al 1981). In addition, IgE transferred with immune thoracic duct lymphocytes induced rapid expulsion in naïve adult rats (Ahmad et al 1991). These findings lend support to a role for IL-4 in resistance to this parasite as parasite induced IgE production appears to be IL-4 dependent (Finkelman et al 1986).

IL-5 is a cytokine associated with a type 2 response and interest in it comes from its role in the production and activation of eosinophils. However, it seems unlikely that eosinophils are active against gut dwelling stages of the parasite *in vivo*. Certainly in mice treated with anti-IL-5 antibody there was no effect on parasite burden although there was a clear depletion of eosinophils (Herndon & Kayes 1992). This suggests neither IL-5 nor eosinophils are important in resistance to infection with *T. spiralis*.

Expulsion is T-cell dependent requiring the presence of CD4+ cells (Grencis et al 1985). However, there are clear differences in the way infections are rejected between mice and rats. In immune rats there is a near total rejection of parasites within hours and immune memory lasts for several months. In comparison, in mice the rejection process is slower. Here, not only are incoming larvae rejected a number of days after infection, they are rejected less efficiently than in rats, and the immune memory is shorter lasting a matter of days (Bell 1998).

Mast cell degranulation is associated with expulsion of the intestinal phases of the parasite. Primary infection in both mice and rats causes mastocytosis. In mice it lasts a short time after infection whereas in rats it lasts longer. Mice require a larger infection to induce mastocytosis compared to rats. However, despite their differences while mast cells numbers remain high, there is rapid subsequent rejection (Alizadeh & Wakelin 1982a). During expulsion there is an increase in the levels of mast cell products; mast cell protease (Woodbury et al 1984), leucotrienes (Moqbel et al 1987), histamine, serotonin and prostaglandin E2 (Castro et al 1987). Blocking stem cell factor receptor (c-kit) by monoclonal

antibody prevents mastocytosis and completely abrogates expulsion in mice (Grencis et al 1993).

Variation in the mast cell response is influenced by both host and parasite control. Comparison between inbred strains of mice has shown that variation between strains involves non-MHC genes (Alizadeh & Wakelin 1982b). The differences between resistant and susceptible strains appear to be a consequence of how rapidly they mount an immune response. Resistant strains mount more rapid responses as measured by IL-2 and IL-3. There appear to be no differences between the strains in absolute cytokine levels (Crook & Wakelin 1994). Mast cell responses are quicker and greater in high responder mice than in intermediate and low responder strains (Tuohy et al 1990). This response is also dependent upon parasite strain. Some strains elicit a greater mucosal mastocytosis than others (Bolas-Fernandez & Wakelin 1989).

Further evidence of a role for immediate type hypersensitivity comes from serum transfer experiments. Transfer of IgE (Ahmad et al 1991) and IgG1 (Appleton et al 1988) is associated with expulsion of adult parasites from rats.

During infection, there is a dramatic change in ion transport across the gut epithelium and a concomitant increase in fluid secretion. This may act as a possible mechanism of rejection through the physical flushing out of larvae (Harari et al 1987). When immune rats are exposed to antigen there is an increase in smooth wall contractility (Vermillion & Collins 1988) which is dependent on mast cell activation and serotonin release although not prostaglandin or histamine (Vermillion et al 1988). In addition, the release of leucotrienes has been associated with increased smooth muscle contractility, vascular permeability, mucus secretion and inflammatory cell activation (Moqbel

et al 1987). However, although serotonin does increase fluid secretion this increase does not appear to reduce larval numbers in rats. The induction of fluid secretion by prostaglandin E2, cholera toxin or hypertonic mannitol does not reduce larval numbers in rats (Zhang & Castro 1990). Although there is an association between mastocytosis and rejection there remains some doubt as to whether this association means that mast cell degranulation is the effector mechanism of rejection or is merely indirectly associated with it (Bell 1998).

Vaccination with larval antigen was able to elicit a normal immune response in resistant strains of mice but could not confer resistance in susceptible strains (Robinson et al 1995c). The mechanism underlying this difference remains unclear, as serum from exposed but susceptible strains is able to confer immunity in resistant strains when transferred (Robinson et al 1995b). Immunisation with 2 antigens identified from infective larvae was able to accelerate expulsion and reduce fecundity in adults (Silberstein & Despommier 1985b). This was similarly seen in hamsters vaccinated with larval stage antigen that showed a marked reduction in fecundity of adult females (Behnke et al 1994).

Eosinophils have been suggested as a possible effector mechanism for the killing of larvae (Gansmuller et al 1987). However, studies supporting this have concentrated on *in vitro* killing. *In vivo* eosinophils appears to have no effect on the course of infection. Eosinophilia can be prevented by treatment with anti-IL-5 monoclonal antibody without any effect on worm burdens and muscle larvae establishment after reinfection (Herndon & Kayes 1992).

There is an increase in goblet cell hyperplasia soon after infection and this may be a Th2 driven response to infection (Ishikawa et al 1997). Certainly

intestinal mucus has been implicated in preventing the establishment of infections through the phenomenon of mucus trapping. It had been suggested that larvae trapped in mucus are unable to penetrate the mucosa. Larvae released from muscle become coated with antibody, which increases entrapment in mucus (Carlisle et al 1991a). However, it appears that mucus entrapment may not be the main mechanism underlying expulsion in rats. *In vitro* entrapment can occur with antibodies of specificities that are not protective *in vivo* (Carlisle et al 1991b). Therefore, although mucus may play a role in helping to prevent infection it is not the primary effector mechanism in the rapid expulsion seen in rats.

Concurrent infection with *H. polygyrus* reduces the protective immune response to *T. spiralis* (Behnke 1987). This may be due to the immunomodulatory effects of adult *H. polygyrus* or may result from the non-specific effects of parasitism causing a general reduction in ability to mount effective immune responses. Certainly a priming infection with *H. polygyrus* will lead to the rapid expulsion of a primary infection of *T. spiralis* in rats previously immunised with larval antigen (Bell & McGregor 1980). Conversely, the immune response to *T. spiralis* infection is capable of removing adult *H. polygyrus* (Behnke et al 1992). Again this may simply result from the release of the products of mast cell degranulation having a general effect. However, there is cross reactivity between antigens on *H. polygyrus* and *T. spiralis* (Robinson et al 1997b). This appears not simply to be due to one conserved immunodominant protein produced by both species as the same epitope is recognised on several dissimilar proteins. The biological significance of this remains unclear although

it has been suggested that this cross-reactivity accounts for some of the interactions seen in concurrent infections.

Adequate Zn nutrition is a necessary mineral for effective cellular immunity (Tizard 1992). When rats were put on Zn deficient diets there was decreased expulsion of infection, which was abrogated when given normal quantities of Zn (Fenwick et al 1990). However the practical significance of this remains uncertain as the animals were severely Zn deficient.

1.2.2.3 *Trichuris muris*

The life cycle of *T. muris* is simple and direct. Non-embryonated eggs are passed in the faeces and develop to the first larval stage, which remains within the egg. After ingestion the larvae hatch and penetrate the mucosa of the small intestine where they develop for 2-10 days. They then emerge back into the lumen and move to the caecum and colon where they again partially penetrate the mucosa. Here they develop to adults 3-4 months later. The females are highly fecund each laying up to 2000 eggs per day.

The mechanisms of resistance remain unclear. For the host to develop resistance it must mount a Th2 response (Else et al 1993). Treatment of resistant mice with anti-CD4+ monoclonal antibody will abrogate resistance allowing the development of fecund adults (Koyama et al 1995). Those strains of mice that mount a Th1 type response become chronically infected. However, this susceptibility can be abrogated by treatment with an anti-IFN-gamma monoclonal antibody (Else et al 1994). Conversely, treatment with IL-4 facilitates expulsion in susceptible strains of mice. This is supported by evidence that treatment of resistant strains with IL-12 leads to susceptibility to chronic

infection (Bancroft et al 1997). IL-12 drives the immune response toward a Th1 type response characterised by the production of interferon gamma.

It is noteworthy that mice lacking a functional gene for IL-4 are still capable of expulsion (Finkelman et al 1997). This is in direct contrast with *H. polygyrus* where mice with a non-functional IL-4 gene are incapable of expelling the infection. These findings suggest that mice could have an alternative pathway to develop an effector mechanism against *T. muris* but not for *H. polygyrus*.

Vaccination with adult worm antigen can lead to effective immunity in some strains of mice. This immunity is associated with a parasite-specific IgA production. Those strains of mice that remain unprotected do not mount an IgA response (Robinson et al 1995a). Work done with monoclonal antibodies raised against antigens on *T. muris* has shown that immunity as measured by worm burdens could be transferred by two IgA monoclonals recognising stichocyte granules (Roach et al 1991). Stichocyte granules are believed to be important in either tissue penetration or in feeding. Interestingly there is a correlation between humans with IgA deficiency and heavy infections with the similar human parasite *Trichuris trichiura* (Bundy 1988). However, it appears that expulsion of infection is not an antibody dependent event. Reconstitution of severely immunodeficient mice with CD4+ T cells led to expulsion of worms without any antibody responses (Else & Grencis 1996). However, this study only looked at IgG1 and IgG2a responses.

Mast cells are thought not to play a significant role in expulsion as their accumulation is similar in resistant and susceptible strains of mice; accumulation

of mast cells occurring approximately 3 days after expulsion (Lee & Wakelin 1982).

Finally it should also be noted that the outcome of infection is not just a product of the host's ability to mount an appropriate immune response. Different isolates of parasite are able to induce different responses in the same mouse strains (Bellaby et al 1995). Some isolates elicited a predominantly Th1 type response characterised by IgG2a and IFN-gamma production whereas other strains led to a Th2 response profile characterised by IgG1 and IL-5 production.

1.2.2.4 *Nippostrongylus brasiliensis*

The life cycle is simple and direct with eggs passed in the faeces. Larvae hatch within 24 hours and develop to infective third stage larvae. The larvae enter the host through skin penetration and migration through the tissues to the lungs. Here they develop to fourth-stage larvae forming eosinophilic granulomas. From there, they emerge into the bronchi and trachea and are subsequently coughed up and swallowed. Within the small intestine, they develop to adults. The pre-patent period is 6-8 days. Infection lasts for approximately 10 days after infection in immunocompetant mice.

Expulsion is a Th2 dependent phenomenon. This is supported by the observation that treatment with IFN-gamma or IL-12 increases fecundity and the time course of the infection (Urban et al 1996). IL-12 promotes IFN-gamma production and it is thought that it is through this mechanism that IL-12 affects infection. Treatment with IL-12 has minimal effects in mice concurrently given anti-IFN-gamma monoclonal antibody (Finkelman et al 1997). This effect of IL-

12 is reversible. Discontinuation of treatment leads to reversion to a Th2 cytokine profile.

The role of IL-4 is somewhat different in infection in *N. brasiliensis* compared to *H. polygyrus* and *T. spiralis*. Treatment with IL-4 does cause expulsion of the parasite in SCID mice (Urban et al 1995) and is B cell, T cell, leukotriene and mast cell independent (Finkelman et al 1997). Treatment with exogenous IL-4 leads to an initial decrease in parasite fecundity followed by caudal migration and expulsion of live parasites (Urban et al 1995). This may suggest that the effect on the parasite is a nutritional one. Certainly expelled parasites are not dead, and can recover if transferred to naïve mice (Kassai et al 1987). However, IL-4 is not necessary for expulsion. Anti-IL-4 monoclonal antibody does not prevent expulsion of adult parasites (Madden et al 1991) and mice deficient in IL-4 are able still to expel larvae as quickly as normal mice (Lawrence et al 1996). Although mice with abrogated CD4+ cell function are unable to expel parasites, when they are treated with IL-4 they can. This suggests that the mouse immune system has at least two separate methods of expelling this parasite, one that is IL-4 dependent and one that is not. Which method is used in normal infections remains unclear.

One possible mechanism whereby IL-4 leads to expulsion would be if it acted directly on the parasite. However, this seems unlikely. Antibody to mouse IL-4 receptor blocks expulsion due to exogenous IL-4 in mice (Urban et al 1993). However, anti-mouse IL-4 receptor does not bind to rat IL-4 receptor. If IL-4 acted directly on the parasite it would be expected that the parasite would have evolved a rat-like IL-4 receptor as rats are its natural host. In addition, exogenous IL-4 fails to cause expulsion in mice treated with anti-CD4+

monoclonal antibody that are defective in the IL-4 signal transduction molecule Stat6 (Urban et al 1998). If IL-4 acted directly on the parasite, exogenous IL-4 would still be expected to cause expulsion. Interestingly it has been suggested that IL-13 which acts through Stat6 may have a similar role in expulsion to IL-4. These 2 cytokines may act similarly to cause expulsion, with IL-13 fulfilling the role of IL-4 in deficient mice. Although anti-IL-4 receptor does block IL-13 receptor it may not do so efficiently. Alternatively Stat6 may signal for an as yet unidentified cytokine (Finkelman et al 1997).

Mast cells are not necessary for expulsion of *N. brasiliensis*. W/Wv mice that are deficient in mast cells are still able to expel parasites although they are slower than normal mice (Mitchell et al 1983). The reason they are slower may not be due simply to their mast cell deficiency as reconstitution of mast cells does not speed up expulsion (Maeda et al 1992). It is known that such mice have other physiological abnormalities. Indeed there is evidence that mast cell hyperplasia may be beneficial to the parasite. In rats given stem cell factor there is an increase in jejunal mast cells and an increase in faecal egg output (Newlands et al 1995). It could be that increased permeability of the mucosa induced by mast cell degranulation increases the food available to the parasite for the short time it is resident in the gut.

During the initial stages of infection, there is a suppression of mucosal mast cells (Haig et al 1994). Initially, there is a Th1 type response characterised by mitogen stimulated mesenteric lymph node cells secreting IFN-gamma. This is quickly followed by a switch to a Th2 response characterised by secretion of IL-5 under the same conditions (Ishikawa et al 1998). This is similar to that seen in *T. spiralis* infection (Goyal et al 1994) and it may represent an

immunomodulation by the parasite to allow for development to take place for the short time it is resident in the host.

As with the other parasites commonly used in models, *N. brasiliensis* induces IL-5 production and eosinophilia. Antibody directed against IL-5 suppressed blood eosinophilia and lung infiltration by eosinophils (Coffman et al 1989). However such treatment had no effect on the numbers of parasites within the gut, although there is some evidence that treatment increased killing of lung stages (Dent L., cited in (Finkelman et al 1997)).

Free radical production has been associated with rejection of the adult worm burden (Smith & Bryant 1989a). Further evidence comes from the observation that treatment with butylated hydroxyanisole, which scavenges for reactive oxygen intermediates inhibits expulsion (Smith & Bryant 1989b) (although butylated hydroxyanisole has other metabolic effects). The source of these free radicals remains unclear. Eosinophils remain a strong candidate for their production and it may be that the free radical response is associated with the eosinophil response to lung stages and is only temporally associated with expulsion.

1.2.2.5 *Schistosomes*

Schistosomes are trematodes of widespread medical and veterinary importance. Although they are biologically very different from the nematodes, a brief review of their host-parasite interactions is valuable in casting light on possible immune mechanisms that may be relevant to the study of both *T. circumcincta* and *H. contortus*.

The life cycles are indirect involving susceptible snail and warm-blooded vertebrate hosts. Eggs are passed in the faeces (in the case of *Schistosoma mansoni* and *Schistosoma japonicum*) or in the urine (*Schistosoma haematobium*). Free-swimming miracidia hatch from the eggs on contact with water. The miracidia penetrate the soft tissues of the snail host where they reproduce asexually to form sporocysts over several generations. These sporocysts develop into free swimming cercariae, which leave the snail and penetrate the skin of the host. After penetrating the skin, they migrate into the blood stream via subdermal capillaries. In the blood stream they are carried throughout the body but preferentially locate in the lungs. From there, they migrate to the liver where they enter the portal blood vessels. From here, they are carried to the vasculature surrounding the intestine or in the case of *S. haematobium*, surrounding the bladder. Once at their preferred site, the mature schistosomes lay their eggs, which are excreted into the intestinal or bladder lumen. Many of the eggs enter the circulation and lodge in various tissues. Here a granulomatous reaction is set up. It is these granuloma that account for much of the associated disease.

An interesting original observation was that thymectomised mice infected with *S. mansoni* passed far fewer eggs than did infected immunocompetant mice (Doenhoff et al 1979). The numbers of faecal eggs could be increased by administering T cells from normal infected animals. It has been suggested that this effect may be a consequence of the T cells producing tumour necrosis factor α (TNF-α). Evidence for this comes from work involving infected SCID mice, which normally fail to excrete faecal eggs. When injected with recombinant TNF-α, faecal egg excretion was restored and egg production of female

schistosomes was increased (Amiri et al 1993). TNF-α analogues have been identified in invertebrates and it is possible that mammalian TNF-α has a direct effect on the parasite (Pearce 1995).

Original work looking at immune sera of rats identified a 28kDa band that was associated with resistance (Capron 1992). It was subsequently identified as a glutathione s-transferase (GST). The most profound effect of using recombinant GST as a vaccine candidate was reduced fecundity of the parasite and reduced survival of eggs passed in the faeces (Xu et al 1993). IgA specific to this antigen appears to be an effector mechanism of resistance associated with this molecule (Grzych et al 1993).

An important corollary of the finding that the immune response to the parasite can reduce fecundity is that faecal egg counts may not be an accurate measure of infection intensity. For example high worm-specific IgE responses have been associated with reduced faecal egg counts amongst people living in endemic areas. The original assumption was that this represented a reduction in worm burden (Butterworth 1994). While it seems likely a parasite-specific IgE response does reduce worm burden (Hagan 1996), the suppression of parasite fecundity by immune individuals would need to be taken into account in order to understand the relative contribution of each to resistance (Pearce 1995).

Interestingly there remains some controversy over the precise role of the IgE response to the parasite. The production of parasite-specific and parasite non-specific IgE is well recognised as often being associated with helminth infections (Jarrett & Miller 1982). However, there is uncertainty concerning the role of helminth induced non-specific IgE. It has been suggested that this may benefit the parasite by saturating Fc_{ε} receptors. Therefore on mast cells this

would reduce parasite induced degranulation, on eosinophils and macrophages it would reduce antibody dependant cellular cytotoxicity, and on B-cells it would prevent them presenting antigen via IgE bound to CD23 (Pritchard 1993). Conversely this increase in non-specific IgE production may be a necessary host response to reduce the risk of anaphylaxis which could result from hyperreactivity to parasite antigen (Hagan 1993).

1.2.3 Immunity to Gastrointestinal Parasites in Sheep

The effectiveness of the mucosal immune response on gastrointestinal parasites is measured by its ability to control worm burdens, worm fecundity and worm development. The control of worm burdens can be through the control of incoming larvae by preventing their establishment (Miller et al 1983), the expulsion of developing larvae (Emery et al 1993) or through the rejection of adults (Miller 1984).

As is evident from work with models, the development of immunity to helminths appears to vary between parasites and hosts in terms of both speed and mechanism. Although the initiation of immune responses is antigen specific, the mechanism underlying worm expulsion can have non-specific effects on other nematodes present in the same or distal part of the alimentary tract (Dineen et al 1977).

It is clear that the immune response to gastrointestinal parasites in ruminants is T cell dependent. Sheep immune to *H. contortus* can have their immune response partially abrogated after treatment with anti-ovine CD4+ monoclonal antibody (Gill et al 1993a). No comparable effect is seen when the sheep are similarly depleted of CD8+ cells. However, there remains little

information on the process of antigen presentation, cellular recruitment and the role of regulatory cytokines in the immune responses to parasites in ruminants. It is generally assumed that the effector immune responses in ruminants follow a Th2 type response as seen in rodent models. This is supported by typical Th2 type responses such as eosinophilia, mastocytosis and IgE production seen in helminth infected sheep and cattle (Miller 1984).

Blood and tissue eosinophilia is a typical characteristic of helminth infections (Rothwell 1989). The function of eosinophils in mucosal parasites remains unclear. In mice their generation in the bone marrow is IL-5 dependent and they have been associated with protection against parasites migrating through tissues (Rothwell 1989). However there remains no conclusive evidence that that they are required for protection against gastrointestinal helminths (Miller 1996). Indeed, they have been associated with some of the detrimental effects of parasitism. There is an association between the levels of infiltration of eosinophils and diarrhoea in *T. colubriformis* infected sheep (Larsen et al 1994). In lambs infected with *T. circumcincta,* the number of submucosal eosinophils is associated with the degree of abomasal pH elevation. Those lambs with more eosinophils have a higher pH (I. Scott personal communication).

Mast cells have often been implicated as an effector mechanism in the control of helminth burdens (Huntley et al 1987). The number of *T. circumcincta* parasites is associated with the numbers of globule leukocytes in animals over 6 months of age. In contrast worm burden is not associated with parasite specific IgM or IgG, numbers of peripheral eosinophils or the numbers of mucosal mast cells (Seaton et al 1989; Stear et al 1995). Those sheep with increased numbers of globule leucocytes have significantly fewer parasites. As

globule leucocytes are generally regarded as discharged mast cells (Murray et al 1968), it would appear that control of *T. circumcincta* worm burdens can be mediated through an immediate hypersensitivity response involving the discharge of mast cells. Although similar accumulations of mast cells have been associated with resistance of sheep to *H. contortus* (Amarante et al 1999), there remains some doubt over their precise role in the control of this parasite. There is no unequivocal evidence of a role for mast cells in immune exclusion (Huntley et al 1992) and there are often poor correlations between numbers of intraepithelial globule leucocytes and mucosal mast cells (Seaton et al 1989). In sheep infected with *T. colubriformis* there is a strong association between the numbers of globule leucocytes (G.L.) and worm burden (Douch et al 1986). Treatment of resistant sheep with dexamethasone abrogated resistance and led to decreased G.L. numbers.

Mucosal mast cells may affect parasite loads in any of three ways. Firstly, they may be directly anti-parasitic. They can release a variety of low molecular weight mediators after attachment to IgE cross-linked with antigen. These mediators may have direct detrimental effects on the parasites' survival. Secondly, chymases released systemically and into the gut lumen increase gut permeability which would allow the leakage of plasma antibody into the gut lumen (Jones et al 1994; Scudamore et al 1995). Thirdly, mast cells are a major source of cytokines and they may play an important role in the coordination of local immune responses through the production of cytokines such as IL-4, IL-5, and IL-6 (Miller 1996).

Sheep possess three IgG subtypes, IgG1, IgG2 and IgG3 (Tizard 1992). IgG1 is the predominant subtype found at mucosal surfaces. Increased levels of

IgG1 have been associated with resistance to *H. contortus* (Gill et al 1993a). The mechanism by which IgG1 may confer immunity remains unclear. In goats infected with *T. colubriformis,* anti-parasite IgG1 was associated with decreased feeding by parasites incubated *in vitro* (Bottjer et al 1985). This effect was reversed after washing the parasites.

IgG can stimulate the degranulation of mast cells. Immunity to *T. colubriformis* was associated with increased levels of mucus IgG1, IgG2 and globule leukocytes (McClure et al 1992). However, mast cell products could cause leakage of proteins into the gut lumen. This could also account for increased gut antibody levels. Nonetheless, IgG1 is regarded as a good marker for resistance to *T. colubriformis.* Indeed parasite-specific IgG1 titres have been suggested as phenotypic markers for the selection of genetically resistant sheep to *T. colubriformis* (Douch et al 1996). However, in cattle infected with *O. ostertagi,* rising IgG1 titres were not associated with immunity (Hilderson et al 1995). Interestingly there was a negative correlation between IgA and IgG1 responses in sheep to *T. circumcincta* (Sinski et al 1995). IgA may have a role in resistance to this parasite in lambs (Stear et al 1995). Therefore, the negative relationship between IgA and IgG1 in animals infected with *T. circumcincta* would cast doubt on whether IgG1 plays a role in immunity to *T. circumcincta* at least in lambs.

IgE has been associated with developing immunity to *H. contortus* (Kooyman et al 1997). In this experiment increased total serum IgE and IgE directed against parasite excretory/secretory product were correlated with decreased worm burdens. However, there was no IgE response detected against

third-stage larvae. This would suggest that immunity involving IgE may not be directed at incoming larvae.

In cattle infected with *O. ostertagi* the influence of IgE on infection is poorly defined. There is evidence that the level of lymph IgE is negatively correlated with parasite burden (Baker & Gershwin 1993). However, there is conflicting evidence for the relationship between infection levels and total serum IgE. A high level of infection resulted in elevated IgE compared to calves exposed to lower levels (Miller et al 1996). In contrast, in a different experiment, higher IgE responses were seen in calves moderately infected compared to calves given higher infections (Baker & Gershwin 1993).

IgA production is increased in lambs selectively bred for resistance to *H. contortus* (Gill et al 1994). In sheep infected with *T. circumcincta,* local parasite-specific IgA was correlated with worm length (Smith et al 1985). Four experiments were recorded, two involving 4.5 month-old lambs and two involving 10 month old lambs. When the data was pooled the correlation between mean worm length and peak lymph IgA was 0.96. However, correlations between age groups can be misleading. Older lambs have shorter worms on average than younger lambs and younger lambs have generally lower immune responses than older lambs. Therefore, any parameter that varies with age will give strong correlations with worm length.

During lactation, resistance to helminth infections is often poorer. However, sheep infected with *T. circumcincta* have increased levels of IgA in gastric lymph during lactation (Smith et al 1983). This would suggest that the depression in immunity seen at this time is not due to decreased IgA production,

although this does not rule out the possibility of impaired transport of IgA to the mucosal surface.

Because of the association between IgA and worm length it is postulated that anti-parasite IgA works through interfering with the feeding of the parasite (Stear et al 1995). IgA may also work indirectly by binding to inflammatory cells in the mucosa provoking the release of cytokines. In humans IgA/antigen complexes induce eosinophils to release IL-5 (Dubucquoi et al 1994). Thus IgA may act indirectly in the orchestration of immune responses at mucosal surfaces.

1.2.4 Factors that Influence the Immune Response in Sheep

1.2.4.1 Sex

In a wide variety of vertebrate hosts the sex of the host influences the infection rate, intensity of infection and rate of development of resistance to parasitic infection (Poulin 1996). Male animals are usually more susceptible to infection and develop resistance less quickly than females (Zuk & McKean 1996). Entire male sheep were found to be more susceptible to infection with *Oesophagostomum columbianum* than entire females (Dobson 1964). Castration of females but not males reduced this sex difference. Other studies have suggested that castration of males does increase resistance to infection (Barger 1993). These sex differences can theoretically be attributed to several factors. They could be due to differences in physiology between the sexes, differences in behaviour, differences in farm animal management or differences in the rate of ingestion of parasites.

Four possible physiological mechanisms have been proposed for the sex differences in susceptibility to parasitism. They are the deleterious effect in

males of being the heterogametic sex, the effect of stress, the direct effects of sex steroids on the parasite, or indirect effects of steroid hormones on the immune system.

The heterogametic hypothesis is based on the supposition that deleterious recessive alleles normally masked in the homogametic sex would have an effect in males because only one of their sex chromosomes is fully functional. However, in birds where the females are heterogametic there still remains the same sex effects as are seen in mammals i.e. female birds are more resistant to infection than males (Poulin 1996).

Stress is widely recognised to have deleterious effects on immune responses. Stress responses have typically measured the release of corticotrophin-releasing hormone from the hypothalamus, which triggers the release of adrenocorticotrophic hormone by the pituitary which in turn act on the adrenal gland to produce glucocorticosteroids such as cortisol (Ganong 1987). Glucocorticosteroids have wide ranging effects on immune responses (Khansari et al 1990), suppressing antibody production, reducing cytokine release, and blocking lymphocyte proliferation. Treating sheep with cortisone can abolish immunity to *T. circumcincta* (Dunsmore 1961). It seems probable that males generally lead more stressful lives, particularly during breeding seasons by undergoing stress through antagonistic encounters with other males and in pursuing and mating females. Whether this may be an underlying reason for sex differences is uncertain and will be difficult to disentangle from other confounding effects of sex steroids on resistance.

Sex steroids could act directly on the parasite or indirectly by acting on the immune system. However, it is difficult to distinguish the two effects in a

parasitised animal. Certainly in laboratory animal models, physiological levels of oestrogens stimulate humoral responses while androgens suppress both cellular and humoral responses (Zuk & McKean 1996). For example, females tend to have higher levels of circulating antibody, and more active cell-mediated immune responses such as graft rejection (Grossman 1989).

In summary, although sex differences in susceptibility and resistance to parasite infection are clear the mechanisms underlying these differences remain to be fully identified.

1.2.4.2 Host Age

Age is widely recognised to influence the ability of animals to mount effective immune responses to a wide variety of pathogens. Ruminants less than six months of age are generally more susceptible to many infectious agents than when mature. They are much more susceptible to viral, bacterial and parasitic intestinal and respiratory pathogens (Colditz et al 1996). The possible reasons for this include not having been previously exposed to the agent to develop active immunity, the suppressive effects of passively acquired maternal antibody or stress associated with early life such as weaning. However when these factors are taken into account there still appears to be a constitutive immunological hyporesponsiveness to infection (Watson & Gill 1991). Young lambs have significantly lower proportions of CD4+ and CD8+ lymphocytes though greater proportions of B cells and T19+ lymphocytes (Watson et al 1994). Sheep less than a year old mount significantly poorer antibody and T cell responses to various antigens and mitogens in comparison to older sheep (Watson et al 1994; Watson & Gill 1991).

However young lambs are able to mount sufficient immune responses to a variety of antigens to confer immunity. For example, vaccination with inactivated clostridial and pasturella vaccines confers solid immunity to these pathogens. Work on mice models has challenged the widely held view that neonates are immunologically privileged but that they are able to generate immunity provided that antigen is correctly presented to T cells (Forsthuber et al 1996). This would suggest that the hyporesponsiveness seen in young animals may not result from their immune system being immunoincompetent but rather from a lack of adult numbers of immune cells.

1.2.4.3 Host genes

It is commonly acknowledged that individuals within a species are not equally susceptible to disease. Some individuals show resistance (either partial or total) while others show increased susceptibility. Variation in disease resistance to a wide variety of pathogens is seen both within and between breeds of most of the domestic animals (Owen & Axford 1991). Variation in resistance to gastrointestinal parasites is well recognised in sheep (Stear & Murray 1994) with substantial differences both within and between breeds. Further to this, selective breeding experiments and laboratory studies have indicated a genetic basis to resistance (Wakelin & Blackwell 1993).

Apparent differences between breeds may be a consequence of genetic variation within breeds. Sampling error might result in selecting relatively resistant sheep from one breed and relatively susceptible sheep from another when in reality both breeds have a similar range of susceptibilities. However, there do appear to be real breed differences particularly in susceptibility to *H.*

contortus. For example, Red Maasai sheep are consistently more resistant to *H. contortus* infection than other East African breeds such as the Dorper and the Blackhead Somali (Mugambi 1994).

Resistance may involve both innate and acquired mechanisms. Innate responses do not involve immunological mechanisms but are a consequence of the host being physiologically unsuitable for the pathogen to either establish or develop (Stear & Wakelin 1998). However, there is no evidence that innate resistance accounts for the genetic resistance seen in sheep to *Teladorsagia circumcincta*. In very young lambs, there is little or no genetic variation in faecal egg counts. As lambs age, heritability estimates increase until at 6 months of age the heritability of faecal egg counts is 0.22 (Bishop et al 1996a). This indicates that breeding for resistance to this parasite involves selecting for those animals more able to mount effective immune responses to the parasite.

1.2.4.4 Nutrition

There is a dynamic relationship between host nutrition and parasitism. Infection has important effects on host nutrition and the plane of host nutrition influences the severity and course of infection.

In parasitic gastroenteritis there is an increased loss of endogenous protein into the gastrointestinal tract through leakage of plasma protein, increased turnover of epithelial cells, and mucoprotein secretion (Parkins & Holmes 1989). In sheep infected with *H. contortus* most of this protein loss will be reabsorbed further down the gastroinestinal tract though partly as non-protein nitrogen (Rowe et al 1988). There is however an energy cost to the host in recycling

endogenous protein and the gross efficiency of use of metabolisable energy is decreased (Sykes & Coop 1977).

Infection also affects host nutrition by reducing voluntary food intake (Symons 1985). The mechanisms for this remain unclear. Interest has recently focused on the effects of gastrointestinal hormones and factors influencing satiety centres in the central nervous system.

Cattle infected with *O. ostertagi* show an association between inappetance and elevated gastrin concentrations (Fox et al 1989a; Fox et al 1989b). This is consistent with the expectation that in infected animals, elevated abomasal pH would stimulate the release of gastrin. When treated with omeprazole, which inhibits gastric acid secretion and thus increases blood-gastrin, worm-free calves exhibited reduced feed intake (Fox et al 1989c).

In cattle infected with *O. ostertagi,* gastrin levels increase at the same time as pH supporting the hypothesis that elevated gastrin is a consequence of elevated pH (Enterocasso et al 1986). However in sheep infected with *T. circumcincta,* gastrin levels become elevated before abomasal pH, and the elevated pH occurs when the sheep are infected with larval and adult stages (Anderson et al 1985). This suggests that elevated pH in sheep is not simply a consequence of loss of parietal cell function due to fourth-stage larvae occupying gastric glands nor that elevated gastrin levels are a consequence of increased abomasal pH. Therefore, caution should be exercised in drawing parallels between cattle infected with *O. ostertagi* and sheep infected with *T. circumcincta.*

Cholecystokinin release had been thought to be a candidate mechanism for depressing food intake but this now seems unlikely. Adding a potent CCK

antagonist had no effect on short-term food intake in infected lambs infected with *T. colubriformis* (Dynes et al 1998).

Parasitism appears to affect the functioning of the hypothatlamus. In rats infected with *Nippostrongylus brasiliensis*, anorexia is accompanied by increased neuropeptide Y gene expression in the hypothalamic arcuate nucleus (Horbury et al 1995). If the satiety centre within the hypothalamus is blocked with a benzodiazepine drug, feed intake is increased in sheep infected with *Trichostrongylus colubriformis* (Coop & Holmes 1996).

Host nutrition has important effects on the parasite and the detrimental effects of parasitism on the host. Improving nutrition improves both resilience to the effects of parasitism and resistance to the parasite.

Improving protein nutrition improves resilience to haemonchosis. In one experiment 3 month-old Finn-Dorset/Dorset Horn castrated male lambs infected with *H. contortus* were put on either a high-protein diet (169g crude protein/kg dry matter) or a low protein diet (88g crude protein/kg dry matter). Those lambs on the low protein diet exhibited more clinical signs of haemonchosis than the high protein diet (Abbott et al 1986b). This was despite the diets having no effects on parasite establishment, worm burden or faecal egg counts.

Although improved nutrition does not appear to improve parasite establishment in parasite-naïve sheep (Coop & Holmes 1996), it does increase the rate of acquisition of resistance. An original observation made with naturally infected sheep was that chronic haemonchosis is associated with reduced feed quality in Kenya (Allonby 1974). This has subsequently been supported by experimental evidence that improving protein nutrition improves resistance to further infection with *H. contortus* (Abbott et al 1988). Similarly in lambs

infected with *Trichostrongylus colubriformis*, protein supplementation improved resistance to infection as measured by worm burdens and faecal egg counts (Kambara et al 1993). In both these cases the improvement was only seen in lambs less than 6 months of age.

The method by which improved diet affects the immune response remains unclear. Protein supplementation did not significantly increase the degree of leucocyte responsiveness to mitogen or parasite antigen in lambs infected with *T. colubriformis* (Kambara et al 1993). However supplementation did increase the numbers of T19+ (CD4-/CD8-) cells (Kambara & McFarlane 1996).

The rate of expulsion of *T. colubriformis* has been associated with the amount of dietary rumen bypass protein in 3 month old Merino lambs. Those with greater proportions of rumen bypass protein had increased rates of expulsion (Van Houtert et al 1995). In this experiment, expulsion was associated with peripheral eosinophilia and increased mucosal mast cell proteases (MCP) although expulsion preceded the increase in MCP. Similarly lambs trickle infected with *T. circumcincta* and directly infused with protein into the abomasum showed increased levels of gastric mast cell protease in the supplemented group compared to the controls (Coop et al 1995). Those lambs with increased MCP had more fourth-stage larvae and lower total worm burdens. In a similar experiment with *T. circumcincta*, protein supplementation increased the numbers of mast cells, globule leucocytes and the concentration of MCP while worm burdens and faecal egg counts were reduced (Coop & Holmes 1996).

In lambs given fish-meal supplementation and infected with *Nematodirus battus* there was an enhanced anti-worm IgG response together with increased

mucosal globule leucocytes and eosinphils although there was no significant effect of supplementation on worm burden (Israf et al 1996).

1.3 OBJECTIVES OF THIS STUDY

T. circumcincta and *H. contortus* are two of the most important parasitic infections of sheep in the world. Although both can be controlled by the use of anthelmintics and grazing management, the rapid evolution of resistance to drugs (Jackson 1993) and the impracticability of control through management necessitates the development of new means of control. A variety of additional methods have been proposed to supplement existing control strategies. These include the use of genetically resistant sheep (Stear & Murray 1994), improved diets (Holmes 1993), the use of nematophagous fungi (Waller et al 1994), and the development of vaccines (Emery & Wagland 1991). The applicability of all of these methods will be greatly helped by a better understanding of the interaction between parasite and host and the identification of the major factors that influence that interaction. It is the general purpose of this thesis to look at some of the host parasite interactions involving sheep infected with *T. circumcincta* and *H. contortus* and to set this work in the context of the wider field of parasitology.

It is well recognised that some lambs develop immunity to infections more quickly and effectively than others as measured by faecal egg counts, and that this resistance is heritable. However, the mechanisms underlying this resistance for each of the parasites remain unclear and controversial. One of the objectives of this work was to attempt to examine whether the local IgA response to *T. circumcincta* was associated with resistance. Further to this was an attempt to

draw conclusions as to whether it was likely to be the controlling mechanism for resistance or merely associated with another as yet unidentified mechanism.

The specificity of the IgA response was examined to attempt to identify antigens that might be associated with resistance or susceptibility. The goal of this work would be either to use antigens associated with resistance as vaccine candidates or to develop more efficient selection criteria for future breeding programmes that incorporated resistance traits.

One of the major factors that allows *T. circumcincta* to be so successful is its ability to go into inhibition. It has often been postulated that two factors involved in larval inhibition are the immune response and the infection intensity. Previously exposed lambs given new infections have a greater proportion of their parasite burden in inhibition than naively infected lambs (Smith et al 1984). This suggests an immunological mechanism underlying inhibition. However, the mechanisms underlying this are unknown. To investigate whether the IgA response may have a role to play in this, the relationship between the quantity and specificity of the IgA response and larval inhibition was studied. In addition, the effect of infection intensity on larval inhibition was also investigated. Finally, the independence of density-dependant effects and local antibody responses were investigated.

H. contortus is a highly pathogenic abomasal parasite. Previous work has shown that as with other nematode parasites there is genetic variability in resistance and resilience to infection (Abbott et al 1985; Abbott et al 1988). However, the mechanisms of resistance to natural infection are uncertain. One of the objectives of the work described here was to investigate if the parasite-specific IgA could be a possible effector mechanism for resistance to this parasite

and whether such a response might act in a similar way to that proposed for the IgA response to *T. circumcincta.*

Improved protein nutrition is known to improve resilience to haemonchosis in genetically susceptible sheep (Wallace et al 1995). Improved protein nutrition may both offset the detrimental effects of infection and improve the immune response to infection. To investigate this, the influence of protein supplementation on the parasite-specific IgA response was studied.

CHAPTER TWO

MATERIALS AND METHODS

2.1 PARASITOLOGICAL METHODS

2.1.1 Modified McMaster Technique

A modified McMaster egg counting method (Gordon & Whitlock 1939) (Bairden 1991) was used to count trichostrongyle eggs in faeces. Three grams of faeces taken directly from the rectum of each animal was examined. The faeces were homogenised with 42ml of water and sieved through a 250 micron sieve and the filtrate collected. After thorough mixing, 15 ml of the filtrate was centrifuged for 5 minutes at 2000 rpm. The supernatant was discarded and the remaining faecal pellet broken up using a whirl mixer. The tube was then filled with saturated sodium chloride solution and inverted six times. A sufficient volume to fill 2 chambers of a McMaster slide was taken off immediately. The total number of eggs within both chambers was counted and the result multiplied by 50 to give the estimated number of eggs per gram of faeces (epg). To help eliminate error, two or four replicate samples were counted for each faecal sample. The numbers of *Nematodirus spp.*, eggs were counted separately. In the natural infection, larval culture showed that the overwhelming majority of trichostrongyle eggs were *T. circumcincta* (Bishop et al.1996a). Over 4 years 74% of adult worms counted at necropsy were *T. circumcincta* (Stear et al. 1998)

2.1.2 Total Worm Counts

Total worm counts were estimated by counting the number of adult larvae in the abomasal contents and the number of larvae in the abomasal digest. Each

abomasum was removed, opened along the greater curvature and the lining washed. The abomasal contents and the washings were collected and made up to two litres. 10, four ml sub-samples were taken and examined to estimate the total adult worm burden.

One half of each washed abomasum was digested for six hours with pepsin-HCl at 42°C (appendix 1). The digest was made up to two litres with deionised water and 10 four ml aliquots were examined to estimate the number of fourth-stage larvae (Armour et al 1966).

2.1.3 Worm Length Measurements

Twenty female worms were randomly selected from each lamb and measured using image analysis (PC-image, Foster Findlay Associates) at x25 magnification to estimate average worm length.

2.1.4 Culture of Third-Stage larvae

The strains of *T. circumcincta* and *H. contortus* larvae used were originally a gift of the Moredun Institute, Pentlands Science Park, Penicuik, Scotland. Sufficient numbers of larvae for the preparation of somatic extracts were obtained by passaging larvae through helminth naïve lambs. Lambs were infected with third stage larvae and three weeks later faeces were collected by a faecal bag attached to a harness. Faeces were stored in cartons for 14 days at 23°C. They were then soaked in water for 3 hours to liberate larvae from the faecal pellets. The contents were then passed through a coarse mesh sieve and the larvae recovered by baermanisation through gauze suspended in PBS. The larvae were subsequently concentrated by sedimentation.

2.1.5 Preparation of Larval Somatic Extract

Fourth-stage *T. circumcincta* larvae were harvested 6 days after infecting helminth naive lambs with 150 000 third-stage larvae. The lambs were killed and the abomasums removed. The abomasa were opened along the greater curvature and the contents discarded. The mucosal surface was washed under a gently running tap and the abomasum cut into strips. The strips were suspended in Baerman funnels containing phosphate buffered saline (PBS) (pH 7.4) at 37°C. The larvae migrated into the PBS and collected at the bottom of the funnels. The larvae were collected at 30 minute intervals until migration had ended. The larvae were rebaermanised into PBS through surgical swabs suspended in 50ml tubes to remove any gross abomasal debris collected initially. The collected larvae were washed by centrifugation (800 *g*) and resuspension of the pellet 5 times in PBS. They were then washed once in PBS containing 100 iu/ml penicillin, 0.1mg/ml streptomycin, 2.5μg/ml amphotericin B and 0.05 mg/ml gentamicin and once in a 10 mM Tris solution containing 1 mM disodium ethylene diamine tetracetic acid (EDTA), 1 mM ethylene glycol bis (2-amino ethyl ether)-*N,N,N',N'*-tetracetic acid (EGTA), 1 mM *N*-ethylmaleimide (NEM), 0.1 μM pepstatin, 1 mM PMSF, and 0.1 mM TPCK as proteinase inhibitors (Tris-inhibitor solution) (Maizels et al 1991). The pellet was resuspended in a 1% w/w solution of deoxycholate in Tris-inhibitor solution and homogenised using a hand-held electric homogeniser (Janke & Kunkel IKA Labortechnik) on ice. The supernatant was filtered through a 0.2μm filter and a protein assay performed using the Pierce BCA protein assay kit. This somatic extract was used as antigen for the ELISA.

Fourth-stage (L4) *H. contortus* larvae were harvested 4 days after infecting helminth naive lambs with 75,000 third-stage larvae. After each lamb was killed, its abomasum was removed and opened along the greater curvature. The abomasal contents were discarded and the mucosal surface washed under a gently running tap. The abomasum was cut into strips which were then suspended in Baerman funnels containing acid-phosphate buffered saline (acid-PBS) (pH1) at 37°C. The larvae migrated into the PBS and were collected at 10 minute intervals from the bottom of the funnels until migration had ended. The larvae were rebaermanised into PBS through surgical swabs suspended in 50ml tubes to remove any gross abomasal debris. The collected larvae were washed three times at 200 g for five minutes in PBS and were then washed once in Hanks balanced salt solution. They were then given a final wash in a 10 mM Tris solution containing 1 mM EDTA, 5 μM pepstatin, 1 mM PMSF, and 1.4 mM TPCK, 5.5 μM antipain, 0.2 mM phenanthroline, 0.7 mM *N*-α-p-tosyl-L-lysine chloromethyl ketone HCl (TLCK), and 5 μM leupeptin as proteinase inhibitors (Tris-inhibitor solution). The pellet was resuspended in a 1% w/w solution of sodium deoxycholate in Tris-inhibitor solution and homogenised using a hand-held electric homogeniser (Janke & Kunkel IKA Labortechnik) on ice. The supernatant was filtered through a 0.2 μm filter and a protein assay performed using the Pierce BCA protein assay kit.

Adult worms were recovered from lambs 21 days after being infected with 15,000 third-stage larvae. Each abomasum was opened along the greater curvature. The abomasal lining was washed and the contents and washings collected. These were sieved through a large pore sieve to remove large particulate debris and then sieved through a fine pore sieve to collect the adult

parasites. This filtrate was put in gauze and suspended in a beaker containing PBS for 4 hours at 37°C. The adults migrated into the PBS and settled at the bottom of the beaker. Thereafter they were treated in the same way as the fourth-stage larvae.

A somatic extract of third-stage (L3) larvae was obtained by washing third stage larvae in a dilute hypochlorite solution to allow exsheathment. A somatic extract was then prepared in the same way as for the fourth-stage larvae.

2.2 SEROLOGICAL METHODS

2.2.1 Plasma samples

Blood was collected from the sheep by jugular venepuncture into heparinised evacuated glass tubes or into EDTA monovette tubes (Starstedt). The tubes were centrifuged for 15 minutes at 1500 rpm and the plasma removed and stored at either -20°C or -80°C.

2.2.2 Cell Culture

Dr. S. Hobbes, Dr. P. Bird and Professor I. McConnell kindly donated the cells used to generate monoclonal IgG anti-sheep IgA (pers., comm.).

The cell culture media used was 10% foetal bovine serum in RPMI with 50µg/ml gentamicin (Gibco). Cells were thawed quickly from liquid nitrogen and suspended in 10ml of cell culture media and centrifuged for 5 minutes to pellet the cells. They were then resuspended in 10 ml of media and 100µl of cell suspension was mixed with 100µl of trypan blue stain made up in phosphate buffered saline. The number of viable cells was estimated by counting 200 viable (unstained) cells using a haemocytometer. The cell concentration was

calculated by the formula; cell count x 2 x 10^4. The cells were made up to a concentration of 3 x 10^5 cells/ml in cell culture media. 10 ml of the cell mixture was put into 25cm^3 cell culture flasks. The flasks were incubated at 37°C in a 5% CO_2 atmosphere. The cultures were examined daily and when the cells were nearing confluence they were subdivided into fresh culture media. The supernatant from the cultures was used for the ELISA and western blots after being titrated for amount of antibody.

Cells were frozen down for storage before they became confluent at a concentration of 5-6 x 10^6 ml^{-1}. Cells were pelleted by centrifugation and the freezing mixture (appendix 2) added dropwise in order to reduce osmotic shock until the required volume was achieved. The mixture was then added to a cryovial and left in the N_2 vapour above liquid nitrogen for two hours before transferring it into liquid nitrogen.

2.2.3 ELISA

2.2.3.1 *Teladorsagia circumcincta*

The wells on a microtitre plate (Nunc) were covered with 100µl of antigen solution in bicarbonate buffer (pH 9.6) at a protein concentration of 5µg/ml, and left overnight at 4°C. The plate was washed 5 times with a 0.05% w/v solution of Tween in PBS (T-PBS) and this was repeated between each subsequent stage. The wells were then filled with 200µl of a 4% w/w solution of skimmed milk powder in T-PBS (blocking buffer) to block any unoccupied sites on the wells and incubated at 37°C for 2 hours on a shaking platform. 100µl of the plasma samples diluted 1:10 in blocking buffer were added in triplicate to the plate and incubated at 37°C for 30 minutes. As there is a limited quantity of IgA in serum a

1:10 dilution was selected to maximise IgA binding. To allow for comparison between plates, and to minimise day to day variation, a strongly positive plasma sample and a negative plasma sample from a lamb unexposed to *T. circumcincta* were included on all plates. 100µl of a monoclonal rat IgG anti-sheep IgA diluted 1:50 in blocking buffer was then added and incubated for 30 minutes at 37°C. The monoclonal antibody was a gift of Dr S. Hobbes, Dr. P. Bird and Professor I. McConnell and its specificity validated by them (pers. Comm). Then 100µl of a goat IgG anti-rat IgG conjugated to alkaline phosphatase (Sigma) diluted 1:1000 in blocking buffer was added and again incubated at 37°C for 30 minutes. Finally 100µl of the alkaline phosphatase substrate 5-bromo-4-chloro-3indoyl-phosphate (KPL) was added and after a 30 minute incubation at 37°C the plate was read on a microtitre plate reader (Titertek) at a wavelength of 635nm.

2.2.3.2 *Haemonchus contortus*

The wells on a microtitre plate (Nunc) were covered with 100 µl of antigen solution in bicarbonate buffer (pH 9.6) at a protein concentration of 5 µg/ml, and left overnight at 4°C. The plate was washed 5 times with a 0.05% w/v solution of Tween (Sigma, Poole, Dorset, UK) in phosphate buffered saline (T-PBS) and this was repeated between each subsequent stage. The wells were then filled with 200 µl of a 4% w/w solution of skimmed milk powder (Safeway, UK) in T-PBS (blocking buffer) to block any unoccupied sites on the wells and incubated at 37°C for 2 hours on a shaking platform; 100 µl of the plasma samples diluted 1:10 in blocking buffer were added in triplicate to the plate and incubated at 37°C for 30 minutes. To simplify comparison between plates, and to minimise day to day variation, a strongly positive plasma sample and a plasma sample

from a helminth-naïve lamb were included on all plates. 100 µl of a monoclonal rat IgG anti-sheep IgA diluted 1:50 in blocking buffer was then added and incubated for 30 minutes at 37°C. Then 100 µl of a goat IgG anti-rat IgG conjugated to alkaline phosphatase (Sigma) diluted 1:1000 in blocking buffer was added and again incubated at 37°C for 30 minutes. Finally, 100 µl of the alkaline phosphatase substrate, *p*-nitrophenyl phosphate (Sigma) was added. After a 30 minute incubation at 37°C the plate was read on a microtitre plate reader (Titertek) at a wavelength of 405 nm.

In both ELISAs, no attempt was made to quantify the amount of parasite-specific antibody. The optical density is dependent upon the amount of antibody, and the avidity and affinity of the antibody for each component of the antigen preparation. Because of the complexity of the antigen preparations, any attempt to estimate absolute antibody concentrations would have been prone to error.

Variation in batches of parasite antigen was avoided by using only one batch of antigen for each ELISA. To ensure an optimal concentration of antibody dilution the monoclonal antibody was titrated against against a postive serum control and a dilution selected where the optical densities had reached a plateau (Appendix 6).

2.2.4 Western Blots

Protein fractionation was accomplished by SDS-PAGE (Laemmli 1970) under reducing conditions (Bio-Rad Protean IIxi Cell). Vertical 7.5% and 15% polyacrylamide gels were used with 40µg protein per track (appendices 3 & 4). Protein standards (Bio-Rad Kaleidoscope Prestained Standards) were run simultaneously.

After electrophoresis the proteins were transferred onto nitrocellulose paper (Bio-Rad) with a pore size of 0.45 μm (Trans blot Electrophoretic Transfer Cell Bio-Rad) (appendix 4) (Bollag & Rozycki 1996). The nitrocellulose paper was stained with Ponceau-s to check for effective protein transfer and to enable accurate cutting of each protein track into strips. Non-specific binding sites were blocked with a 4% skimmed milk solution in PBS with 0.05% Tween (T-PBS) overnight. Following three washes, each of 10 minutes, in de-ionised water the strips were incubated in a 1:10 dilution of plasma in T-PBS for 3 hours. The strips were again washed three times in de-ionised water and incubated for 1 hour with a 1:40 dilution of a monoclonal rat anti-sheep IgA antibody. The strips were again washed as before and incubated for one hour in a 1:1000 dilution of an alkaline phosphatase-conjugated mouse anti-rat IgG monoclonal antibody (Sigma). After another three, 10 minute washes the strips were placed in a solution containing 5-bromo-4-chloro-3-indoyl phosphate (BCIP, KPL laboratories) to visualise the protein bands recognised by sheep antibodies. For convenience, each band is referred to as an antigen, although separate bands may not always represent distinct molecular species.

No attempt was made to quantify the intensity of the bands. Bands were noted if observed and to limit day to day variation a strongly reactive plasma sample was used on each blot.

2.3 STATISTICAL ANALYSIS

For clarity the relevant statistical analyses are described in each chapter.

CHAPTER THREE

THE RELATIONSHIP BETWEEN IgA DIRECTED AGAINST FOURTH-STAGE T. CIRCUMCINCTA LARVAE AND WORM LENGTH IN OUTBRED SHEEP

3.1 INTRODUCTION

Teladorsagia circumcincta is a major constraint on sheep production in temperate regions of the world (Urquhart et al 1987). Current methods of controlling nematode infections in livestock rely heavily on anthelmintic treatment, but these methods are threatened by the increasing frequency of anthelmintic resistance among nematode populations (Jackson 1993). There is an urgent and growing need for additional control strategies. One of the most promising strategies is the selective breeding of sheep for increased resistance to infection (Stear & Murray 1994).

There is no doubt that there is substantial variation in resistance to many parasitic infections (Wakelin 1988). Some of this variation is due to host genetic factors. Resistance is usually measured by faecal egg counts. Most estimated heritabilities for single faecal egg counts in mixed natural infections have been approximately 0.3 (Stear & Murray 1994). Because there is a good genetic correlation between egg counts from 3-6 month old lambs (mean = 0.87), it is possible to combine these counts and thus increase the heritability estimate. The estimated heritability can be further increased by decreasing the measurement error by taking several counts per faecal sample (Stear et al 1997a).

Heritability estimates increase with age of the lamb. This strongly suggests that genetic resistance to *T. circumcincta* is an acquired response. There remains some debate over the mechanisms underlying genetic variation. One view is that resistance is highly complex with many different mechanisms having many different effects on the parasite (Miller 1984). An alternative view is that resistance to *T. circumcincta* is relatively simple; the most important manifestation of immunity in growing lambs being the control of worm growth causing a reduction in worm fecundity (Stear et al 1997b).

Young lambs appear to be unable to control worm burdens (Stear et al 1996a). It is known that sheep regulate worm length before they regulate worm numbers (Seaton et al 1989). The only mechanism to have been consistently associated with reduced worm length is the local IgA response (Smith et al 1985; Stear et al 1995). Worm length is correlated with worm fecundity (Stear et al 1999). Thus there is evidence that young lambs control worm fecundity through a local IgA response. Previous work has shown a strong relationship between IgA directed against fourth-stage larvae and adult worm fecundity (Stear et al 1995) and that this parasite-specific IgA response was inversely related to parasite specigic IgG_1 (Sinski et al 1995).

The work described in this chapter was designed to examine on a large data set the relationship between parasite-specific IgA and the control of worm fecundity for *T. circumcincta* in lambs. 933, six-month old Scottish Blackface lambs were sampled for blood and faeces over 5 consecutive years. The lambs had a mixed nematode parasite burden but were overwhelmingly infected with *T. circumcincta*. An ELISA was developed to quantify IgA responses to fourth-

stage larvae and faecal egg counts were performed. Heritability estimates were performed on the IgA response.

3.2 MATERIALS AND METHODS

3.2.1 Animals

The lambs studied were straightbred Scottish Blackface lambs kept on an 800 ewe commercial upland farm in Ayrshire, Scotland. The ewes lambed outdoors over three weeks between April and May and those ewes with twin lambs were brought onto two fields of improved pasture. These lambs were studied from 5 successive years, a total of 933. The lambs were weaned in July and kept on the larger of the improved fields. They were given an anthelmintic monthly, the dose appropriate for the heaviest lamb being given to all the lambs. The efficacy of the anthelmintic treatment was tested by faecal egg reduction tests.

3.2.2 Parasitological methods

The parasitological methods are described in Chapter 2.

3.2.3 ELISA

The ELISA method is described in Chapter 2. To avoid variation in the composition of antigen the same batch of antigen was used.

3.2.4 Statistical analysis

The Univariate program on the SAS package (SAS Institute, Cary, NC, USA) was used to estimate the means and variances of the parasitological data.

The optical densities were transformed into an optical density index for each animal using the following formula;

$$ODI = \frac{Mean\ OD - Mean\ negative\ OD}{Mean\ positive\ OD - Mean\ negative\ OD}$$

The associations between amount of parasite-specific IgA, adult worm burden and worm length were estimated by multiple regression analysis using the GLM program of the SAS package (SAS Institute). The sire and dam variance components of the IgA response were estimated using a mixed models analysis. The heritability of the IgA response was estimated as four times the sire variance component divided by the sum of the sire, dam and residual variances.

3.3 RESULTS

The most prevalent species found was *T. circumcincta* which accounted for over 74% of all nematodes recovered, although there was considerable variation between years with 96% of all nematodes in 1992 being *T. circumcincta* compared to, 77% in 1993, 71% in 1994 and 51% in 1995. The mean intensity of *T. circumcincta* infection, as measured by arithmetic mean counts of larvae and adult nematodes, varied between years with 13,000 in 1992, 3,400 in 1993, 2,300 in 1994 and 6,300 in 1995. Similarly, the numbers of adult worms recovered at necropsy varied between years (table 3.1). Six other categories of parasite were found, *Trichostrongylus axei, Haemonchus contortus, Cooperia* spp., *Trichostrongylus vitrinus, Nematodirus* spp., and *Bunostomum trigonocephalum* (Stear et al. 1998). The distribution of *T. circumcincta* was positively skewed with the majority of parasites being found in a minority of lambs. *T. circumcincta* was found in nearly all of the lambs examined (513 out of 514). The length of adult worms varied between 0.6 and 1.2 cm.

The IgA optical density indices (IgA-ODI) ranged from 0-0.75 (appendix 5). The distribution of IgA-ODI over the 5 years was highly variable and positively skewed (Figure 3.1). A small proportion of the lambs (12 out of 933) appeared to raise no detectable IgA to fourth-stage larvae.

There was a significant relationship between the IgA-ODI and adult worm length ($p<0.0001$). It appeared that those lambs with greater IgA responses had on average shorter adult worms. The relationship appeared to be linear (figure 3.2).

The degree of variance of the IgA-ODI response was significantly influenced by the year ($p<0.0001$) and the sex of lamb ($p<0.0010$) with female lambs having the highest responses followed by castrated males. A small proportion of the lambs had retained testicles (rigs) and these lambs had the lowest IgA responses (figure 3.3).

There was a significant relationship between the number of adult worms and the length of adult female worms ($p<0.01$). It was of interest to examine whether the effect of the IgA response acted independently of the adult worm burden. To investigate this a multiple regression model that fitted the log transformed numbers of adult worms as well as the IgA-ODI response to fourth-stage larvae against the adult female worm length was used. Both traits remained significant and appeared to act at least in part independently of each other. The regression coefficient for the number of adults was -0.034 ± 0.012 when analysed on its own and -0.029 ± 0.012 when analysed jointly with IgA-ODI. For the IgA-ODI response the regression coefficient was -0.084 ± 0.016 when analysed on its own and -0.081 ± 0.016 when analysed in conjunction with the number of adult worms. The r-square values were 0.016 for the number of adult

worms and 0.056 for the IgA-ODI response alone. When analysed together, the r-square was 0.068. These suggest that the two mechanisms were acting in part independently.

The components of variation in the IgA-ODI response could be partitioned into additive genetic effects (34%), maternal effects (13%) and a residual (53%) (figure 3.4).

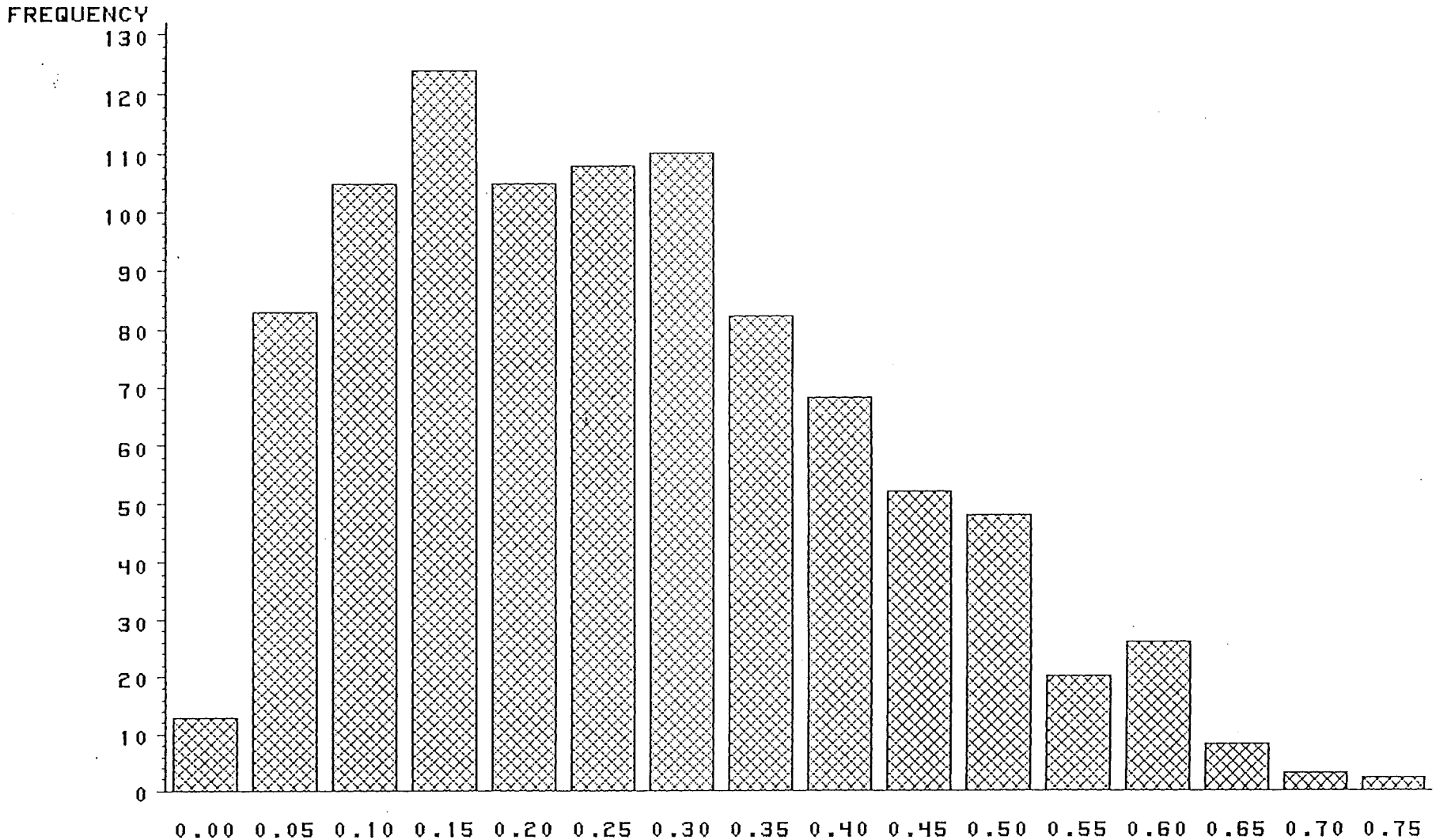

Figure 3.1. The Distribution of IgA responses over five years.

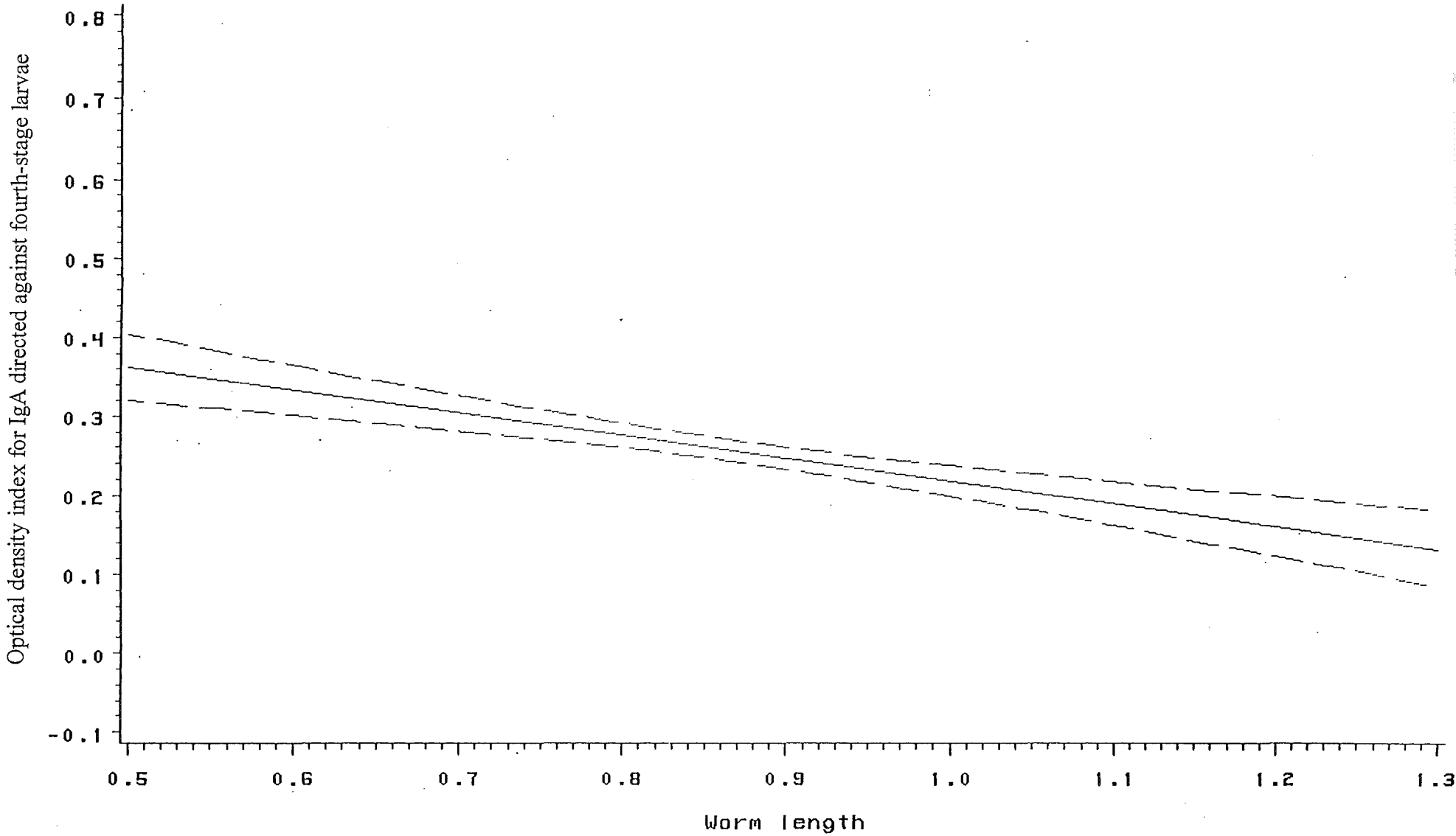

Figure 3.2. The relationship between Parasite-specific IgA and Adult Female Worm Length

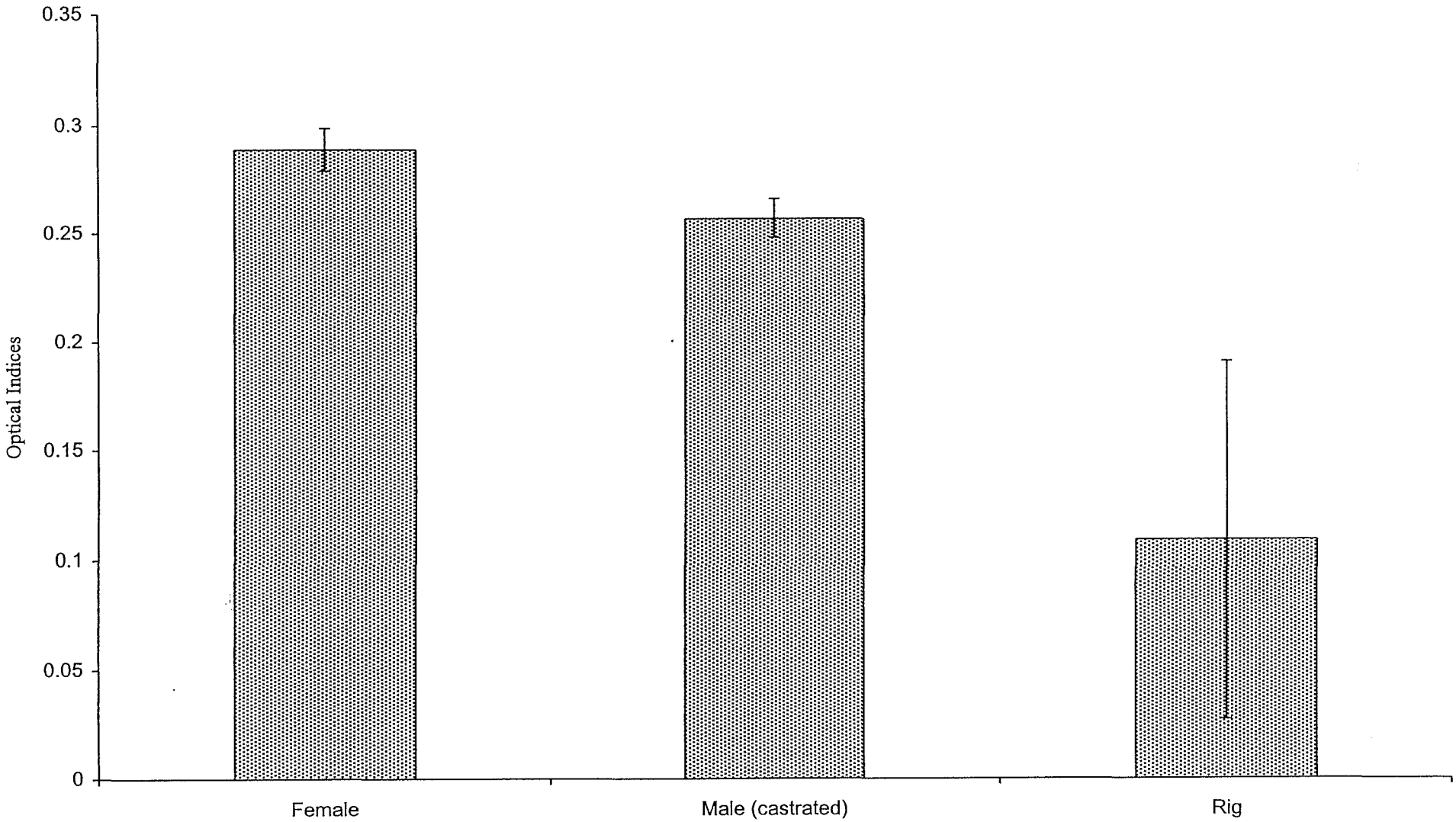

Figure 3.3. The Effect of Sex on the Parasite-Specific IgA Response

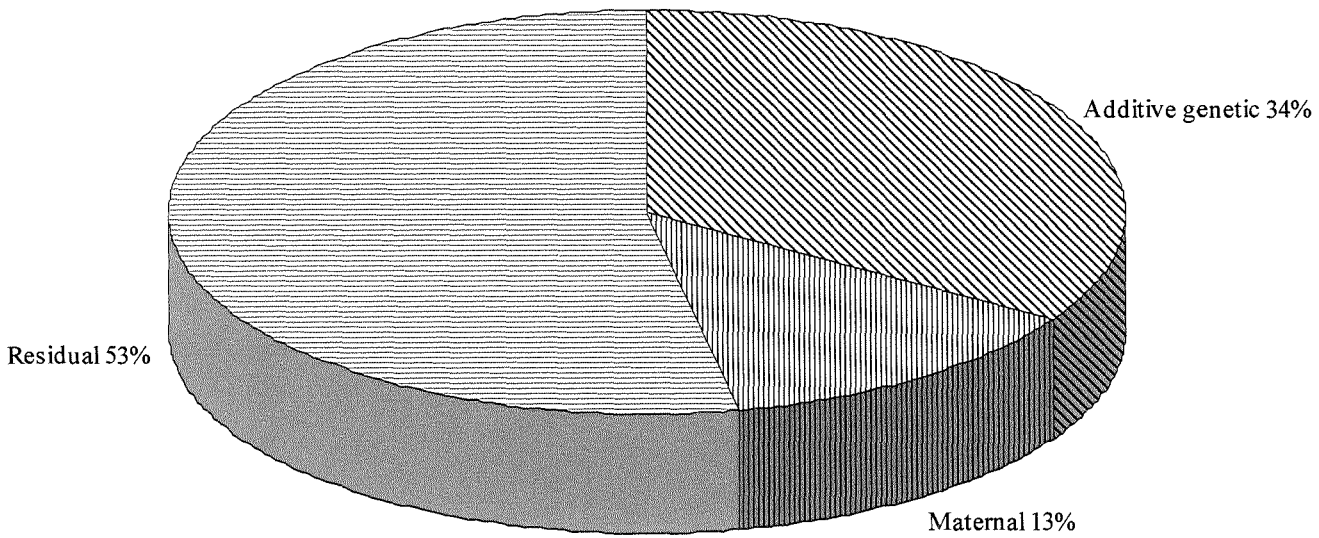

Figure 3.4 The Components of Variation in the Parasite-Specific IgA Response

	1992 (n = 109)	1993 (n = 100)	1994 (n = 153)	1995 (n = 152)
Mean number of adult worms	6570	2778	1548	2992
The negative binomial distribution parameter *k*	1.90	1.79	1.63	1.90

Table 3.1 The number of adult *T. circumcincta* among lambs that had been naturally infected.

3.4 DISCUSSION

This study supports the hypothesis that IgA is the major immunological controlling mechanism of the fecundity of *T. circumcincta* (Stear et al 1995). There was a large variation between lambs in the IgA responses directed against fourth-stage larvae. The IgA response was very closely associated with adult worm length. This response was under genetic control and was influenced significantly by the year of sampling and the sex of the lamb. Male lambs had poorer IgA responses and longer, more fecund adult female worms. In addition, the number of adult worms influenced the length of adult female worms although the mechanism for this appeared to act only partially through the IgA response.

No attempt was made to quantify the amount of IgA in the plasma samples. The optical density is dependent upon the amount of antibody, its avidity and affinity to each component of the antigen preparation. Correlations between responses do not therefore necessarily indicate similar amounts of antibody, but rather higher-than average and lower-than average responses.

There was extensive variation in the IgA responses among years and lambs. The distribution of IgA responses was positively skewed. Several factors may be responsible for this. The distribution is similar to the distribution of *T. circumcincta* worm burdens (Stear et al 1998) and worm lengths (Stear & Bishop in press). It would be expected that antigenic exposure would have an effect on the distribution of the immune response; those animals exposed to more antigen would be expected to mount greater immune responses. Although this could theoretically be avoided in deliberate infections, differences in exposure between groups infected naturally and groups infected artificially may not be as great as commonly assumed. The variance of faecal egg counts appears to be generally

similar irrespective of the infection being natural or deliberate. This will presumably be due to unavoidable differences in individual infection doses and non-specific differences between sheep that influence establishment and worm development. The IgA response was heritable and so some of the variation will be genetic in origin.

The marked differences between castrated lambs and rigs could indicate either a direct depressive effect of testicular hormones on the IgA response or an indirect effect for example through dietary metabolism. Entire male lambs grow more quickly than castrated ones through increased feed intake and more efficient metabolic use of food for liveweight gain. Supplementing protein intake in susceptible lambs infected with *Haemonchus contortus* improves their immunological response (see chapter 6) (Wallace et al 1995). It could be that entire male lambs are directing dietary protein more towards growth than towards an effective IgA response.

There was a strong and consistent correlation between IgA directed against fourth-stage larvae and length of adult female worms. Although this does not give conclusive evidence for a causal link, it strongly implies that the IgA response is controlling worm length. It could be that the IgA response is acting as a marker for some other response that is the effector mechanism. However, it is hard to envisage a response that would be more closely correlated to worm length than the IgA response. This finding is consistent with transfer experiments where local IgA responses were transferred from immune, infected sheep to parasite-naïve infected sheep and resistance, as measured by larval growth retardation, was transferred (Smith et al 1986). However this study could

not rule out a role for other substances secreted from the adoptively transferred cells.

Female lambs mounted significantly greater IgA responses to fourth-stage larvae than castrated male lambs which in turn had significantly greater responses than lambs with 1 or 2 retained testicles (rigs). This finding supports the widely acknowledged sex differences in host-parasite interactions in laboratory animals (Waddell et al 1971), and in sheep (Barger 1993).

There was a significant effect of the number of adult worms on the length of female worms. Those lambs with large worm populations had on average shorter worms. This could be working through a dose dependent mechanism where more parasites lead to a greater antigenic challenge and therefore a greater IgA response. However, the result of the multiple regression analysis suggests that although this does appear to happen, it does not explain the entire effect seen. If this density-dependant effect worked solely through an increased IgA response then the effect of adult worm burden would cease to be significant in the combined analysis and the r-squares would not be partially additive. Other possible mechanisms by which the number of adult worms might influence worm length are through competition for food reserves or through the action of pheromones that retard development. These considerations are addressed further in chapter five.

In conclusion there was considerable variation in IgA response to fourth-stage larvae. IgA appears to be the major mechanism of resistance to *T. circumcincta* in 6-month old lambs. Historically, selection criteria for breeding for resistance have been based on faecal egg counts, which are a product of worm burden, which is not under genetic control, and worm fecundity, which is.

This work suggests that the IgA responses to fourth-stage larvae is a more accurate criterion for future breeding for resistance.

CHAPTER FOUR

THE RECOGNITION OF MOLECULES FROM FOURTH-STAGE LARVAE OF *TELADORSAGIA CIRCUMCINCTA* BY IgA FROM INFECTED SHEEP

4.1 INTRODUCTION

Genetic resistance to *T. circumcincta* is an acquired response (Stear et al 1997a). There remains some debate over the mechanisms underlying genetic variation. One view is that resistance is highly complex with many different mechanisms having many different effects on the parasite (Miller 1984). An alternative view is that resistance to *T. circumcincta* is relatively simple; the most important manifestation of immunity in growing lambs being the control of worm growth causing a reduction in worm fecundity (Stear et al 1997b). Previous work (Stear et al 1995) and the work described in chapter three suggest that the major mechanism regulating worm growth is the IgA response to fourth-stage larvae, or something closely related to this response. In adult sheep, additional mechanisms appear to regulate worm burdens (Stear et al 1995).

A number of antigens have been identified on third-stage larvae and adult *T. circumcincta* and some of these have been associated with protection or susceptibility to infection (McGillivery et al 1992). A comparison of IgA responses to third-stage, fourth-stage and adult *T. circumcincta* indicated that the strongest association with reduced worm length was with increased responses to fourth-stage larvae (Stear et al., 1995). The present study was designed to

identify those molecules on fourth-stage larvae that are recognised by sheep IgA and to investigate any associations between their recognition and the control of worm length and fecundity.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design

The experimental design has been described previously (Stear et al. 1995). Briefly, 30 six-month old sheep were taken from a farm where they had been naturally exposed to a predominantly *T. circumcincta* infection. They were kept in helminth-free conditions for another three months until their faecal egg counts were very low. Then, 24 of them were infected with a single dose of 50,000 third-stage larvae (L3). Eight weeks later all 30 lambs were treated with two broad-spectrum anthelmintics, levamisole hydrochloride (Levacide, Norbrook Animal Health, London, UK) and albendazole sulphoxide (Rycoben, Young's Animal Health, Leyland, UK), at the recommended dose rates. After four weeks, when faecal egg counts were zero, the 24 previously infected lambs together with 3 previously uninfected sheep were infected with 50,000 *T. circumcincta* L3. The remaining 3 lambs acted as previously exposed controls. All the lambs were necropsied eight weeks after the last infection.

4.2.2 Plasma samples

Blood samples were collected immediately before slaughter by jugular venepuncture into evacuated glass tubes containing 20mM disodium EDTA (Becton Dickinson UK Ltd., Oxford, UK). Plasma was obtained by centrifugation at 1000g for 20 minutes and stored at -20°C.

4.2.3 Necropsy

The method for measuring adult female worm lengths is described in chapter two.

4.2.4 Preparation of parasite somatic extract

The preparation of parasite somatic extracts is described in chapter two.

4.2.5 Western blotting

The Western blotting technique is described in chapter two.

4.2.6 Statistical analysis

The similarity between the reactions of different sheep against protein bands was assessed by Pearson product-moment correlation coefficients using the Corr procedure in the SAS package (SAS Institute, Cary, NC. USA).

The influence of antigen recognition was tested by comparing mean female worm lengths in those animals that recognised a particular band against those animals that failed to recognise the same band. Those bands recognised by less than 5 or by more than 18 of the sheep were not analysed because of the unreliability of any conclusions drawn from their recognition. Separate one way analyses of variance were done for each of the 36 remaining bands.

A single statistical model was then used to estimate the total amount of variation in worm length that could be attributed to variation in density-dependent effects together with variation in IgA quantity and specificity along with their interactions. Density-dependent effects were estimated by fitting the log-transformed adult worm burden. IgA quantity was the concentration of IgA

in the abomasal mucosa, as estimated from the mean of 8 sets of three replicates against fourth-stage somatic extracts in a simple indirect ELISA (Stear et al 1995). Recognition of the adult parasite molecule of 28,000 Da (McCririe et al 1997) was strongly correlated with recognition of the fourth-stage parasite molecule of 87,000 Da and the analysis did not include the 28,000 Da molecule.

4.3 Results

In total, 49 bands were recognised by some or all of the sheep and ranged in molecular weight from 3,000 to 184,000 Da. The frequency of recognition ranged from 9% to 100%; only 3 sheep recognised a band of 75,000 Da whereas all 30 sheep recognised a band with a molecular weight of 18,000 Da (Table 4.1). The parasite naïve lambs recognised two bands of approximate molecular weights 77,000 Da and 184,000 Da.

There was considerable heterogeneity in the recognition patterns amongst the sheep (Appendix 7). No pair of sheep recognised the same set of bands and no sheep recognised all 49 bands.

The mean adult worm lengths from each animal included in the analysis ranged from 0.751 to 1.095 cm. The recognition of two bands of approximate molecular weights 87,000 Da (p87) and 129,000 Da (p129) was associated with a reduction in adult worm length (Table 4.1). Those lambs that recognised p129 had adult female worms 0.10cm shorter than those lambs that failed to recognise this molecule. Those lambs that recognised p87 had worms 0.12cm shorter. Recognition of both molecules accounted for 37% of the total variation in worm length.

A single statistical model was used to assess the total variation in adult worm length attributable to IgA quantity, antibody specificity and density-dependant effects. This model fitted recognition of p87, p129, the 37,000 Da adult worm molecule, total mucosal IgA (range 0 to 1.2 ODI), log transformed adult worm burden and their interactions. This model accounted, in a statistical sense, for 93% of the total variation in worm length. This is a remarkably high value and corresponds to a correlation of over 0.96.

Table 4.1. The recognition of fourth-stage larval antigens by IgA and their associations with adult female worm length

MOLECULAR MASS	FREQUENCY (%)	INFLUENCE ON WORM LENGTH (CM)	PROBABILITY
3 000	86	NT	
5 000	68		
7 000	91	NT	
8 000	36		
14 000	45		
15 000	45		
16 000	68		
17 000	95	NT	
18 000	100	NT	
19 000	95	NT	
20 000	91	NT	
21 000	91	NT	
22 000	45		
24 000	82		
26 000	23		
28 000	59		
32 000	83	NT	
32 500	87	NT	
33 000	17	NT	
36 000	35		
37 000	61		
38 000	48		
39 000	21		
40 000	30		

MOLECULAR MASS	FREQUENCY (%)	INFLUENCE ON WORM LENGTH (CM)	PROBABILITY
41 000	39		
70 000	48		
71 000	34		
75 000	9	NT	
77 000	91	NT	
81 000	22		
84 000	30		
87 000	39	-0.12 +/- 0.04	0.011
94 000	43		
98 000	57		
100 000	65		
102 000	74		
103 000	74		
108 000	65		
109 000	65		
115 000	83		
117 000	74		
118 000	70		
129 000	57	-0.10 +/- 0.04	0.008
141 000	56		
145 000	74		
153 000	44		
165 000	78		
172 000	61		
184 000	96	NT	

NT not tested

Table 4.1. The recognition of fourth-stage larval antigens by IgA and their associations with adult female worm length

4.4 DISCUSSION

Sheep varied considerably in the recognition of antigens present on fourth-stage larvae of *T. circumcincta.* No single sheep recognised all the bands detected and only one antigen was recognised by all the sheep. The recognition of two bands with molecular weights of 87,000 and 129,000 Da was associated with decreased worm length. Variation in the amount of local fourth-stage specific IgA, in antibody specificity and in the number of adult worms present in individual sheep and their interactions accounted, in a statistical sense, for most of the observed variation in worm length.

Considerable heterogeneity among sheep in the recognition of antigens by antibody has previously been reported for extracts of third stage larvae and adult *T. circumcincta* (McCririe et al 1997), among humans for excretory-secretory materials of *Ascaris lumbricoides* (Kennedy et al 1990) and among cattle for extracts of *Ostertagia ostertagi* (Hilderson et al 1993) and excretory-secretory materials from *Dictyocaulus viviparus* (Britton et al 1992). Therefore, variation among outbred individuals in recognition of parasite molecules appears to be a widespread phenomenon.

The variation among animals in antigen recognition is relevant to understanding resistance to nematode infection. Research in experimental models has clearly shown that resistant and susceptible strains of mice recognise different molecules on parasitic nematodes (Kennedy 1989). This differential recognition could be partly responsible for the differences in resistance. Interestingly, while recognition of most molecules differs among but not within strains, the recognition of other molecules differs within strains (Kwan-Lim & Maizels 1990). These results imply that differences in antigen recognition are

due to both genetic and non-genetic components. Further research is necessary to determine the relative importance of genetic and non-genetic elements in outbred species.

Female worm length is an excellent indicator of worm fecundity. Female worm length is strongly correlated with mean number of eggs per female (r = 0.97; $p < 0.0001$) (Stear et al. 1995). Of the 49 bands recognised in fourth-stage larval extracts, only 27 occurred at a suitable frequency for analysis. The recognition of only 2 of these 27 was associated with a reduction in worm length. Therefore, responses against most parasite molecules appear to have little if any effect on resistance to infection. The results of the present study suggest that anti-larval responses reduce the length and hence the size and fecundity of adult nematodes. This concurs with the observation that immune responses against *Trichinella spiralis* larvae reduce adult fecundity (Silberstein & Despommier 1985a). Further research is necessary to characterise these bands and determine exactly how the immune response influences worm growth.

The variation among animals in antigen recognition also has implications for the future development of vaccines. If only some sheep are able to mount effective immune responses against each parasite antigen then vaccines are unlikely to be effective in all sheep tested. However, it has been argued that a successful vaccine does not need to invoke a protective response in all sheep; an effective immune response in some sheep could reduce pasture contamination and be beneficial to the flock as a whole (Barnes et al 1995).

A remarkably high proportion (93%) of the variation in worm length could be accounted for by combining the effects on worm length of adult worm burden, IgA quantity and the recognition of the 87,000 Da, 129,000 Da bands from

fourth-stage larvae and the 37,000 Da band from adult parasites. In other words, after allowing for the influence of worm burdens, most of the variation in worm length among sheep can be accounted for by differences in IgA quantity and specificity. This observation implies that a single immunological mechanism plays a major role in regulating worm length and fecundity. This mechanism appears to be the parasite-specific, local IgA response or something that is very strongly associated with the quantity and specificity of this IgA response.

It is known that allels of the class II *DRB1* locus are associated with reduced egg counts (Schwaiger et al 1995). The function of these molecules is to present parasite molecules to helper T lymphocytes. These helper T lymphocytes can potentially turn on a host of immune responses, any combination of which could in theory be associated with reduced egg counts. One possible response could be the IgA response. The current study, using a statistical approach suggests that over 90% of the variation in worm length can be accounted for in variation of parasite-specfic IgA. For another mechanism to be at work, where IgA was acting merely as a marker, even more of the measured variation would have to be accounted for. As no known immunological response appears sufficiently strongly associated with both IgA quantity and specificity, our working hypothesis is that the observed association with worm length is directly due to the IgA response.

In conclusion, following infection sheep showed considerable variation in recognition of antigens on fourth-stage larvae. The recognition of two bands was strongly associated with a reduction in mean length of adult female worms. Variation among sheep in the recognition of these two bands, together with variation in the recognition of a previously described band in adult parasite

extracts, accounted for a substantial proportion of the variation in worm length. The combination of variation in antigen recognition, variation in the production of IgA specific for fourth-stage larvae and the influence of variation in adult worm burdens accounted for over 90% of the total variation among sheep in adult female worm length. These results suggest that a single immunological mechanism is responsible for most of the variation in adult female worm length and that this mechanism is the IgA response to fourth-stage larvae or a response that is extremely strongly associated with this IgA response.

CHAPTER FIVE

THE MECHANISMS OF INHIBITION OF *TELADORSAGIA CIRCUMCINCTA* LARVAE IN SHEEP

5.1 INTRODUCTION

Teladorsagia circumcincta is a major constraint on sheep production in temperate regions of the world (Urquhart et al 1987). It is remarkably well adapted to its host and is able to suspend development by going into inhibition within the host shortly after moulting to the fourth-stage (Eysker 1997). The ability of *Teladorsagia circumcincta* larvae to go into inhibition in adverse conditions may contribute to the extraordinary success of this parasite. This suspension of development may allow it to avoid development during periods that would be disadvantageous, for example due to reduced survival of larval progeny on pasture during cold weather. These inhibited larvae are then able to come out of inhibition and continue development to adults. Simultaneous development of large numbers of inhibited larvae can precipitate Type II teladorsagiasis.

The mechanisms underlying inhibition and emergence from inhibition are poorly understood. There appear to be four possible mechanisms. The genetic makeup of a parasite strain influences the tendency to go into inhibition. Some isolates of the parasite can go into inhibition while others cannot (Borgsteede & Eysker 1987). The season influences the proportion of ingested parasites that go into inhibition. A larger proportion of third stage larvae ingested during the autumn will go into inhibition compared to those ingested earlier in the year.

This may be due to the effect of larval chilling (Armour & Bruce 1974). Thirdly, the development of immunity has been associated with inhibition. Lambs previously infected are more likely to have larvae going into inhibition than naïve lambs (Smith et al 1984). This is supported by the observation that sheep immunosuppressed by treatment with cortisone or whole-body irradiated have fewer inhibited larvae than controls (Dunsmore 1961). Finally, there appears to be a density-dependent component where animals given heavy infections are more likely to have inhibited larvae than lambs given moderate infections. There is uncertainty as to whether the density-dependent component acts independently, for example through competition for food resources, or through an immune mechanism where a larger antigenic dose causes a greater immunological response (Anderson & Michel 1977).

Animals with increased concentrations of parasite-specific IgA have shorter and less fecund worms (Stear et al 1995). IgA may inhibit larval development (Smith et al 1985). The present work was designed to investigate in two separate data sets whether variation in parasite-specific IgA was associated with variation among sheep in the extent of larval inhibition and to study the density-dependant effects of adult worm burden on larval inhibition. Sheep were studied over a 5 year period to avoid variation in numbers of larvae due to variation between years. A single farm was studied to avoid any major strain effects on inhibition. In addition, a group of deliberately infected sheep were studied where all fourth-stage larvae recovered after necropsy could be assumed to be inhibited and not recently ingested.

5.2 MATERIALS & METHODS

5.2.1 Experimental design

5.2.1.1 Deliberate infection

The experimental design has been described previously in chapter 4

5.2.1.2 Natural Infection

A group of naturally infected lambs were studied the details of which are described in chapter 4.

5.2.2 Plasma samples

Blood samples were collected immediately before slaughter by jugular venepuncture into evacuated glass tubes containing 20mM disodium EDTA (Becton Dickinson UK Ltd., Oxford, UK). Plasma was obtained by centrifugation at 1000g for 20 minutes and stored at -20°C.

5.2.3 Necropsy

The methods of determining the numbers of adult and fourth-stage larvae are described in chapter two.

5.2.4 Parasitology

The parasitological methods are described in chapter two.

5.2.5 Preparation of Parasite Somatic Extract

The preparation of parasite somatic extract is described in chapter two.

5.2.6 ELISA

The ELISA method is described in chapter two.

5.2.7 Western Blot

The Western blot method is described in chapter two. In addition to those bands identified in chapter 4 bands identified in previous studies (McCririe et al 1997) on third-stage and adult *T. circumcincta* were included in the analyses.

5.2.8 Statistical analysis

The optical densities were transformed into an optical density index for each animal using the following formula;

$$ODI = \frac{Mean\,OD - Mean\,negative\,OD}{Mean\,positive\,OD - Mean\,negative\,OD}$$

The numbers of fourth-stage larvae and adult worm burdens from both the naturally and deliberately infected sheep followed a negative binomial distribution. Taylor's power law was used to find the appropriate transformation of data in order to satisfy the assumption of homogeneity of variance for analysis of variance. The appropriate transformation was logarithmic to the base ten.

The correlations between parasite-specific IgA, bands from fourth-stage and adult worms recognised by western blotting, and log transformed adult worm burdens, and numbers of fourth-stage larvae, were estimated by using the GLM procedure on the SAS package (SAS Institute).

5.3 RESULTS

There was substantial variation among sheep in the number of fourth-stage larvae (L4) present, ranging from 0 in 130 sheep to a maximum of 27,100 in one animal. The arithmetic mean was 2,492 L4, but this value masked considerable variation among years (Table 5.1). The mean number of L4 per sheep varied more than ten-fold from a high of 6170 in 1992 to a low of 542 in 1993. The number of larvae in these sheep followed a negative binomial distribution (Table 5.1). The index of overdispersion (k) was only 0.223 and 0.280 in 1993 and 1994 when the mean intensity of infection was relatively low but 0.516 and 0.549 in 1995 and 1992 when the mean intensity of infection was relatively high. As k is an inverse index, these values indicate that the distribution of L4 is more variable in years with relatively low levels of infection. The ratio of L4 to adult *T. circumcincta* also varied among years. The ratio was relatively low in years with low infection intensity, being only 0.16 and 0.32 in 1993 and 1994 compared to 0.48 and 0.50 in 1992 and 1995. These ratios suggested that there was a positive relationship between the number of adults and L4 across years.

There was a close relationship between the mean and the variance in different years. The variance was equal to the mean to the power 2.09 $\pm$ 0.04 ($p < 0.0001$). As the exponent was close to 2, the result suggests that a logarithmic transformation is most appropriate prior to parametric analyses.

The relationship between the number of adult and L4 was further examined by regression analysis of the logarithms of both traits. The relationship was highly significant ($p < 0.0001$). The gradient was not significantly different from one (1.21 $\pm$ 0.13). As gradients of one suggest a linear relationship, the untransformed data were then plotted. To minimise extraneous variation the

animals were grouped into classes ranging from 0-5000, 5001-10,000 fourth-stage larvae and so on. Again, these data were consistent with a linear relationship between the number of adult and fourth-stage *T. circumcincta* (Figure 5.1). As the number of adults within a lamb increased, so did the number of larvae. This density-dependent relationship suggests that development of incoming larvae is retarded or inhibited in heavily-infected abomasa. However, the regression analysis on the transformed data indicated that the number of adults only accounted for a small proportion of the variation among sheep in numbers of fourth-stage larvae ($r^2 = 0.14$).

There was also a positive relationship between the number of L4 and of adults in the deliberately infected sheep ($p<0.05$). Again the gradient was not significantly different from one (1.77 ± 0.85) and the relationship accounted for a relatively small proportion of the total variation ($r^2 = 0.17$).

There was a significant association between parasite-specific IgA and the number of inhibited larvae in the deliberate infection ($p<0.01$). Those lambs with greater IgA responses had increased numbers of inhibited larvae. The relationship between IgA and numbers of inhibited larvae appeared to be linear (Figure 5.2).

As the density-dependent relationship accounted for only a small proportion of the total variation, the association between immune responses and larval development was then investigated. In a multiple regression model that fitted the log-transformed number of adults as well as the IgA response to L4 following deliberate infection, both traits were significant at the 5% level. Interestingly, the traits appeared statistically independent of each other. For the number of adults the regression coefficient was 1.77 ± 0.85 when the trait was

analysed on its own and 1.80 $\pm$ 0.78 when analysed jointly with the IgA response. For the IgA response the regression coefficient was 2.40 $\pm$ 1.24 when IgA was examined singly and 2.45 $\pm$ 1.13 when analysed in conjunction with the number of adult worms. The r-square values were 0.17 for the number of adult worms alone and 0.15 for the IgA response alone. When analysed together, the r-square value was 0.33. These analyses suggested that the mechanisms underlying density-dependence and immune regulation were independent of each other and that the mechanisms were acting additively to regulate the numbers of inhibited larvae.

Among naturally infected sheep, multiple regression indicated that there was again a positive relationship between the number of adult and fourth-stage larvae (1.59 $\pm$ 0.28; $p < 0.0001$). The relationship between the number of fourth-stage larvae and the IgA response was not quite significant (5.46 $\pm$ 2.81; $p = 0.053$). The interaction term was also not quite significant (-0.65 $\pm$ 0.36; $p = 0.068$). The presence of the interaction term indicates that the IgA response and the density-dependent regulation are not acting completely additively in the naturally-infected animals, possibly because the intensity of infection was much higher in the naturally-infected individuals.

Western blotting revealed 98 distinct bands that were recognised by some but not all sheep; 18 bands were recognised in preparations of third-stage larvae, 49 in L4 and 31 in adult material. Separate multiple regression analyses were carried out for each band. These analyses also included the transformed number of adult parasites and the mean mucosal IgA response to L4. The recognition of one or more of five bands was tentatively associated with the number of L4 ($p < 0.10$). Sheep that recognised antigens in adult parasites with Mr of 16.5 kDa or

26 kDa had fewer L4. Sheep that recognised a 108 kDa band in the L4 preparation had fewer L4 while those sheep that recognised a band at 41 kDa in the same L4 preparation had more L4 than those sheep that failed to recognise this band. Sheep that recognised a band with an Mr of 43 kDa in third-stage larvae also had fewer L4.

A combined analysis included the number of adult *T. circumcincta*, the mean mucosal IgA responses to third and fourth-stage larvae and the presence or absence of each band. Each effect was significant at the 5% level and together these variables accounted for over 91% of the variation in the transformed number of fourth-stage larvae.

Year	Number	Mean	S.E.M.	K	S.E.	L4:Adult
1992	110	6170	810	0.549	0.066	0.48
1993	99	542	106	0.280	0.045	0.16
1994	165	726	122	0.223	0.027	0.32
1995	156	3165	356	0.516	0.055	0.50

Table 5.1. Distribution and variation of fourth-stage larvae among naturally-infected sheep

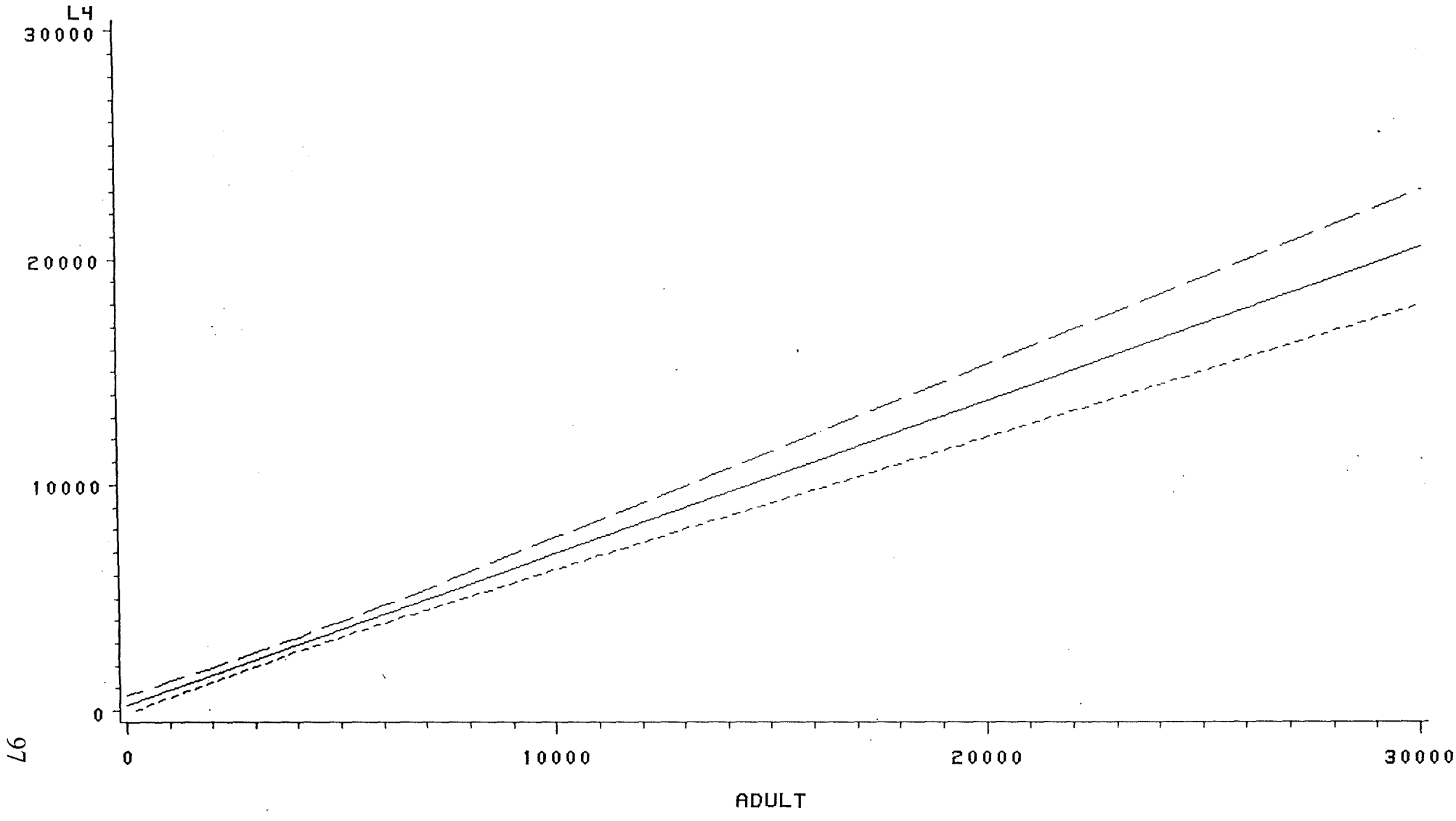

Figure 5.1. The relationship between the number of fourth-stage larvae (L4) and adult worm burden (Dashed lines represent the standard error).

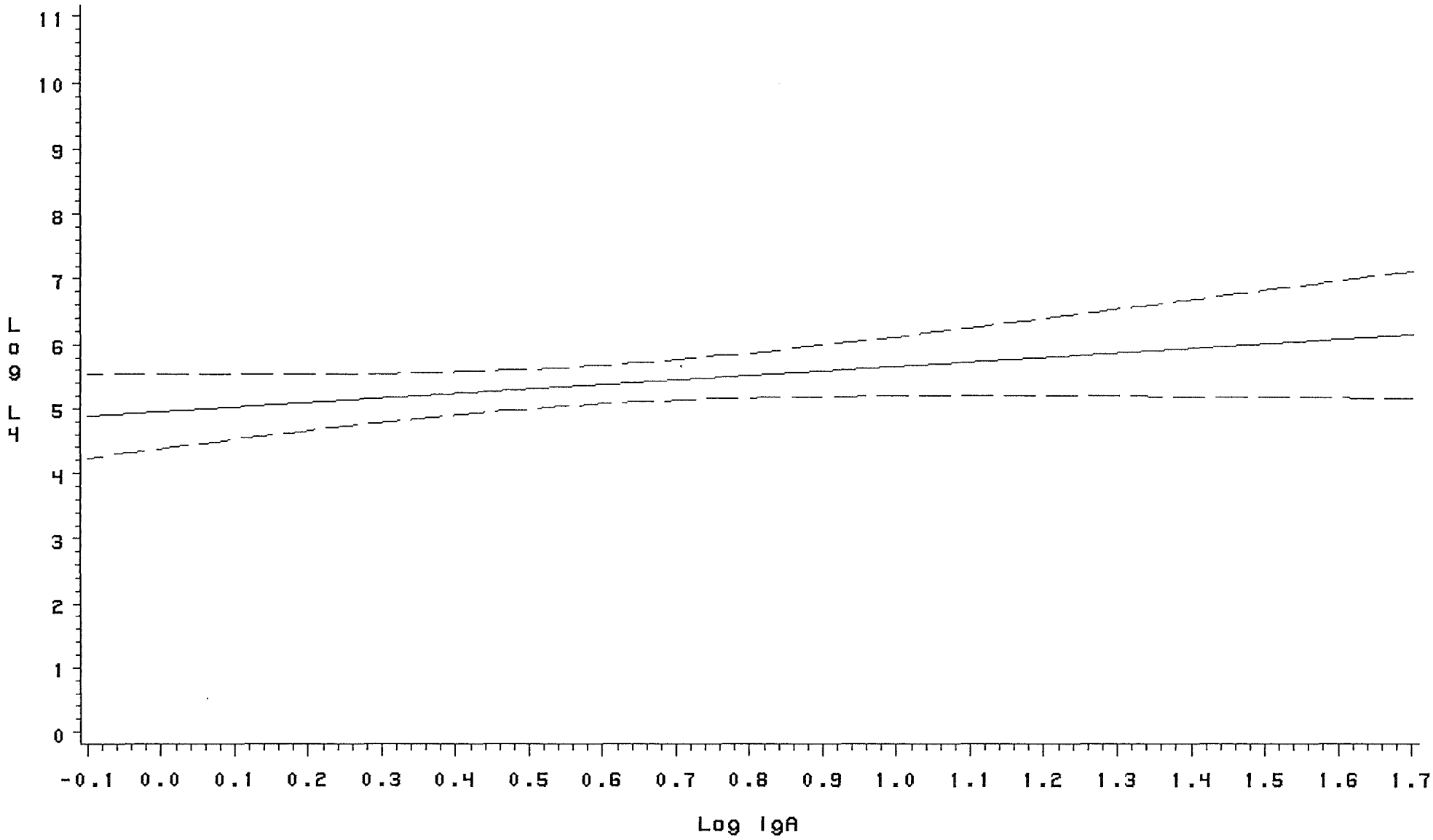

Figure 5.2. The relationship between IgAdirected against fourth-stage larvae measured by optical density indices and the number of fourth-stage larvae (Dashed lines represent the standard error).

5.4 DISCUSSION

This chapter describes the distribution of fourth-stage larvae among infected sheep, the relationship between the parasite-specific IgA response and larval inhibition, the effect of the recognition of specific bands by antibody on variation in the numbers of inhibited larvae and the relationship between the number of adult worms and inhibited larvae. Two possible mechanisms of inhibition are identified; a parasite-specific IgA response and density-dependence. It appears that at high levels of infection these act independently of each other.

The distribution of fourth-stage larvae indicates that there was wide variability in the numbers of larvae in an outbred flock exposed to natural infection. The variation in the distributions were less in years where there were high levels of infection compared to years with low infection intensity. This is presumably a consequence of fewer animals having no fourth-stage larvae in high intensity years.

There was a linear relationship between the numbers of adults and fourth-stage larvae. Those sheep with greater adult burdens of parasites also had greater numbers of fourth-stage larvae. In the naturally infected sheep we were not able to distinguish recently ingested fourth-stage larvae from those in inhibition. Any association between numbers of fourth-stage larvae and numbers of adults could simply be a consequence of increased exposure where those lambs ingesting more larvae would be expected to have more adults as well. To overcome this a deliberately infected group of sheep were studied where all fourth-stage larvae could be assumed to be inhibited or retarded as the sheep had been infected eight weeks previously. Thus it could be concluded that the association between

adults and fourth-stage was not simply a consequence of increased parasite intake. Although in both data sets the effect of adult worm burden on numbers of L4 was significant this density-dependant effect on larval inhibition accounted for only a small proportion of the total variation in the numbers of L4, indicating other factors responsible for larval inhibition.

The mechanism underlying the density-dependant relationship between the number of adult worms and of fourth-stage larvae remains unclear. Parasites might secrete a substance that allows incoming larvae to estimate the size of the existing population. In crowded or unfavourable conditions, some larvae might suspend development. Just such a facultative response is seen in the dauer response of *Caenorhabitis elegans*, where larvae are able to go into the dauer phase in the face of reduced food resources and under the influence of a pheromone (Wood 1988).

Immunity has long been thought of as a possible mechanism whereby larvae are made to go into inhibition. Increased production of parasite-specific IgA is significantly associated with decreased worm length (Stear et al 1995), and in passive transfer experiments IgA appeared to have an effect of stunting adult worms, (Smith et al 1986). However in this experiment it is possible that the stunting was due to other factors secreted by the whole cells which were transferred. It could be that the association between IgA and number of fourth-stage larvae is an exaggerated effect of this reduction in worm length (McKellar 1993). Alternatively, it may be a response of the parasite to unfavourable conditions for growth, perhaps a consequence of IgA binding to parasite molecules that influence parasite feeding or metabolism.

The recognition of five bands by antibody was associated with numbers of inhibited larvae in the deliberately infected sheep. Each was of a different molecular weight and had different recognition patterns indicating that they are distinct from each other. Interestingly four of the bands were associated with decreased numbers of larvae. It is tempting to speculate on the possible mechanism of such molecules particularly as two were recognised on adults. It could be that these molecules are acting as signals recognised by incoming larvae which communicate the presence of adults and by blocking these molecules by antibody these signals fail and so incoming larvae continue to develop. Such a mechanism could underlie density-dependence. However, the present results suggest that the IgA response and density-dependence act independently. More work is required to confirm that recognition of these molecules is associated with reduced inhibition and to determine the mechanism by which they act on incoming larvae.

Recognition of one molecule on fourth-stage larvae was associated with increased larval inhibition. The recognition of two bands on L4 by IgA has been associated with decreased worm length and therefore reduced worm fecundity (chapter 4). However, the bands on L4 associated with numbers of inhibited larvae were different. No association between inhibition and the recognition of the two bands associated with reduced worm length were found. This may indicate separate immunological mechanisms for inhibition compared to those associated with reduced worm growth.

The combined analysis of the effects of adult worm burden, IgA quantity and specificity accounted for over 90% of the variation in numbers of L4 in the deliberately infected sheep. This is a remarkably high figure when it is

considered there are inevitably inaccuracies in estimating parasite burdens and that the IgA response was measured in the plasma and not in the mucus. It appears that virtually all of the variation can be accounted for in this model.

In conclusion the present study has described two possible mechanisms underlying larval inhibition of *T. circumcincta* at the fourth-stage. The strength and specificity of the IgA response and the adult worm burden are associated with inhibition. These two mechanisms appear to work largely independently. The mechanism of how this IgA response works remains unknown. It may work through interferring with feeding, digestion, metabolism or population regulation. Further work will be required to confirm the associations seen here and to identify and define those molecules involved. This will involve N-terminal sequencing, immuno-localisation through immunohistochemistry, and screening for enzymatic activity. In addition further work is required to determine how incoming larvae interact with established infections.

CHAPTER SIX

THE CONTROL OF WORM LENGTH IN LAMBS INFECTED WITH *HAEMONCHUS CONTORTUS*

6.1 INTRODUCTION

Haemonchus contortus is a highly pathogenic parasite of small ruminants of tropical and sub-tropical regions in the world (Urquhart et al 1987). The adult parasite is a blood feeder. Consequently, infection causes a protein losing gastropathy exacerbated by anaemia.

Nutrition has a major effect on both resistance to *H. contortus* and resilience to the effects of infection (Coop & Holmes 1996). Supplementation with protein is associated with reduced faecal egg counts in lambs given trickle infections (Shaw et al 1995) and increases the rate of acquisition of immunity to a variety of parasites (Coop & Holmes 1996). Protein supplementation also offsets the increase in protein turnover and inappetance seen in haemonchosis (Abbott et al 1986a; Wallace et al 1996; Wallace et al 1995). However, it has been reported that it does not have an effect on parasite establishment in the cases of *T. circumcincta* (Coop & Holmes 1996), or *T. colubriformis* (Van Houtert et al 1995). However, in this last study there was evidence that protein supplementation was associated with decreased faecal egg counts.

In addition, the influence of dietary protein on the course of infection depends on the breed of sheep under study. Susceptible breeds, such as the Hampshire Down, show greater improvements than resistant breeds such as the Scottish Blackface (Abbott et al 1985; Wallace et al 1996).

Although much work has been done on the development of vaccines to control *H. contortus* (Munn 1993; Schallig & Van Leeuwen 1997; Smith et al 1994), there remains uncertainty over the effector mechanisms of immunity and the genetic and non-genetic factors that influence the development of effective immunity. Effective immunity is dependent on CD4+ T-cells (Gill et al 1993b). Both IgG1 and IgA have been associated with a reduction in faecal egg counts and worm burdens (Gill et al 1993a). Work with another abomasal parasite *Teladorsagia circumcincta* has indicated a very strong correlation between parasite-specific IgA and worm fecundity (Stear et al 1995; Chapter 3).

The purpose of the work described in this chapter was to determine if increased IgA is associated with decreased fecundity in *H. contortus* and whether the magnitude of the IgA response is influenced by protein nutrition.

6.2 MATERIALS AND METHODS

6.2.1 Experimental animals

The experimental design has been described previously (Wallace et al 1995). Briefly, 16, five-month-old pedigree Hampshire down lambs reared in helminth-free conditions were divided by stratified random sampling into two groups according to bodyweight and sex. One group of twelve was given a basal diet containing 98g metabolisable protein (MP) kg^{-1} dry matter (DM) and the other group of twelve, a supplemented diet containing 173g MP kg^{-1} DM. Both diets were isocaloric. The additional protein was supplied in the form of soyabean meal. Each animal consumed 1.4 kg of fresh matter daily, divided over two feeds except for one lamb in the basal dietary group which consumed 95% and 97% of the feed on the first two days of the experiment. The two groups

were maintained on their diets for two months before eight of the twelve animals in each group were infected. Each animal was housed individually throughout the duration of the experiment.

6.2.2 Parasitological technique

Third stage *Haemonchus contortus* larvae of a strain originally obtained from the Moredun Research Institute (Edinburgh) were used. Each lamb received an initial infecting dose of 100 larvae kg bodyweight^{-1}. Subsequently they were given a trickle infection of 200 larvae three times a week for 10 weeks. Immediately after this period all the infected lambs were necropsied.

6.2.3 Plasma samples

Blood samples were collected from each lamb by jugular venepuncture into heparinised evacuated glass tubes on the day of slaughter. The tubes were centrifuged and the plasma removed and stored at -20°C.

6.2.4 Necropsy

The methods for determining total worm burden, average female worm length and number of eggs *in utero* are described in chapter two.

6.2.5 Larval antigen preparation

The method of preparing parasite somatic extracts is described in chapter two.

6.2.6 ELISA

The ELISA method is described in chapter two

6.2.7 Statistical Analysis

The optical densities (OD) were transformed into an optical density index (ODI) for each animal using the following formula;

$$ODI = \frac{Mean\,OD - Mean\,negative\,OD}{Mean\,positive\,OD - Mean\,negative\,OD}$$

The associations between parasite-specific IgA and worm-length, and IgA and dietary group were examined with the general linear model procedure on the SAS package (SAS Institute).

A multiple regression model was used to examine the effects of adult worm burden and IgA directed against L4 on adult female parasite worm length.

6.3 Results

Infected lambs fed the protein-supplemented diet appeared to produce significantly more parasite-specific IgA ($p<0.005$). They had on average 140% more parasite-specific IgA than the positive control whereas those lambs on the basal diet averaged only 80% of the positive control value. Figure 6.1 shows that the supplemented group had more IgA against all three stages of the parasitic life-cycle: third-stage, fourth-stage and adult parasites.

Reduced adult worm length was significantly associated with increased IgA against L3 ($p<0.05$) and against L4 ($p<0.05$) (Figure 6.2). However, it was not significantly correlated with adult-specific IgA.

The mean length of adult female worms was 2.13 cm in the basal dietary group and 2.00 cm in the supplemented group (Wallace et al 1995). This

difference in worm length between the two groups could be almost entirely attributed to the difference in parasite-specific IgA (appendix 8). The multiple regression model fitting the effect of worm burden and IgA directed against L4, predicted a decrease in worm length of 5.74mm for each unit increase in IgA. The difference between the groups in the L4 response was 0.19 units IgA. This difference would be expected to lead to a reduction in length of 1.1mm. The actual measured difference between the two groups was 1.3mm. Therefore, the difference between the two groups in their worm length could, in a statistical sense, be explained almost entirely by differences in the amount of IgA against fourth stage larvae.

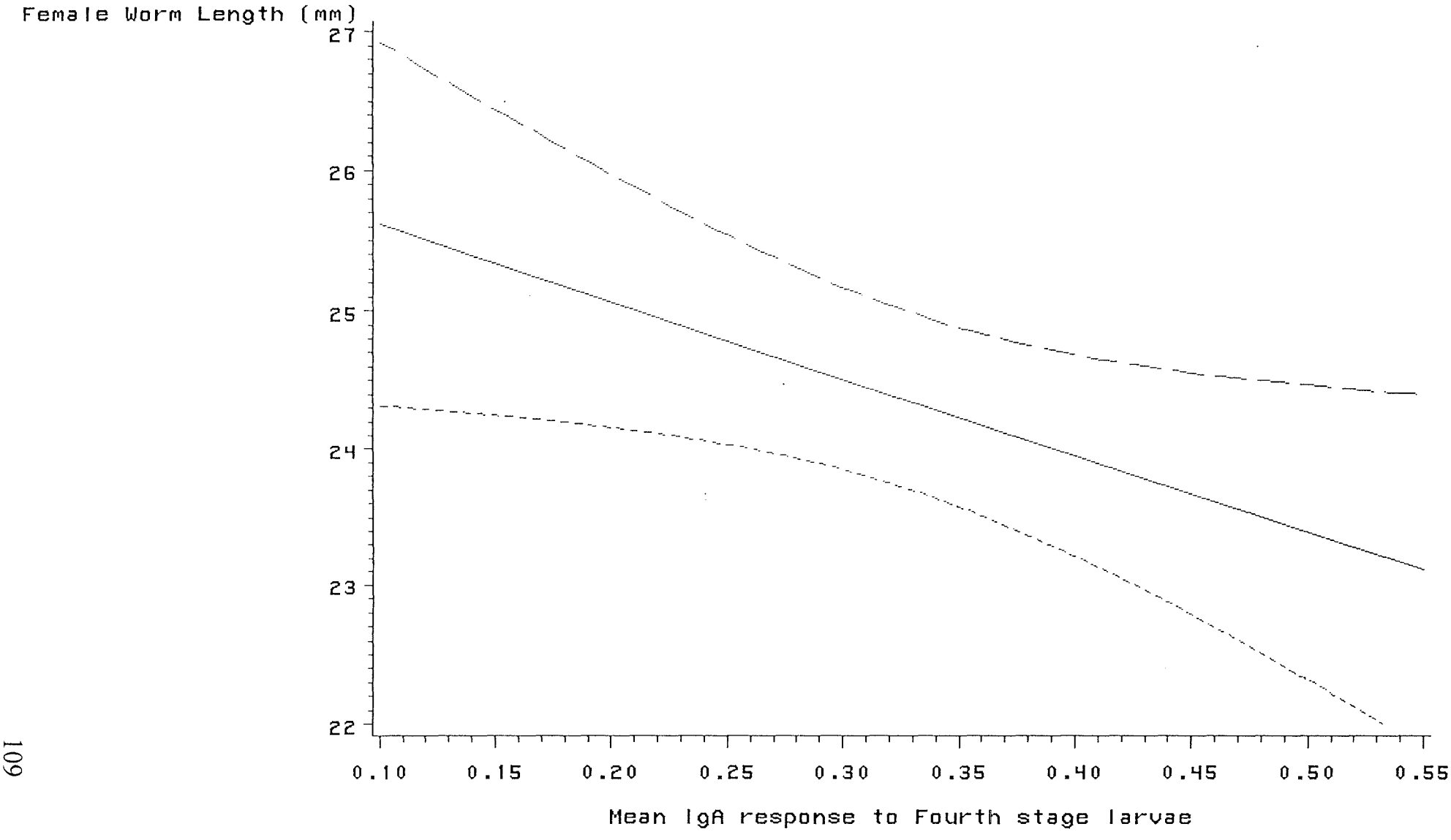

Figure 6.2. The relationship between parasite-specific IgA as measured by optical density indices and adult female worm length (Dashed lines represent the standard error).

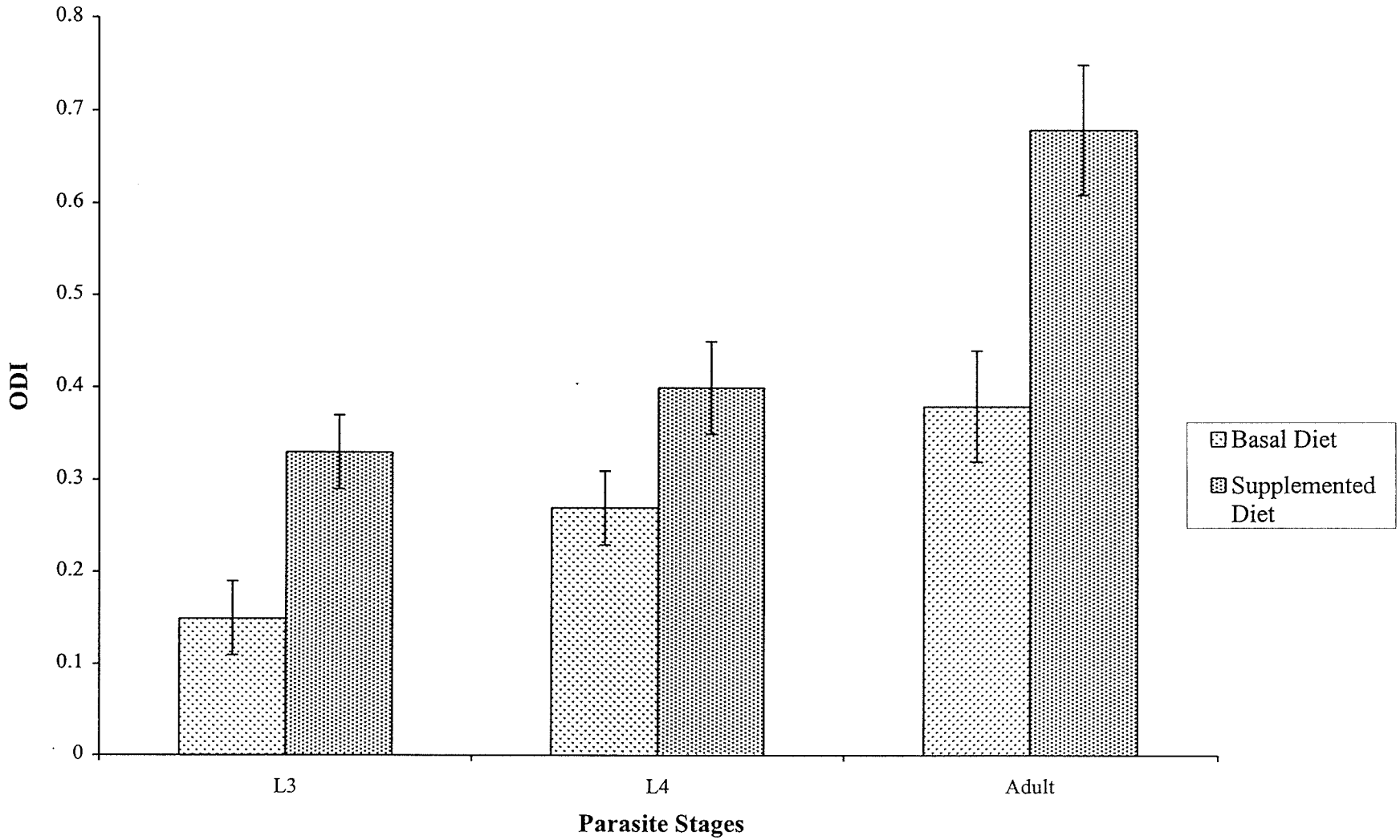

Figure 6.1. The Effect of Dietary Group on the Parasite-Specific IgA Responses

6.4 DISCUSSION

Previous work has shown that lambs fed a protein-supplemented diet had a lower concentration of nematode eggs in their faeces following infection with *H. contortus*. Lambs on a basal diet had worms with a mean of 669 eggs *in utero* while lambs on the supplemented diet had worms with a mean of 539 eggs *in utero*. Those worms from the protein-supplemented lambs were significantly shorter although there was no significant difference in the numbers of worms recovered at necropsy. As shorter worms had fewer eggs *in utero* and sheep with shorter worms had lower faecal egg counts, adult female worms infecting lambs given the supplemented diet appeared to be less fecund.

The purpose of the present study was to identify a possible effector mechanism for this apparent difference in fecundity. This work has shown that those lambs offered the supplemented diet produced more parasite-specific IgA and that there was a significant association between the amount of parasite-specific IgA to third and fourth-stage larvae and adult worm length. Further, the increase in IgA was sufficient to account, in a statistical sense, for virtually all the difference in worm lengths between the two dietary groups. These results suggest that a major protective mechanism in lambs infected with *H. contortus* is the IgA mediated suppression of worm growth and fecundity. Therefore, resistance to *H. contortus* and *T. circumcincta* appear to be remarkably similar.

The present experiment did not provide any evidence that young animals can control worm burdens following infection with *H. contortus* as there was no significant differences in the total worm burdens between the two dietary groups (Wallace et al 1995). There is no convincing evidence in the literature that sheep younger than 7 months can mount effective responses to control the number of

H. contortus or *T. circumcincta.* In contrast, lambs appear able to influence the number of the small intestinal nematodes *Trichostrongylus colubriformis* and *Nematodirus battus* (Dineen & Windon 1980) (McClure et al 1998). These differences may reflect the inability of lambs to mount effective responses in the abomasum or different susceptibility of the different nematode species to immune responses.

There is some confusion concerning the effects of protein nutrition on the immune responses to *T. colubriformis.* In one study looking at the effects in a long-term sub-clinical infection no demonstrable effect was seen (Kyriazakis et al 1996). This is in comparison to another study where an effect was seen in the T-cell population in sheep exposed to *T. colubriformis* (Kambara & McFarlane 1996). Here an increase in T19+ cells was detected in those on protein supplementation compared to those not supplemented. It may be that this subset of T cells is associated with resistance to this parasite. Certainly no direct comparison between the two experiments can be made as infection conditions, breed of sheep and diets were different between the two experiments.

No significant effect was seen in protein supplementation of lambs exposed to *N. battus* (Israf et al 1996). It was concluded in this study that when diet was adequate animals could be segregated into high responders and low responders in relation to their worm burdens and that genetic susceptibility was not overcome by improved nutrition.

Although the present study indicates a correlation between IgA and worm fecundity, this need not mean that IgA is the controlling mechanism. With *T. circumcincta* there is such a strong and consistent correlation between IgA specific to fourth-stage larvae and fecundity it is hard to envisage a mechanism

for which IgA would merely be a marker. However, the present study had a relatively small number of animals and only IgA was measured. It could be that with *H. contortus* increased parasite-specfic IgA is only a marker for the true controlling mechanism which is influenced by dietary protein. In addition the ELISA used does not directly measure quantity of IgA but will be influnenced by binding avidity of the IgA to parasite molecules. Consequently, conclusions drawn concerning the role of IgA in *H. contortus* infections remain tentative until they can be confirmed in other, larger studies or through passive transfer experiments.

In the original study, the lambs on the basal diet seemed less able to withstand the pathophysiological effects of infection as measured by packed cell volume and liveweight gain (Wallace et al 1995). However, it may be that the effect of protein supplementation was not solely to offset the resulting protein loss from parasitism. Animals on lower protein diets mounted a less effective immune response as measured by IgA. It is plausible that shorter, less fecund worms are less active feeders and therefore less pathogenic. If this is the case then protein supplementation not only reduces the pathophysiological effects of infection, but also reduces the pathogenic effects of each parasite.

The mechanism whereby IgA controls fecundity remains unknown although it would be reasonable to postulate that it affects the feeding, digestion or metabolism of the parasite. To elucidate this further will require the identification of those specific molecules recognised by IgA that are associated with reduced worm length.

In conclusion, this study indicates that parasite-specific IgA may be the major mechanism controlling fecundity of *H. contortus,* and that in a relatively susceptible breed, this response is dependent on adequate dietary protein.

CHAPTER SEVEN

GENERAL DISCUSSION

The work described in this thesis was designed to elucidate the effect of the host IgA response to *Teladorsagia circumcincta*, as a mechanism of genetic resistance, and to identify recognised antigenic targets on the parasite associated with resistance. Evidence was also developed to determine if a similar response was seen in sheep infected with *Haemonchus contortus* and to see if this response could be improved by dietary supplementation.

The salient findings were:

- Genetic resistance to *T. circumcincta* in lambs is mediated through the control of worm fecundity and not worm burden.
- The IgA response to fourth-stage *T. circumcincta* (estimated by optical density indices) appears to be the major immunological controlling mechanism in lambs.
- This IgA response is heritable.
- Most of the variation in *T. circumcincta* fecundity is attributable to three factors, IgA quantity, IgA specificity, and density dependence.
- IgA appears to have a similar effect in lambs infected with *H. contortus* where IgA levels are correlated with worm length.
- IgA response is associated with larval inhibition.
- Protein supplementation improves the IgA response.

- The number of adult *T. circumcincta* has a density-dependant effect on the number of inhibited larvae. Those animals with large adult worm burdens have increased numbers of inhibited larvae.

The faecal egg count of sheep infected with *T. circumcincta* is heritable. Residual maximum likelihood fitting an animal model with all known pedigree relationships resulted in a heritability estimate of 0.22 (Bishop et al 1996b). This heritability is sufficiently high to make selective breeding for reduced faecal egg counts feasible. This estimate is similar to heritability estimates for infections with *H. contortus*, *T. colubriformis*, and mixed natural infections (Morris et al 1997; Windon 1990; Woolaston & Piper 1996).

Genetic resistance to *Teladorsagia circumcincta* is an acquired rather than an innate response. Heritability estimates of faecal egg counts are not significantly different from zero in young lambs (1-2 months of age) but increase with age to approximately 0.33 at six months of age (Stear et al 1999). This implies that genetic resistance is acting through an acquired response to the parasite. The most likely response is immunological. This is borne out by the correlations between certain MHC type I and II alleles and low and high faecal egg counts in mixed, predominantly *T. circumcincta* infections (Schwaiger et al 1995; Buitkamp et al 1996).

Logically there are three factors that will influence faecal egg counts; worm numbers, average worm fecundity, and the dilution effect of faeces. Although there is considerable variation in worm burdens amongst individuals within a flock, there is no evidence that young lambs (less than 6 months of age) are able to control worm burdens. Mixed model analysis of variance revealed no

sire component in the variance of worm burdens (Stear et al 1998). However in the same analysis, there was a remarkably high heritability (0.62) for the mean length of adult female worms of *T. circumcincta*. Resistance must therefore work through the control of average worm length in this age of lamb. Indeed, this suggests that most of the variation in average worm length is genetic in origin.

There is considerable variation in mean female worm length between infected animals, with a twofold range between the shortest and longest worms (0.6-1.2 cm). Worms within the same lamb have similar lengths and the length of males, though on average shorter than females, has a strong and positive correlation with female worms within the same animal. Worm length is a good indicator of worm fecundity. Fecundity can be defined as the eggs per worm per gram of faeces. The relationship between fecundity and worm length is curvilinear and is consistent in both natural and deliberate infections. In both cases fecundity = $1.1(\text{worm length})^{0.4}$ (Stear et al 1999). Thus it appears that genetic resistance in lambs is working through an immunological response to the parasite, which controls worm growth and thus reduces worm fecundity.

For some time the dominant factor controlling fecundity has been assumed to be worm burden whether working through an immune mechanism or through competition for food. If worm burden is plotted against worm fecundity the resulting relationship is a declining exponential (Anderson & Michel 1977). However, such a graph is in fact plotting worm burden against its inverse multiplied by the egg count. The relationship between a number and its inverse is so strong that it may obscure the true biological relationship. Therefore because worm length is a good marker for worm fecundity for *T. circumcincta* it

seems more appropriate to look at the effects of worm burden on worm length. There is a significant correlation between worm length and worm burden ($p<0.001$) (Stear et al 1999). As worm burden increases, the average worm length decreases. However this correlation is relatively weak ($r=-0.23$) indicating that there is substantial residual variation not accounted for by this density-dependant effect. Because worm burden is not under genetic control in lambs some of the residual variation must be accounted for by an immune response that is heritable.

Only the IgA response to *T. circumcincta* has been consistently associated with reduced worm length. When results from four separate experiments involving 4.5 month old and 10 month old lambs experiments were pooled, a very strong correlation ($r=0.96$) between peak lymph IgA concentration and mean worm length was found (Smith et al 1985). A similar correlation ($r=0.95$) was found when the two experiments involving the younger lambs were pooled. However, correlations between groups can give misleading results due to the increased likelihood of finding spurious associations. For example, many immune responses are stronger in adults than in lambs and any of them could be correlated with mean worm length. It is statistically more reliable to look for correlations within groups. Previous work, which examined associations between various possible immune effector mechanisms and worm length, concluded that IgA directed against fourth-stage larvae was most strongly associated with reduced fecundity (Stear et al 1995). The correlation between mucus IgA and mean adult worm length was 0.62. The work described in chapter three was designed to examine the relationship between IgA and worm

length in a larger data set. It confirmed a strong association between levels of fourth-stage parasite specific IgA and worm length and therefore fecundity.

However, there appear to be a small number of sheep that though able to mount strong IgA responses are nonetheless unable to control worm length. It is not surprising that the specificity of the immune response is important in determining the effectiveness of the response to the parasite. The work described in chapter four indicates the wide variety of antigens recognised by lambs but the relatively few that are associated with resistance. Of a total of 99 bands from third-stage, fourth-stage and adult *T. circumcincta* only four were associated with resistance. For a lamb to be resistant it must produce a sufficiently strong IgA response with the necessary antigen specificity. Some lambs with strong IgA responses did not recognise those bands associated with resistance. When taken together the effects of worm burden, IgA quantity and specificity account for over 90% of the variation in worm length. It would appear that the fecundity of *T. circumcincta* in lambs is mediated through a relatively simple set of mechanisms.

However, statistical associations do not conclusively prove cause and effect link. The most extreme example of this objection came from the philosopher David Hume who described such associations as no more than "constant conjunctions:" we can observe that one thing follows another but it can never be asserted that it must follow. Put differently, the IgA response is consistently associated with reduced adult female worm length in lambs infected with *T. circumcincta.* This could simply mean that the IgA response is acting as a marker or is associated with a different mechanism which is itself the controlling mechanism. For example, sheep may be producing a substance that

stunts worms and also stimulates the production of IgA. In such a case IgA might be found to have a significant effect on worm length without it actually being the effector mechanism.

There is no certain way of refuting this objection to statistical associations, however a number of observations can be made. There appear to be three major factors that influence worm length; IgA quantity, IgA specificity, and the adult worm burden. Work described in chapter four suggests that taken together these account for over 90% of the variation in worm length seen. For another immune mechanism to be the effector mechanism it would have to be very strongly associated with IgA responses. To the best of the author's knowledge no such response is known. The actual effector mechanism would have to account for more of the variation than is being accounted for by the IgA response. It seems unlikely that such a mechanism could exist. Support for the view that the parasite-specific IgA response is the effector mechanism comes from the observation that plasma IgA is only a moderately good marker for mucosal IgA (Sinski et al 1995). This showed a weak though statistically significant correlation between plasma and mucus IgA ($p<0.001$ $r=0.48$-0.63). However, this study gave no evidence of a non-linear relationship between plasma and mucus IgA. Consequently, plasma IgA can be taken to be a reasonable gauge of mucosal IgA. However only 25% of the variation in the plasma IgA could be accounted for by variation in the mucus IgA. Therefore, the association between plasma IgA and worm length is likely to be an underestimate of the true relationship between mucus IgA and worm length.

More evidence of a role for IgA in immunity to *T. circumcincta* comes from work that involved the transfer of lymphocytes between sheep (Smith et al

1986). Here the local IgA response was transferred to previously unexposed sheep that were subsequently infected with *T. circumcincta*. The transferral of the mucosal IgA producing cells was associated with stunting of worms. Thus, a good working hypothesis is that worm fecundity is controlled by the local IgA response in lambs. To conclusively prove IgA as the effector mechanism would require transfer experiments where parasite-specific IgA was transferred to naively infected sheep and worm growth measured.

The parasite-specific IgA response was heritable (0.34). This estimated heritability is consistent with the hypothesis that genetic resistance to *T. circumcincta* is almost entirely a result of the parasite-specific IgA response. Thus it is feasible to breed sheep for increased IgA responsiveness. This heritability estimate did not take into account IgA specificity. If the antigens associated with resistance could be confirmed, then the heritability of the IgA response to these molecules would be expected to be higher. However, whether breeding sheep for increased IgA responsiveness is commercially viable is dependent on its genetic correlations with production traits used in current selection procedures. In naturally infected Scottish Blackface sheep, the genetic correlation between decreased faecal egg counts and liveweight gain is strongly negative (-0.85) (Stear et al 1996a). This suggests that those genes that influence resistance also influence growth rate and that selection for an increased IgA response to *T. circumcincta* is both feasible and desirable. Studies in Australia and New Zealand have found weaker associations between faecal egg counts and production traits but these may reflect different parasite species, different breeds of sheep, and different management systems (Eady 1998). Indeed breeding for resistance may have previously unforeseen benefits to the longer-term control of

this parasite. Resistant sheep will excrete fewer eggs. This will lead to a lower level of contamination and thus lower numbers of infective larvae. Therefore, selection for resistance would be expected to lead to year on year reductions in pasture contamination and so to a reduced degree of parasitism (Bishop & Stear 1997).

The means by which the IgA response stunts larvae remains unknown. It seems reasonable to speculate that the response in some way interferes with the parasite's feeding or metabolism of food. However, surprisingly little is known about the parasite's feeding, metabolism or development. It is the IgA response to fourth-stage larvae rather than to third-stage or adults that is most closely associated with decreased adult worm length but it is unclear what the mechanism for this is. It is also unclear whether the stunting of adult parasites is permanent or temporary. It could be that stunted fourth-stage larvae are slower at growing once they have moulted to adults. The details of this will only be elucidated when the target molecules are definitively identified and characterised.

One of the factors that contribute to the remarkable success of *T. circumcincta* is its ability to go into inhibition. Although it is known that the strain of parasite, previous larval chilling, previous exposure to parasite and the intensity of infection all contribute to inhibition, little is known about the mechanisms underlying it. Larval inhibition may be an extreme example of immune driven adult worm stunting. Because the IgA response is closely associated with adult worm stunting it is clearly a candidate mechanism for immune induced inhibition. Work described in chapter five described a significant association between the IgA response and larval inhibition in

deliberately infected lambs. Five bands recognised by antibody from third-stage, fourth-stage and adult *T. circumcincta* were significantly associated with numbers of inhibited larvae. However, although increased levels of fourth-stage specific IgA were associated with increased numbers of inhibited larvae, recognition of four bands was associated with decreased numbers of inhibited larvae while only one band on fourth-stage larvae was associated with increased numbers. How the recognition of certain molecules by IgA could prevent larval inhibition is uncertain. It could be that the antibody response is blocking messenger or receptor molecules that regulate parasite population development. In the free living nematode, *Caenorhabditis elegans,* larvae can be induced to go into the dauer phase under the influence of a pheromone (Wood 1988). A food signal (a carbohydrate like substance) produced by *E. coli* has an opposite effect, inhibiting dauer larva formation and enhancing recovery from the dauer phase. It could be that if such a signalling system was involved in the regulation of *T. circumcincta* populations then it could be interfered with by antibody. Clearly more work would need to be done to further identify and characterise such molecules.

A similar though non-significant association between parasite-specific IgA and increased numbers of fourth-stage larvae was found in naturally infected sheep. In this experiment, the fourth-stage larvae recovered would have consisted not only of inhibited or retarded larvae but also of recently acquired larvae. It may be that this contributed to the association being non-significant ($p=0.053$).

However, there was a significant effect of infection intensity on larval inhibition in both the naturally and deliberately infected animals. In the

deliberately infected animals where the number of adult worms was lower the effects of adult worm burden and the IgA response acted independently. It appears that at this level of infection the density of adults does not increase the number of inhibited larvae through the IgA response. However, at increased infection levels there is an interaction between the adult worm burden and the IgA response. It could be that in part the heavier antigenic load is provoking a greater antibody response. Nonetheless, only a small proportion of the effect of parasite burden on larval inhibition could be explained through the IgA response. Other possible mechanisms for these density-dependant effects could include the release of pheromones, or limited food resources.

It was of interest to investigate whether a similar mechanism might be at work with another abomasal parasite of sheep, *H. contortus*. Resistance to *H. contortus* is slow to develop and as with *T. circumcincta* there is no evidence that young animals are able to control worm burdens. A group of Hampshire Down lambs had previously been studied at Glasgow University Veterinary School to examine whether improving dietary protein could improve resilience to the effects of disease (Wallace et al 1995). This study showed that dietary supplementation ameliorated the effects of sub-clinical haemonchosis. Interestingly the only parasitological differences between the supplemented and non-supplemented groups were the faecal egg counts and the average length of worms. In the light of these findings, these animals were studied to determine whether parasite-specific IgA might be involved in controlling worm growth in this parasite. The results are described in chapter six and they show that there is a strong association between the parasite-specific IgA response and length of the

adult female parasite. This is consistent with the hypothesis that parasite-specific IgA may be involved in the control of the fecundity of *H. contortus* in lambs.

The only other parasites where a parasite-specific IgA response has been shown to be important in parasite regulation are schistosomes (Capron 1998). Here an IgA response has been associated with reduced parasite fecundity and egg viability (Grzych et al. 1993). In addition extensive work has indicated glutathione-s-transferase (GST) as a putative protective antigen. Recombinant *Salmonella typhimurium* expressing GST has been used to successfully immunise mice. This immunity was associated with a secretory IgA response (Capron 1998). Moreover, significant anti-fecundity effects have been demonstrated in all vaccine experiments performed with GSTs in all schistosome species, suggesting that GST has a central role to play in female worm fecundity. It would be of interest to investigate if the apparent anti-fecundity effect of IgA seen in *T. circumcincta* and *H. contortus* was associated with a similar molecule. However, the GST of schistosomes has a molecular weight of 28 kDa and no such band of a similar molecular weight was associated with reduced fecundity in the present study. This does not rule out a role for a GST-like molecule in these species but this cannot be confirmed until the protective antigens are more fully characterised.

Protein supplementation significantly improved the IgA response to *H. contortus*. This has a number of important implications for the control of parasites. It gives further support to the view that for an animal to mount an adequate immune response it must be on an adequate diet. Hampshire Down sheep were studied in this experiment because they are particularly susceptible to haemonchosis (Loggins et al 1965; Preston & Allonby 1979). This susceptibility

can in part be ameliorated by dietary supplementation. However, the adequacy of the diet for a susceptible breed may differ from that required for a more resistant breed which is mounting a better immune response to the parasite under the same conditions. Different breeds clearly utilise dietary intake differently leading to different growth rates. It could be that a fast growing breed needs more food intake to raise an adequate immune response. Thus advice concerning the adequacy of diets in terms of growth rate and immune responsiveness would need to be tailored to the individual breed and take into account the type and degree of parasitism the animal is subjected to.

Virtually all of the parasitological differences between the two dietary groups could be accounted for in a statistical sense by the differences in the IgA responses between the two groups. This may have important implications for how dietary supplementation affects the course of infections. Dietary supplementation may help infected animals by offsetting the nutritional losses caused by the parasite or by improving the immune response to the parasite or both. If the IgA response interferes with the feeding of the parasite then the parasite will be less pathogenic than if no such response was being mounted. If dietary supplementation improves the IgA response then it would be expected that the effect of this on the parasite population would be to make it less pathogenic by slowing its feeding. Normally the degree of parasitism of an animal is related to the worm burden in that animal. It may be that this assumption needs to be revisited and that the pathogenic effect of an infection is more closely related to total parasite mass. Put differently, shorter worms appear to be less pathogenic than longer ones. For example, it is plausible to envisage a situation where two animals might have the same parasite burden. However, if

one of them had a greater IgA response the average worm length in that animal would be shorter and the pathogenic effects of the infection would be less.

Interestingly, there appear to be substantial differences between young and older animals in their ability to control both *T. circumcincta* and *H. contortus*. Older animals tend to have substantially lower faecal egg counts than younger ones. This appears to be a consequence of their greater ability to control worm burden. There is substantial evidence that mast cell degranulation is associated with reduction in worm burdens in older sheep infected with *T. circumcincta* or *H. contortus*. Previous studies have shown that although there was not a significant association between worm burden and the numbers of mucosal mast cells in sheep infected with *T. circumcincta,* there was a significant association with globule leucocytes (Stear et al 1995; Seaton et al 1989). It also appears that the number of these cells recovered is related to the parasite burden (Huntley et al 1982). As there is considerable evidence that globule leucocytes are discharged mast cells (Murray et al 1968; Huntley et al 1984) these findings support the hypothesis that mast cell degranulation is the mechanism for expelling established or incoming larvae through immediate type hypersensitivity responses (Rothwell 1989). Similarly, sheep bred from a resistant sire and infected with *H. contortus* had fewer worms and more mast cells and globule leucocytes than randomly bred sheep (Gill 1991). Also, lambs of the more resistant St. Croix breed have lower worm burdens and greater numbers of globule leucocytes than the more susceptible Dorset breed (Gamble & Zajac 1992). Therefore, it appears that immunity to both *T. circumcincta* and *H. contortus* develops in two stages. Young animals are able to mount antibody responses to gastrointestinal nematodes that in resistant animals infected with *T.*

circumcincta or *H. contortus* causes stunting. As the animals mature, they develop the ability to control worm burdens and this appears to work through an immediate type hypersensitivity reaction.

Although it is interesting to find the same mechanism at work in both *T. circumcincta* and *H. contortus* care needs to be exercised in drawing conclusions to other parasites. Work with laboratory models indicates that generalisations from one parasite species to another cannot be made. Different parasites are controlled by different immune responses. An antibody response in young animals may have the effect of stunting in one species of parasite but expulsion or immune exclusion in another. Therefore, findings that indicate that an IgA response to *T. circumcincta* or *H. contortus* inhibits worm development and growth does not indicate that such a response would have the same or any effect on a different gastrointestinal parasite. For example, there is good evidence to suggest that some young lambs are able to control the number of *T. colubriformis*. Following vaccination with irradiated *T. colubriformis* larvae and then deliberate infection, lambs could be segregated into low-responder and high-responder groups. High-responder groups had lower faecal egg counts and lower worm burdens (Windon et al 1980).

It is of interest to speculate on why lambs appear incapable of controlling *T. circumcincta* and *H. contortus* worm burdens. Clearly adult animals are capable of expelling adults although they never achieve sterile immunity. Expulsion appears to be associated with mast cell degranulation. Although young lambs do have mucosal mast cells they appear incapable of controlling worm burdens. It is generally recognised that mast cell degranulation is potentially detrimental to the host as it allows damage to the mucosa and leakage

of proteins into the gut. It may be that in young lambs the cost of an adult type mast cell degranulation is too great and would significantly delay the growth and therefore the sexual maturity of the lamb. Perhaps only more mature animals can 'afford' the cost of widespread mast cell degranulation.

There may be a similar evolutionary reason why *T. circumcincta* infection causes such a surprisingly large protein loss in the host. Clearly, the degree of protein loss far outweighs the nutritional demands of the parasite. In causing a relative protein deficiency the parasite is slowing the development of the host. In so doing not only does it reduce the antibody responses against it but it may be maximising the time before the host is capable of mast cell degranulation and therefore expulsion.

These speculations illustrate the need for further work to elucidate the interactions between the host and each of these parasites. The role of IgA in the control of worm fecundity will need to be further defined. In the case of *T. circumcincta* work needs to be done to investigate whether a similar mechanism is involved in resistance in other breeds of sheep and the degree of variation in worm length that can be attributed to it. This is of particular importance in the further selection of sheep for resistance. It may be that there is a strong and favourable association between the IgA response and liveweight gain. Certainly the genetic correlation between nematode egg counts in sheep predominately infected with *T. circumcincta* and liveweight gain is –0.8 (Bishop el al 1996a). This indicates that the most important genes for growth rate in grazing lambs are those that control parasite growth. Those breeds that have been most intensively selected for increased growth may have been inadvertently selected for increased IgA production. Thus there may be limited scope for further selection in some

breeds. This will be dependent upon whether such animals were selected under heavy or light parasite challenge. Under heavy parasite challenge it could be assumed that those animals that grew quickest were those that controlled the parasite best. However under limited challenge those animals selected for increased productivity may have an increased susceptibility to disease as they direct more of their nutritional resources toward liveweight gain as opposed to an effective immune response.

An interesting corollary of the strong genetic correlation between liveweight and nematode egg counts is that it may be better for lambs to control worm growth than worm numbers. Put another way worm growth may be more closely associated with pathogenicity than worm numbers. This is supported by the finding that increased plasma pepsinogen is associated with worm size ($p<0.001$) rather than with worm burden ($p>0.05$) (Stear and Bishop 1999). As increased plasma pepsinogen is regarded as an indicator of the severity of infection this would seem to indicate that it is in the lambs best interests to control worm growth.

Those molecules on *T. circumcincta* that are recognised by resistant animals will need to be confirmed on a larger data set to avoid spurious statistical associations with resistance. Similarly, those molecules on *H. contortus* associated with resistance remain to be identified. In addition, knowledge concerning the function of these molecules would help to identify how resistance is working. Such work would not only help in developing better assays for the identification of resistant animals but would also help in the development of specifically targeted drugs. Indeed much remains to be discovered concerning the basic biology of these parasites. Nothing is known at the molecular level

about the mechanisms underlying inhibition. If inhibition is regulated by pheromones, as chapter five suggests, then these would be obvious targets for the development of vaccines or drugs.

Based on the findings of this thesis, a number of areas require futher work. More work is required to answer remaining questions over the role of IgA and parasite control. Specifically further studies will be required to confirm the role of IgA and the mechanisms of its action on the two parasite species studied. These would involve further studies of large numbers of sheep of different breeds kept under differing conditions to identify whether IgA was involved generally in resistance. It would be informative to quantify the IgA responses as the present work measured only optical density indices. To conclusively indicate a role for IgA would require passive transfer experiments of recently infected sheep.

In order to confirm which antigens are important in genetic resistance, studies involving larger numbers of animals will be required to avoid possible spurious statistical associations between recognition of an antigen and resistance. Candidate resistance antigens will then need to be biochemically characterised and their position within the parasites identified through the development of monoclonal antibodies to the antigens and immunohistochemistry on the parasites. Ultimately experimental vaccination may be attempted with the candidate vaccines although the present work is based on the observation that some animals are unable to mount effective immune responses. To take this into account will require work to identify those genes important in determining antigen recognition.

Finally for this work to be of practical use in the development of new strategies for the control of these parasites field studies will need to set up

looking at ways of incorporating genetic resistance perhaps as measured by IgA responses to whole parasite somatic extract or to the relevant antigens with the normal selection criteria used for breeding programmes.

In conclusion, the present work suggests that IgA is the major mechanism of resistance to *T. circumcincta* in lambs and has identified several molecules that may be targets for this immune response. In addition, the IgA response is one possible mechanism for the development of larval inhibition in resistant animals and several molecules have been identified associated with this. Also, the IgA response appears also to play a role in resistance to *H. contortus*. In this case the IgA response is greatly influenced by protein nutrition. This finding may have important implications in the control of this parasite as sufficient nutrition may overcome to some extent genetic susceptibility.

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Appendix 1. Abomasal digest solution

300ml HCl concentrate

100g Pepsin A powder

Make up to 10 litres with dd H_2O

Appendix 2. Mixture for freezing down hybridoma cells

10% DMSO

20% Foetal Bovine Serum

70% RPMI 1640 cell culture medium

Appendix 3. Solutions used for gel electrophoresis

4 x Running Gel Buffer (1.5M Tris-Cl pH 8.8)

36.3g Tris (FW 121.1) made up to 150ml with dd H_2O.

pH is adjusted to 8.8 with concentrated HCl and then made up to a total volume of 200ml with dd H_2O.

4 x Stacking Gel Buffer (0.5 M Tris-Cl pH 6.8)

3.0g Tris (FW 121.1) made up to 40 ml with dd H_2O

pH is adjusted to 6.8 with concentrated HCl and then made up to a total volume of 50ml with dd H_2O.

10% SDS

10g SDS made up to 100ml with dd H_2O.

Running Gel Overlay

1.0ml of 10% SDS

25ml running gel buffer

Solution is made up to 100ml with dd H_2O

2 x Treatment Buffer (0.125 M Tris-Cl, 4% SDS, 20% v/v Glycerol, 0.2M Dithiotreitol, 0.02% Bromophenol Blue pH 6.8).

2.5ml 4 x Stacking gel buffer

4.0ml 10% SDS

2.0ml glycerol

2.0mg bromophenol blue

0.31g Dithiothreitol (FW 154.2)

Solution made up to 10ml with ddH_2O.

Electrophoresis Buffer (0,025 M Tris, 0,192 M Glycine, 0.1% SDS, pH 8.3)

30.28g Tris (FW 121.1)

144.13g glycine

10g SDS H_2O

Solution made up to 10l with ddH_2O.

Western Blot Buffer

1.93g Tris base (15.5mM)

9g Glycine (120mM)

Solution made up to 1l with ddH_2O. (pH without adjustment 8.1-8.4)

Appendix 4 Ingredients for gels.

7.5% Gel

7.5ml Monomer solution.

7.5ml 4 x Running gel buffer.

0.3ml 10% SDS.

14.6ml dd H_2O.

15 µl 10% Ammonium persulfate.

10µl *N,N,N',N'*-TEMED.

12.5% Gel

12.5ml Monomer solution.

7.5ml 4 x Running gel buffer.

0.3ml 10% SDS.

9.6ml dd H_2O.

15 µl 10% Ammonium persulfate.

10µl *N,N,N',N'*-TEMED.

Stacking Gel (4% acrylimide)

1.33ml Mnomer solution.

2.5ml Stacking gel buffer.

0.1ml 10% SDS

6.0ml dd H_2O

50 µl 10% Ammonium persulfate.

5µl *N,N,N',N'*-TEMED.

Appendix 5. Optical densities for IgA directed against fourth-stage *T. circumcincta.*

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects Coefficient of Variation
1 (95)	425	0.185	0.148
2 (95)	287.5	0.406	0.070
3 (95)	375	0.254	0.097
4 (95)	250	0.230	0.051
5 (95)	337.5	0.205	0.084
6 (95)	1100	0.495	0.049
7 (95)	200	0.099	0.095
8 (95)	362.5	0.534	0.064
9 (95)	212.5	0.071	0.051
10 (95)	550	0.467	0.014
11 (95)	887.5	0.422	0.006
12 (95)	250	0.316	0.028
13 (95)	200	0.166	0.049
14 (95)	212.5	0.900	0.077
15 (95)	75	0.313	0.059
16 (95)	487.5	0.696	0.029
17 (95)	25	0.212	0.028
18 (95)	100	0.208	0.039
19 (95)	100	0.801	0.025
20 (95)	62.5	0.221	0.013
21 (95)	87.5	0.226	0.020
22 (95)	700	1.194	0.015
23 (95)	362.5	0.208	0.019
24 (95)	50	0.131	0.015
25 (95)	62.5	0.534	0.029
26 (95)	0	0.486	0.056
27 (95)	137.5	0.270	0.088
28 (95)	75	0.698	0.099
29 (95)	50	0.294	0.073
30 (95)	50	0.274	0.066
Positive Control		0.560	0.061
Negative Control		0.043	0.075

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
31 (95)	287.5	0.692	0.067
32 (95)	112.5	0.373	0.067
33 (95)	187.5	0.108	0.164
34 (95)	637.5	0.096	0.229
35 (95)	187.5	0.349	0.070
36 (95)	362.5	0.384	0.064
37 (95)	125	0.507	0.047
38 (95)	100	0.958	0.036
39 (95)	425	0.429	0.043
40 (95)	100	0.249	0.018
41 (95)	400	0.974	0.014
42 (95)	75	0.263	0.027
43 (95)	575	0.437	0.011
44 (95)	212.5	0.355	0.150
45 (95)	912.5	0.217	0.019
46 (95)	312.5	0.321	0.034
47 (95)	925	0.845	0.038
48 (95)	50	0.270	0.020
49 (95)	337.5	0.517	0.052
50 (95)	25	0.975	0.012
52 (95)	50	0.122	0.066
53 (95)	300	0.089	0.026
54 (95)	200	0.201	0.092
55 (95)	212.5	0.299	0.021
57 (95)	312.5	0.260	0.081
58 (95)	237.5	0.080	0.144
59 (95)	25	0.851	0.105
60 (95)	225	0.128	0.098
61 (95)	350	0.608	0.081
Positive Control		0.641	0.049
Negative Control		0.053	0.050

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
62 (95)	612.5	0.356	0.010
63 (95)	212.5	0.696	0.071
64 (95)	37.5	0.578	0.059
65 (95)	125	0.508	0.051
66 (95)	437.5	1.271	0.027
67 (95)	87.5	2.145	0.006
68 (95)	87.5	0.550	0.021
69 (95)	262.5	0.691	0.033
71 (95)	375	0.310	0.037
72 (95)	562.5	0.723	0.036
73 (95)	200	1.529	0.054
74 (95)	350	1.124	0.037
75 (95)	937.5	0.910	0.033
76 (95)	200	0.908	0.012
77 (95)	562.5	0.335	0.088
78 (95)	500	0.635	0.021
80 (95)	87.5	0.278	0.034
81 (95)	412.5	0.803	0.049
82 (95)	137.5	1.076	0.051
83 (95)	162.5	0.814	0.028
84 (95)	825	0.861	0.012
85 (95)	412.5	0.823	0.014
86 (95)	550	0.828	0.014
87 (95)	100	1.585	0.028
88 (95)	437.5	2.558	0.016
89 (95)	550	1.349	0.068
90 (95)	212.5	0.524	0.070
93 (95)	512.5	0.196	0.013
94 (95)	62.5	0.948	0.028
Positive Control		1.043	0.017
Negative Control		0.092	0.031

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
95 (95)	137.5	2.098	0.032
96 (95)	200	1.901	0.015
97 (95)	875	0.649	0.004
98 (95)	275	1.019	0.038
100 (95)	437.5	1.135	0.029
101 (95)	300	0.622	0.034
102 (95)	725	2.131	0.012
103 (95)	75	0.867	0.015
104 (95)	375	0.391	0.030
107 (95)	737.5	0.373	0.051
108 (95)	575	0.996	0.024
109 (95)	362.5	0.744	0.007
112 (95)	87.5	1.254	0.021
113 (95)	112.5	0.315	0.029
114 (95)	650	0.434	0.065
115 (95)	200	1.605	0.053
116 (95)	437.5	0.394	0.034
117 (95)	250	0.753	0.014
118 (95)	300	0.512	0.041
119 (95)	200	0.719	0.047
121 (95)	25	0.797	0.041
122 (95)	37.5	1.418	0.008
123 (95)	200	1.685	0.054
124 (95)	200	0.974	0.066
126 (95)	162.5	2.160	0.014
127 (95)	550	0.541	0.067
128 (95)	762.5	0.325	0.056
129 (95)	175	1.023	0.066
130 (95)	362.5	0.921	0.049
Positive Control		1.000	0.013
Negative Control		0.090	0.104

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
131 (95)	125	0.346	0.116
132 (95)	12.5	0.335	0.100
133 (95)	337.5	0.074	0.289
134 (95)	425	0.225	0.081
135 (95)	575	0.135	0.094
136 (95)	575	0.176	0.066
137 (95)	350	0.309	0.076
138 (95)	600	0.940	0.057
139 (95)	75	0.763	0.036
140 (95)	112.5	1.167	0.003
141 (95)	200	0.440	0.031
142 (95)	175	0.624	0.034
143 (95)	250	0.360	0.050
144 (95)	512.5	0.455	0.039
145 (95)	250	0.686	0.021
147 (95)	162.5	0.573	0.034
149 (95)	450	0.419	0.039
150 (95)	237.5	1.060	0.018
152 (95)	800	0.695	0.040
153 (95)	650	0.288	0.020
154 (95)	87.5	0.396	0.019
155 (95)	175	0.973	0.023
156 (95)	237.5	1.453	0.012
157 (95)	112.5	0.330	0.042
158 (95)	375	0.543	0.018
159 (95)	1475	0.606	0.070
160 (95)	125	0.864	0.004
161 (95)	325	0.265	0.010
Positive Control		0.673	0.011
Negative Control		0.059	0.081

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
162 (95)	775	0.741	0.064
163 (95)	750	0.244	0.101
164 (95)	112.5	0.248	0.083
165 (95)	437.5	0.521	0.077
166 (95)	2350	0.539	0.014
167 (95)	612.5	0.184	0.114
168 (95)	187.5	0.251	0.094
169 (95)	362.5	0.304	0.085
170 (95)	212.5	0.330	0.119
171 (95)	487.5	0.105	0.083
172 (95)	587.5	0.135	0.065
173 (95)	337.5	0.306	0.019
175 (95)	275	0.539	0.013
177 (95)	412.5	0.144	0.052
179 (95)	300	0.238	0.037
180 (95)	275	0.389	0.068
181 (95)	62.5	0.503	0.003
182 (95)	87.5	0.203	0.038
183 (95)	87.5	0.592	0.016
184 (95)	300	0.227	0.024
185 (95)	100	0.866	0.026
186 (95)	162.5	0.425	0.027
187 (95)	137.5	0.645	0.021
189 (95)	600	0.771	0.027
190 (95)	137.5	0.540	0.026
192 (95)	37.5	0.553	0.093
193 (95)	550	0.094	0.063
195 (95)	512.5	1.628	0.053
Positive Control		0.737	0.044
Negative Control		0.059	0.068

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
196 (95)	437.5	0.726	0.056
197 (95)	212.5	0.426	0.039
198 (95)	412.5	0.457	0.088
199 (95)	775	0.691	0.049
200 (95)	812.5	0.740	0.040
Positive Control		0.811	0.066
Negative Control		0.092	0.073

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
1 (96)	412.5	0.393	0.065
2 (96)	162.5	0.427	0.054
3 (96)	0	1.348	0.020
4 (96)	100	0.338	0.027
5 (96)	450	0.195	0.031
6 (96)	250	0.947	0.009
7 (96)	387.5	0.290	0.027
8 (96)	37.5	0.277	0.006
9 (96)	562.5	0.489	0.020
10 (96)	150	0.492	0.004
11 (96)	50	0.813	0.048
12 (96)	325	1.429	0.041
13 (96)	387.5	0.365	0.029
14 (96)	375	0.210	0.069
15 (96)	112.5	0.245	0.015
16 (96)	87.5	0.445	0.017
17 (96)	1075	0.284	0.032
19 (96)	650	1.384	0.009
20 (96)	362.5	0.174	0.047
21 (96)	825	0.307	0.008
22 (96)	300	0.177	0.054
23 (96)	737.5	0.807	0.013
Positive Control		0.811	0.066
Negative Control		0.092	0.073

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
25 (96)	12.5	0.593	0.040
26 (96)	325	0.463	0.019
27 (96)	1025	0.733	0.038
28 (96)	25	1.478	0.044
29 (96)	550	0.854	0.042
30 (96)	550	0.213	0.075
32 (96)	87.5	0.689	0.044
33 (96)	225	0.304	0.037
34 (96)	137.5	0.743	0.015
35 (96)	1662.5	0.553	0.025
36 (96)	475	0.283	0.023
37 (96)	150	0.531	0.025
38 (96)	637.5	0.600	0.038
39 (96)	25	0.614	0.038
40 (96)	287.5	0.597	0.013
41 (96)	112.5	0.270	0.024
42 (96)	175	0.715	0.016
43 (96)	312.5	0.137	0.057
44 (96)	450	0.403	0.007
46 (96)	500	1.220	0.013
47 (96)	650	0.457	0.056
48 (96)	462.5	0.891	0.026
50 (96)	2300	0.370	0.014
51 (96)	812.5	0.348	0.031
53 (96)	412.5	0.209	0.048
54 (96)	475	0.891	0.043
55 (96)	2200	1.194	0.025
56 (96)	187.5	0.305	0.008
57 (96)	925	1.055	0.013
Positive Control		0.778	0.014
Negative Control		0.092	0.076

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
58 (96)	1087.5	1.201	0.049
59 (96)	1175	1.148	0.052
60 (96)	312.5	0.344	0.108
61 (96)	262.5	0.556	0.063
62 (96)	337.5	0.709	0.071
64 (96)	875	0.776	0.067
65 (96)	312.5	1.879	0.011
66 (96)	975	0.262	0.064
67 (96)	350	0.624	0.041
68 (96)	287.5	0.619	0.067
69 (96)	1300	0.176	0.031
71 (96)	650	0.647	0.046
72 (96)	0	0.672	0.020
73 (96)	925	0.186	0.026
74 (96)	125	0.459	0.036
75 (96)	1625	0.613	0.041
76 (96)	150	0.677	0.030
78 (96)	150	0.969	0.030
79 (96)	187.5	0.479	0.021
80 (96)	200	0.215	0.022
81 (96)	25	1.012	0.004
82 (96)	412.5	0.564	0.042
83 (96)	612.5	0.187	0.031
84 (96)	237.5	1.052	0.020
85 (96)	1012.5	1.903	0.025
86 (96)	450	1.534	0.035
87 (96)	187.5	0.740	0.056
88 (96)	50	0.424	0.036
89 (96)	212.5	0.296	0.038
90 (96)	812.5	0.286	0.070
Positive Control		0.685	0.033
Negative Control		0.076	0.118

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
91 (96)	112.5	0.629	0.076
92 (96)	275	1.360	0.040
93 (96)	550	0.747	0.041
94 (96)	1200	0.388	0.087
95 (96)	387.5	0.835	0.015
96 (96)	687.5	1.057	0.023
97 (96)	1087.5	0.278	0.049
98 (96)	450	0.549	0.044
99 (96)	75	0.966	0.013
100 (96)	300	1.103	0.030
101 (96)	650	0.372	0.008
102 (96)	525	0.116	0.040
103 (96)	400	0.370	0.027
104 (96)	150	1.659	0.004
105 (96)	437.5	0.280	0.007
106 (96)	1450	0.516	0.017
108 (96)	175	0.341	0.045
109 (96)	562.5	0.240	0.031
110 (96)	975	0.825	0.014
111 (96)	1225	0.156	0.061
113 (96)	200	0.384	0.052
116 (96)	650	0.902	0.054
117 (96)	275	0.310	0.007
118 (96)	150	0.225	0.045
119 (96)	375	0.319	0.036
120 (96)	62.5	0.658	0.054
121 (96)	87.5	0.635	0.065
Positive Control		0.765	0.046
Negative Control		0.087	0.020

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
122 (96)	825	0.804	0.024
124 (96)	475	0.930	0.044
125 (96)	250	0.244	0.085
126 (96)	37.5	0.717	0.080
127 (96)	487.5	0.297	0.025
130 (96)	300	0.298	0.083
132 (96)	250	0.420	0.031
134 (96)	387.5	0.351	0.044
135 (96)	250	0.323	0.088
136 (96)	687.5	0.846	0.054
137 (96)	1300	1.081	0.048
138 (96)	500	0.841	0.046
139 (96)	562.5	0.573	0.041
140 (96)	237.5	0.348	0.047
141 (96)	775	0.852	0.023
142 (96)	525	0.171	0.024
143 (96)	175	0.282	0.046
144 (96)	1200	0.401	0.047
145 (96)	925	0.942	0.029
146 (96)	425	0.495	0.053
147 (96)	87.5	0.571	0.039
148 (96)	437.5	0.181	0.039
150 (96)	437.5	0.115	0.041
151 (96)	162.5	0.747	0.008
152 (96)	300	0.688	0.059
155 (96)	1337.5	0.662	0.041
156 (96)	25	0.591	0.003
Positive Control		0.793	0.023
Negative Control		0.069	0.075

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
157 (96)	425	0.139	0.148
158 (96)	550	0.497	0.054
159 (96)	612.5	0.268	0.035
160 (96)	637.5	0.170	0.060
161 (96)	1050	1.183	0.020
162 (96)	537.5	1.031	0.027
163 (96)	612.5	0.863	0.048
164 (96)	12.5	0.400	0.020
165 (96)	500	0.219	0.025
166 (96)	212.5	1.589	0.011
168 (96)	225	0.474	0.013
169 (96)	1537.5	0.276	0.034
170 (96)	312.5	0.631	0.038
171 (96)	900	0.916	0.003
172 (96)	150	0.804	0.081
173 (96)	250	1.251	0.020
174 (96)	162.5	1.267	0.005
175 (96)	525	0.211	0.020
177 (96)	150	0.212	0.096
178 (96)	562.5	0.526	0.029
179 (96)	612.5	1.013	0.018
180 (96)	62.5	0.488	0.039
181 (96)	0	1.006	0.010
182 (96)	112.5	1.022	0.039
183 (96)	712.5	0.427	0.070
184 (96)	675	1.635	0.025
185 (96)	337.5	0.864	0.067
186 (96)	87.5	0.474	0.041
187 (96)	775	0.407	0.031
Positive Control		0.823	0.048
Negative Control		0.080	0.044

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
189 (96)	362.5	0.152	0.162
190 (96)	175	0.443	0.049
191 (96)	137.5	0.960	0.035
192 (96)	900	0.997	0.042
193 (96)	312.5	0.708	0.070
194 (96)	25	1.008	0.057
196 (96)	1612.5	0.369	0.122
197 (96)	475	1.240	0.049
198 (96)	462.5	2.070	0.007
199 (96)	37.5	0.815	0.013
200 (96)	62.5	1.091	0.011
Positive Control		0.655	0.034
Negative Control		0.076	0.066

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
1 (93)	25	0.162	0.121
2 (93)	475	0.660	0.023
3 (93)	300	0.787	0.009
4 (93)	300	2.348	0.006
5 (93)	400	1.975	0.012
6 (93)	0	1.518	0.019
7 (93)	100	1.028	0.036
9 (93)	100	2.167	0.008
10 (93)	325	0.525	0.027
11 (93)	25	1.368	0.027
12 (93)	25	0.252	0.034
13 (93)	0	0.952	0.045
14 (93)	150	1.281	0.004
Positive Control		0.655	0.034
Negative Control		0.076	0.066

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
15 (93)	125	0.260	0.158
16 (93)	75	1.146	0.052
17 (93)	125	0.175	0.094
18 (93)	0	0.149	0.083
19 (93)	275	0.506	0.108
20 (93)	25	0.377	0.090
21 (93)	275	0.407	0.142
22 (93)	300	0.521	0.104
23 (93)	450	0.985	0.038
24 (93)	75	1.705	0.034
25 (93)	225	1.102	0.022
26 (93)	50	0.490	0.010
27 (93)	25	0.175	0.056
29 (93)	0	0.881	0.038
30 (93)	0	0.690	0.061
31 (93)	75	1.693	0.015
32 (93)	50	0.403	0.014
33 (93)	450	0.567	0.013
34 (93)	0	1.122	0.013
35 (93)	0	1.812	0.012
36 (93)	0	1.098	0.055
37 (93)	75	1.922	0.007
38 (93)	275	0.883	0.014
39 (93)	100	1.214	0.025
42 (93)	50	0.644	0.012
43 (93)	50	0.944	0.031
44 (93)	250	1.115	0.045
45 (93)	500	0.326	0.060
46 (93)	150	0.403	0.047
Positive Control		0.644	0.012
Negative Control		0.071	0.036

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
47 (93)	25	1.247	0.028
48 (93)	550	1.269	0.026
50 (93)	0	0.816	0.019
51 (93)	700	1.850	0.013
52 (93)	600	1.809	0.008
53 (93)	100	1.008	0.040
55 (93)	175	0.794	0.076
56 (93)	250	0.808	0.064
57 (93)	600	0.814	0.037
58 (93)	350	0.829	0.031
59 (93)	0	0.870	0.005
60 (93)	25	1.863	0.032
61 (93)	0	1.674	0.041
62 (93)	25	0.842	0.027
63 (93)	50	1.366	0.014
64 (93)	75	1.820	0.026
65 (93)	0	0.605	0.052
66 (93)	25	0.888	0.055
67 (93)	25	0.888	0.014
68 (93)	150	0.410	0.059
69 (93)	0	1.416	0.022
70 (93)	0	1.338	0.006
72 (93)	100	1.425	0.026
73 (93)	75	1.331	0.008
74 (93)	50	1.272	0.020
75 (93)	50	0.574	0.051
76 (93)	250	0.194	0.057
77 (93)	0	1.012	0.024
78 (93)	125	1.788	0.029
79 (93)	75	0.299	0.034
Positive Control		0.836	0.021
Negative Control		0.073	0.060

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
80 (93)	50	0.318	0.128
82 (93)	25	0.842	0.027
83 (93)	100	0.684	0.072
84 (93)	800	0.465	0.078
85 (93)	175	0.874	0.054
86 (93)	225	0.548	0.054
87 (93)	50	0.695	0.091
88 (93)	125	0.999	0.060
90 (93)	825	1.064	0.015
92 (93)	325	0.153	0.004
93 (93)	550	0.416	0.030
94 (93)	125	0.925	0.023
95 (93)	75	0.736	0.068
96 (93)	125	0.773	0.016
97 (93)	50	1.114	0.032
98 (93)	450	0.478	0.027
99 (93)	75	0.534	0.030
100 (93)	25	1.592	0.037
Y1 (93)	150	1.038	0.018
Y2 (93)	125	0.519	0.006
Y3 (93)	600	0.740	0.019
Y4 (93)	100	1.212	0.024
Y5 (93)	100	0.817	0.008
Y6 (93)	0	1.847	0.004
Y7 (93)	100	0.593	0.053
Y8 (93)	50	0.207	0.066
Y9 (93)	100	0.875	0.015
Y12 (93)	50	0.257	0.071
Y13 (93)	50	2.118	0.018
Positive Control		0.800	0.051
Negative Control		0.081	0.086

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
Y14 (93)	500	1.164	0.042
Y15 (93)	0	2.116	0.009
Y17 (93)	50	1.291	0.039
Y18 (93)	200	0.150	0.112
Y19 (93)	600	0.458	0.073
Y20 (93)	75	1.068	0.062
Y21 (93)	475	0.087	0.183
Y22 (93)	0	1.307	0.031
Y23 (93)	50	1.358	0.104
Y24 (93)	100	0.204	0.033
Y26 (93)	225	0.333	0.008
Y27 (93)	550	1.070	0.023
Y28 (93)	1050	0.457	0.043
Y30 (93)	1875	0.388	0.052
Y31 (93)	375	0.982	0.011
Y32 (93)	75	0.750	0.007
Y33 (93)	625	0.310	0.040
Y35 (93)	0	0.587	0.040
Y36 (93)	0	0.690	0.031
Y37 (93)	200	0.400	0.031
Y38 (93)	0	0.137	0.015
Y39 (93)	50	1.645	0.022
Y40 (93)	175	0.233	0.014
Y41 (93)	375	0.482	0.015
Y42 (93)	975	0.801	0.019
Y43 (93)	575	0.289	0.031
Y44 (93)	325	0.127	0.048
Y45 (93)	100	0.298	0.092
Y46 (93)	400	1.115	0.022
Positive Control		0.623	0.040
Negative Control		0.088	0.020

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
Y47 (93)	425	0.286	0.049
Y48 (93)	25	1.314	0.022
Y50 (93)	250	0.404	0.053
Y51 (93)	525	0.780	0.020
Y52 (93)	75	0.983	0.026
Y53 (93)	225	0.998	0.008
Y54 (93)	175	0.645	0.006
Y55 (93)	125	0.785	0.031
Y56 (93)	100	1.503	0.056
Y57 (93)	525	0.353	0.010
Y59 (93)	25	0.385	0.004
Y60 (93)	350	1.528	0.019
B47 (93)	75	1.081	0.015
B48 (93)	425	1.426	0.005
B49 (93)	0	1.480	0.066
B50 (93)	150	1.521	0.010
B51 (93)	50	0.760	0.038
B52 (93)	0	1.326	0.032
B54 (93)	0	2.103	0.017
B55 (93)	125	0.542	0.025
B56 (93)	200	0.474	0.033
B57 (93)	375	1.028	0.003
B58 (93)	325	0.681	0.032
B60 (93)	475	0.783	0.071
B63 (93)	100	1.228	0.034
B64 (93)	80	1.329	0.027
B65 (93)	325	0.141	0.042
Positive Control		0.809	0.017
Negative Control		0.074	0.061

Sample Number	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
B66 (93)	25	0.428	0.056
B67 (93)	25	1.385	0.045
B69 (93)	25	1.037	0.032
B70 (93)	325	0.348	0.008
B71 (93)	0	1.595	0.025
B72 (93)	150	0.710	0.051
B73 (93)	50	1.870	0.059
B74 (93)	300	0.281	0.067
B75 (93)	50	2.563	0.001
B76 (93)	0	0.661	0.020
B77 (93)	0	1.397	0.028
B78 (93)	25	2.466	0.007
B79 (93)	175	0.328	0.022
B80 (93)	75	0.835	0.010
B81 (93)	325	0.326	0.021
B82 (93)	100	1.073	0.013
B83 (93)	75	1.682	0.020
B84 (93)	200	0.433	0.042
B85 (93)	175	1.726	0.030
B87 (93)	25	2.004	0.051
B88 (93)	150	1.326	0.013
B89 (93)	125	2.175	0.009
B90 (93)	200	0.971	0.026
B91 (93)	175	0.209	0.010
B92 (93)	100	1.246	0.007
B93 (93)	125	0.243	0.025
B94 (93)	25	0.217	0.115
B95 (93)	25	1.497	0.019
B96 (93)	75	1.201	0.059
Positive Control		0.730	0.049
Negative Control		0.065	0.023

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
B97 (93)	600	0.942	0.023
B98 (93)	75	0.787	0.026
Positive Control		0.579	0.047
Negative Control		0.047	0.074

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
B57 (94)	150	0.381	0.038
B58 (94)	0	0.387	0.061
B59 (94)	0	0.245	0.040
B60 (94)	50	0.124	0.014
B61 (94)	200	0.109	0.054
B63 (94)	0	1.034	0.047
B64 (94)	0	0.443	0.106
B65 (94)	350	0.156	0.026
B66 (94)	50	0.079	0.022
B67 (94)	50	1.279	0.048
B68 (94)	0	0.219	0.072
B69 (94)	0	0.522	0.064
B71 (94)	250	0.083	0.071
B72 (94)	100	0.739	0.055
B75 (94)	150	1.331	0.049
B76 (94)	300	0.608	0.016
B78 (94)	300	0.303	0.074
B79 (94)	0	0.771	0.064
B80 (94)	100	0.604	0.073
B81 (94)	100	0.203	0.079
B82 (94)	100	0.125	0.068
Y124 (94)	0	0.720	0.044
Y125 (94)	0	0.220	0.027
Y126 (94)	50	0.244	0.038
Y127 (94)	150	0.482	0.068
Y128 (94)	100	0.246	0.088
Y129 (94)	100	0.147	0.107
Y131 (94)	0	0.242	0.062
Positive Control		0.579	0.047
Negative Control		0.047	0.074

Sample Number	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
Y132 (94)	0	0.257	0.042
Y133 (94)	0	0.226	0.043
Y134 (94)	0	0.189	0.026
Y135 (94)	150	0.234	0.032
Y136 (94)	150	0.094	0.048
Y137 (94)	200	0.429	0.015
Y138 (94)	200	0.895	0.040
Y139 (94)	100	0.605	0.061
Y140 (94)	0	0.377	0.008
Y141 (94)	0	0.232	0.065
Y142 (94)	50	0.198	0.020
Y143 (94)	50	0.328	0.015
Y144 (94)	650	0.508	0.013
Y146 (94)	50	1.809	0.019
Y147 (94)	0	0.191	0.022
Y148 (94)	50	0.260	0.012
Y149 (94)	0	0.275	0.208
Y150 (94)	0	1.798	0.013
Y151 (94)	0	0.168	0.030
Y152 (94)	50	0.064	0.027
Y153 (94)	0	0.500	0.038
Y154 (94)	0	0.261	0.016
Y156 (94)	150	0.634	0.039
Y158 (94)	100	0.304	0.041
Y160 (94)	150	0.856	0.053
Y161 (94)	200	0.144	0.045
Y162 (94)	0	0.234	0.038
Y163 (94)	300	1.044	0.017
Y164 (94)	50	0.486	0.089
Y165 (94)	0	0.525	0.023
Positive Control		0.764	0.051
Negative Control		0.053	0.038

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
Y166 (94)	0	1.312	0.093
Y167 (94)	0	0.494	0.023
Y168 (94)	150	0.864	0.048
B14 (94)	50	0.703	0.058
B15 (94)	300	0.324	0.024
B16 (94)	200	0.681	0.044
B17 (94)	0	0.151	0.046
B18 (94)	0	1.881	0.012
B19 (94)	400	0.307	0.028
B20 (94)	350	1.273	0.033
B21 (94)	50	0.522	0.029
B22 (94)	50	0.159	0.071
B23 (94)	0	0.215	0.015
B24 (94)	0	0.643	0.035
B25 (94)	50	0.538	0.043
B26 (94)	50	0.933	0.054
B27 (94)	0	1.738	0.037
B28 (94)	150	0.144	0.028
B29 (94)	0	0.321	0.015
B30 (94)	200	0.327	0.025
B31 (94)	150	0.215	0.033
B32 (94)	350	0.419	0.090
B33 (94)	100	0.363	0.075
B35 (94)	50	0.075	0.035
B36 (94)	0	1.233	0.018
B37 (94)	0	0.893	0.028
Positive Control		0.678	0.032
Negative Control		0.049	0.041

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
B39 (94)	550	0.238	0.052
B40 (94)	50	0.314	0.054
B41 (94)	50	0.081	0.077
B42 (94)	150	0.389	0.076
B43 (94)	50	0.756	0.032
B44 (94)	0	0.483	0.012
B45 (94)	0	1.847	0.053
B46 (94)	400	0.805	0.034
B47 (94)	0	0.124	0.040
B48 (94)	250	0.118	0.061
B49 (94)	0	0.171	0.059
B50 (94)	150	0.580	0.080
B51 (94)	0	0.359	0.045
B52 (94)	50	0.528	0.022
B53 (94)	0	0.047	0.025
B54 (94)	550	0.219	0.029
B55 (94)	0	0.649	0.013
B56 (94)	400	0.516	0.012
B83 (94)	250	0.230	0.048
B84 (94)	150	0.263	0.025
B85 (94)	50	1.503	0.020
B87 (94)	100	1.168	0.014
B89 (94)	0	0.346	0.028
B90 (94)	300	0.113	0.044
B91 (94)	0	0.232	0.013
B92 (94)	0	0.550	0.021
B93 (94)	150	0.305	0.023
B95 (94)	0	0.358	0.021
B96 (94)	200	0.208	0.027
Positive Control		0.623	0.025
Negative Control		0.045	0.072

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
B97 (94)	150	0.852	0.047
B98 (94)	0	0.274	0.015
P78 (94)	100	0.173	0.053
P79 (94)	550	0.635	0.040
Y62 (94)	150	0.497	0.079
Y63 (94)	350	0.213	0.081
Y64 (94)	150	0.543	0.106
Y65 (94)	0	1.321	0.069
Y66 (94)	0	0.342	0.043
Y67 (94)	50	0.202	0.065
Y68 (94)	300	0.201	0.026
Y69 (94)	100	0.117	0.023
Y70 (94)	0	0.231	0.026
Y71 (94)	0	0.132	0.012
Y72 (94)	200	0.096	0.028
Y73 (94)	0	0.089	0.077
Y76 (94)	0	0.108	0.054
Y77 (94)	250	0.241	0.025
Y80 (94)	0	0.326	0.057
Y81 (94)	0	1.560	0.045
Y82 (94)	0	0.217	0.066
Y83 (94)	0	0.345	0.061
Y85 (94)	0	0.119	0.078
Y86 (94)	150	0.451	0.033
Y87 (94)	0	0.232	0.024
Y88 (94)	0	0.627	0.053
Y89 (94)	0	0.297	0.072
Y90 (94)	0	0.269	0.069
Y91 (94)	50	0.160	0.029
Y92 (94)	300	0.117	0.039
Positive Control		0.497	0.029
Negative Control		0.035	0.059

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
Y94 (94)	0	0.627	0.054
Y95 (94)	0	0.385	0.039
Y96 (94)	0	0.470	0.054
Y97 (94)	0	1.487	0.027
Y100 (94)	0	0.150	0.028
Y101 (94)	0	0.112	0.059
Y103 (94)	0	0.295	0.033
Y104 (94)	0	0.112	0.066
Y105 (94)	100	0.407	0.013
Y106 (94)	100	0.275	0.022
Y107 (94)	150	0.256	0.045
Y108 (94)	0	0.284	0.007
Y109 (94)	0	0.189	0.003
Y110 (94)	0	0.171	0.032
Y111 (94)	0	0.466	0.018
Y112 (94)	200	0.248	0.053
Y113 (94)	150	1.333	0.045
Y114 (94)	200	0.224	0.071
Y115 (94)	0	0.606	0.008
Y116 (94)	0	0.104	0.028
Y117 (94)	150	0.213	0.024
Y118 (94)	50	0.456	0.068
Y119 (94)	200	0.207	0.052
Y120 (94)	900	1.611	0.034
Y121 (94)	50	0.279	0.061
Y122 (94)	0	0.273	0.035
Y123 (94)	0	0.616	0.010
Y176 (94)	0	0.804	0.047
Positive Control		0.540	0.046
Negative Control		0.036	0.141

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
Y177 (94)	0	0.283	0.037
Y178 (94)	0	0.594	0.058
Y179 (94)	0	0.452	0.045
Y180 (94)	0	0.169	0.036
Y181 (94)	100	0.216	0.042
Y182 (94)	0	0.464	0.045
Y183 (94)	250	1.319	0.064
Y185 (94)	100	2.046	0.008
Y186 (94)	50	1.667	0.026
Y187 (94)	350	0.153	0.026
Y188 (94)	0	0.152	0.060
Y189 (94)	100	1.377	0.003
Y191 (94)	0	0.298	0.017
Y192 (94)	250	0.154	0.034
Positive Control		0.493	0.042
Negative Control		0.032	0.018

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
Y1 (92)	0	0.576	0.082
Y2 (92)	0	0.426	0.018
Y3 (92)	0	0.453	0.029
Y4 (92)	550	0.666	0.088
Y5 (92)	250	0.207	0.150
Y6 (92)	2700	0.112	0.105
Y7 (92)	200	0.116	0.083
Y8 (92)	50	0.492	0.053
Y10 (92)	0	0.524	0.058
Y11 (92)	300	0.758	0.060
Y12 (92)	200	1.013	0.058
Y13 (92)	1550	0.958	0.125
Y14 (92)	0	0.265	0.049
Positive Control		0.493	0.042
Negative Control		0.032	0.018

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
Y15 (92)	100	0.646	0.019
Y16 (92)	50	0.991	0.059
Y17 (92)	250	0.388	0.042
Y18 (92)	0	0.274	0.048
Y19 (92)	0	1.262	0.044
Y20 (92)	150	0.754	0.039
Y21 (92)	50	0.167	0.024
Y22 (92)	150	0.247	0.027
Y23 (92)	500	0.097	0.114
Y24 (92)	250	0.137	0.101
Y25 (92)	0	0.472	0.022
Y26 (92)	0	0.218	0.030
Y27 (92)	350	0.373	0.039
Y28 (92)	300	0.967	0.040
Y29 (92)	0	0.312	0.109
Y30 (92)	0	0.226	0.019
Y31 (92)	50	0.498	0.055
Y32 (92)	400	0.268	0.069
Y33 (92)	200	1.229	0.018
Y34 (92)	450	0.545	0.034
Y35 (92)	300	0.438	0.040
Y36 (92)	250	0.656	0.030
Y37 (92)	800	0.622	0.060
Y38 (92)	200	1.947	0.039
Y39 (92)	200	0.371	0.088
Y41 (92)	0	0.917	0.040
Y42 (92)	650	0.299	0.079
Y43 (92)	150	0.842	0.054
Positive Control		0.468	0.067
Negative Control		0.028	0.137

Sample Number	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
Y45 (92)	0	0.258	0.068
Y46 (92)	150	1.162	0.034
Y47 (92)	300	0.482	0.062
Y48 (92)	250	0.305	0.060
Y49 (92)	100	0.479	0.044
Y50 (92)	200	0.583	0.042
Y51 (92)	50	0.702	0.037
Y52 (92)	100	0.677	0.049
Y53 (92)	550	0.630	0.013
Y54 (92)	700	0.541	0.020
Y55 (92)	850	1.341	0.033
Y56 (92)	300	1.647	0.025
Y58 (92)	50	0.174	0.046
Y59 (92)	150	2.000	0.020
Y60 (92)	100	1.248	0.065
Y61 (92)	700	0.111	0.037
Y62 (92)	300	0.222	0.018
Y63 (92)	50	0.516	0.035
Y64 (92)	350	0.737	0.017
Y65 (92)	350	0.556	0.011
Y66 (92)	650	0.583	0.045
Y67 (92)	0	1.465	0.018
Y68 (92)	0	0.492	0.030
Y69 (92)	300	2.062	0.011
Y70 (92)	0	0.842	0.028
Y71 (92)	750	2.012	0.016
Y72 (92)	50	0.406	0.002
Y73 (92)	250	0.595	0.037
Y74 (92)	150	0.756	0.028
Positive Control		0.704	0.025
Negative Control		0.065	0.032

Sample Number	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
Y76 (92)	50	0.536	0.035
Y77 (92)	0	0.700	0.046
Y78 (92)	0	1.065	0.037
Y79 (92)	0	0.320	0.028
Y80 (92)	950	0.083	0.062
Y81 (92)	450	1.433	0.016
Y82 (92)	50	0.791	0.058
Y83 (92)	100	0.422	0.026
Y85 (92)	0	0.660	0.039
Y86 (92)	100	0.573	0.047
Y87 (92)	0	0.659	0.002
Y88 (92)	0	0.600	0.013
Y89 (92)	50	0.139	0.011
Y90 (92)	50	0.782	0.005
Y91 (92)	50	1.167	0.022
Y94 (92)	350	0.720	0.019
Y95 (92)	150	0.553	0.013
Y96 (92)	800	0.207	0.029
Y98 (92)	50	0.829	0.022
Y99 (92)	50	0.319	0.050
Y100 (92)	0	1.065	0.017
B15 (92)	150	0.333	0.029
B17 (92)	2400	0.254	0.075
B18 (92)	150	0.826	0.049
B19 (92)	600	0.397	0.031
B20 (92)	150	0.164	0.079
B21 (92)	100	1.359	0.058
B22 (92)	2150	1.656	0.056
Positive Control		0.709	0.123
Negative Control		0.062	0.147

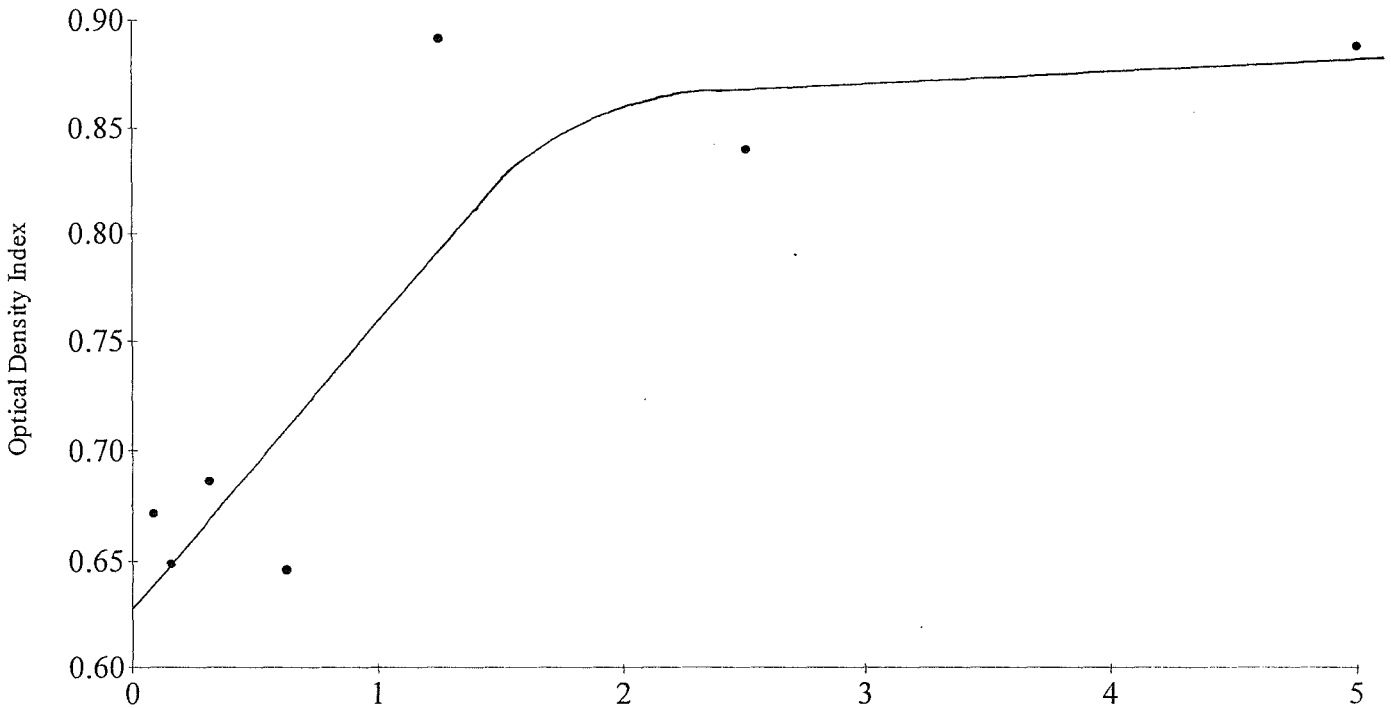
Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
Y936 (92)	0	1.010	0.057
Y937 (92)	300	0.609	0.016
Y938 (92)	50	1.068	0.045
Y940 (92)	100	0.829	0.043
Y941 (92)	500	0.104	0.034
Y942 (92)	50	0.081	0.093
Y943 (92)	0	0.367	0.044
Y944 (92)	0	1.093	0.072
Y945 (92)	100	1.382	0.015
Y946 (92)	300	0.840	0.014
Y947 (92)	50	0.406	0.027
Y948 (92)	0	0.607	0.032
Y949 (92)	50	0.253	0.005
Y950 (92)	50	0.540	0.017
Y951 (92)	100	0.630	0.029
Y952 (92)	0	0.916	0.040
Y953 (92)	100	0.680	0.042
Y954 (92)	0	1.927	0.041
Y955 (92)	800	0.726	0.006
Y956 (92)	1200	0.325	0.016
Y957 (92)	0	2.134	0.008
Y958 (92)	0	0.660	0.024
Y959 (92)	50	0.276	0.016
Y960 (92)	1250	0.193	0.008
Y961 (92)	150	0.968	0.085
Y962 (92)	100	1.610	0.044
Y963 (92)	50	0.174	0.072
Y964 (92)	0	0.512	0.010
Y965 (92)	100	1.154	0.024
Y966 (92)	50	0.463	0.011
Positive Control		0.724	0.044
Negative Control		0.062	0.049

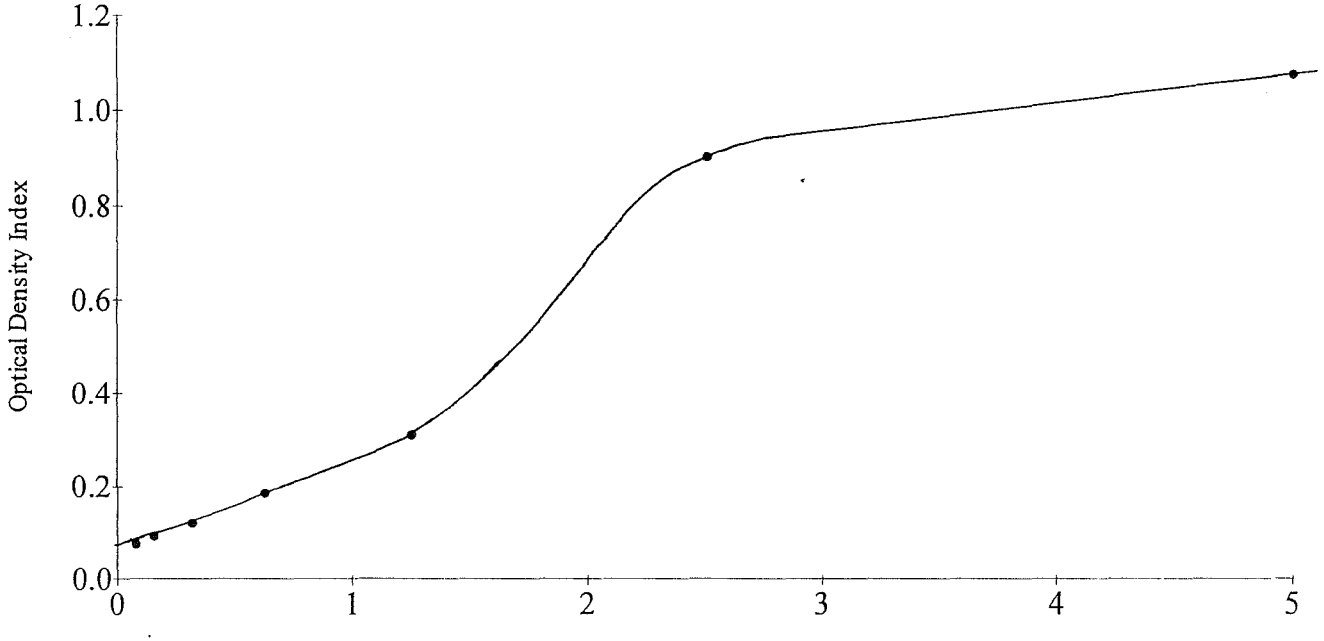
Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
B23 (92)	1600	0.214	0.104
B24 (92)	0	0.839	0.040
B25 (92)	0	0.784	0.010
B26 (92)	450	0.112	0.070
B27 (92)	0	1.894	0.019
B29 (92)	450	1.170	0.033
B30 (92)	0	1.269	0.029
B31 (92)	0	1.060	0.029
B32 (92)	0	0.662	0.002
B34 (92)	50	1.039	0.030
B35 (92)	0	0.249	0.108
B36 (92)	1200	1.735	0.012
B37 (92)	50	0.983	0.015
B39 (92)	0	0.632	0.014
B40 (92)	350	0.429	0.007
B41 (92)	500	1.130	0.037
B45 (92)	200	0.387	0.068
Y926 (92)	0	0.506	0.015
Y927 (92)	0	1.038	0.006
Y928 (92)	150	0.923	0.033
Y929 (92)	100	2.266	0.019
Y930 (92)	900	1.520	0.014
Y932 (92)	200	0.996	0.058
Y933 (92)	300	0.766	0.022
Y934 (92)	0	1.251	0.060
Y935 (92)	0	0.577	0.038
Positive Control		0.809	0.031
Negative Control		0.068	0.039

Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
Y968 (92)	250	0.393	0.029
Y969 (92)	100	1.372	0.064
Y970 (92)	100	1.283	0.046
Y971 (92)	0	1.050	0.040
Y972 (92)	0	0.148	0.043
Y975 (92)	50	1.448	0.039
Y976 (92)	200	1.230	0.009
Y977 (92)	100	0.756	0.021
Y978 (92)	300	0.345	0.022
Y979 (92)	100	1.295	0.033
Y980 (92)	150	0.695	0.030
Y981 (92)	0	0.295	0.048
Y982 (92)	100	0.305	0.029
Y983 (92)	0	0.914	0.019
Y984 (92)	0	0.704	0.030
Y987 (92)	150	0.327	0.077
Y988 (92)	1000	0.721	0.036
Y989 (92)	0	0.432	0.015
Y990 (92)	0	0.566	0.042
Y991 (92)	100	0.598	0.041
Y992 (92)	50	0.255	0.126
Y993 (92)	250	0.561	0.033
Y994 (92)	400	0.710	0.071
Y995 (92)	300	0.283	0.012
Positive Control		0.594	0.008
Negative Control		0.053	0.057

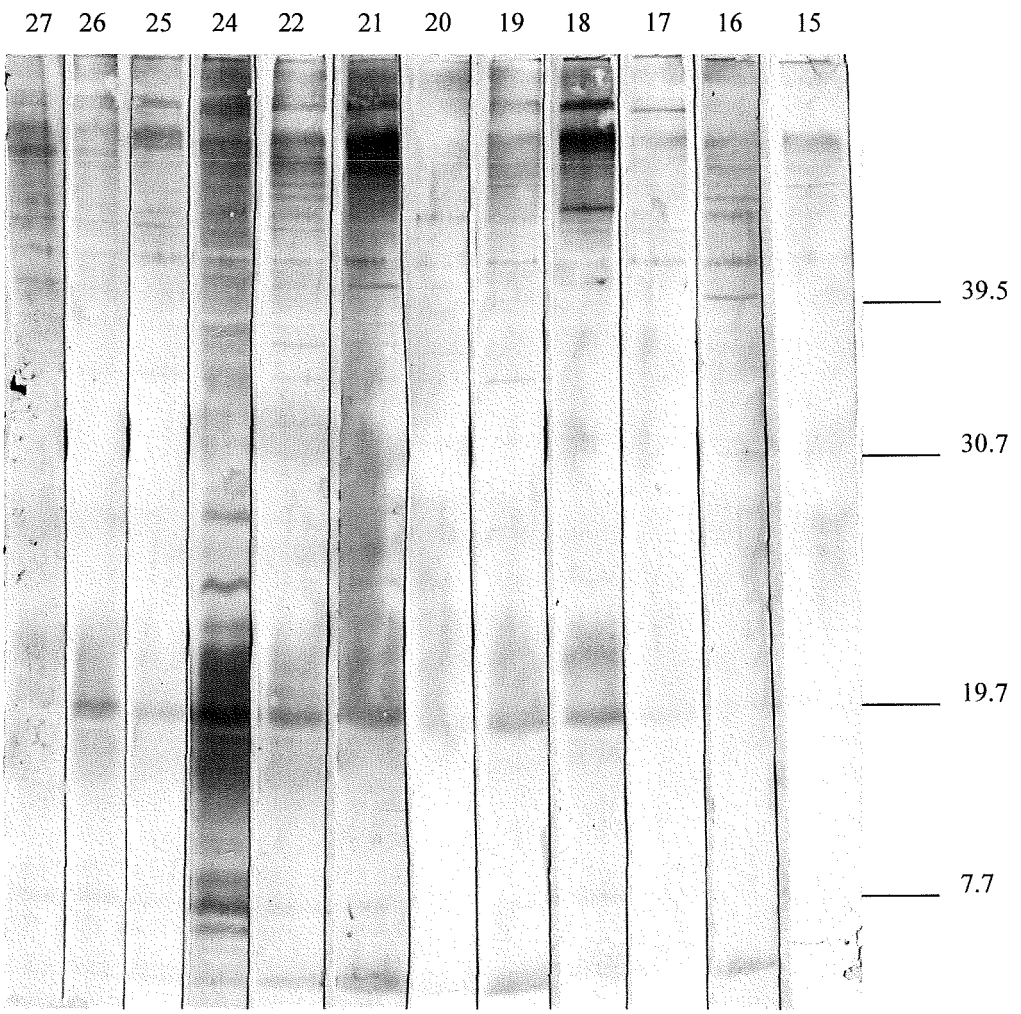
Sample Number (Year)	Mean Faecal Egg Count	Optical Density	Within Subjects coefficient of Variation
Y996 (92)	0	0.385	0.607
Y997 (92)	150	0.886	0.034
Y998 (92)	150	1.139	0.024
Positive Control		0.652	0.044
Negative Control		0.056	0.031

Appendix 6. Titration curves for Monoclonal antibody used in ELISAs

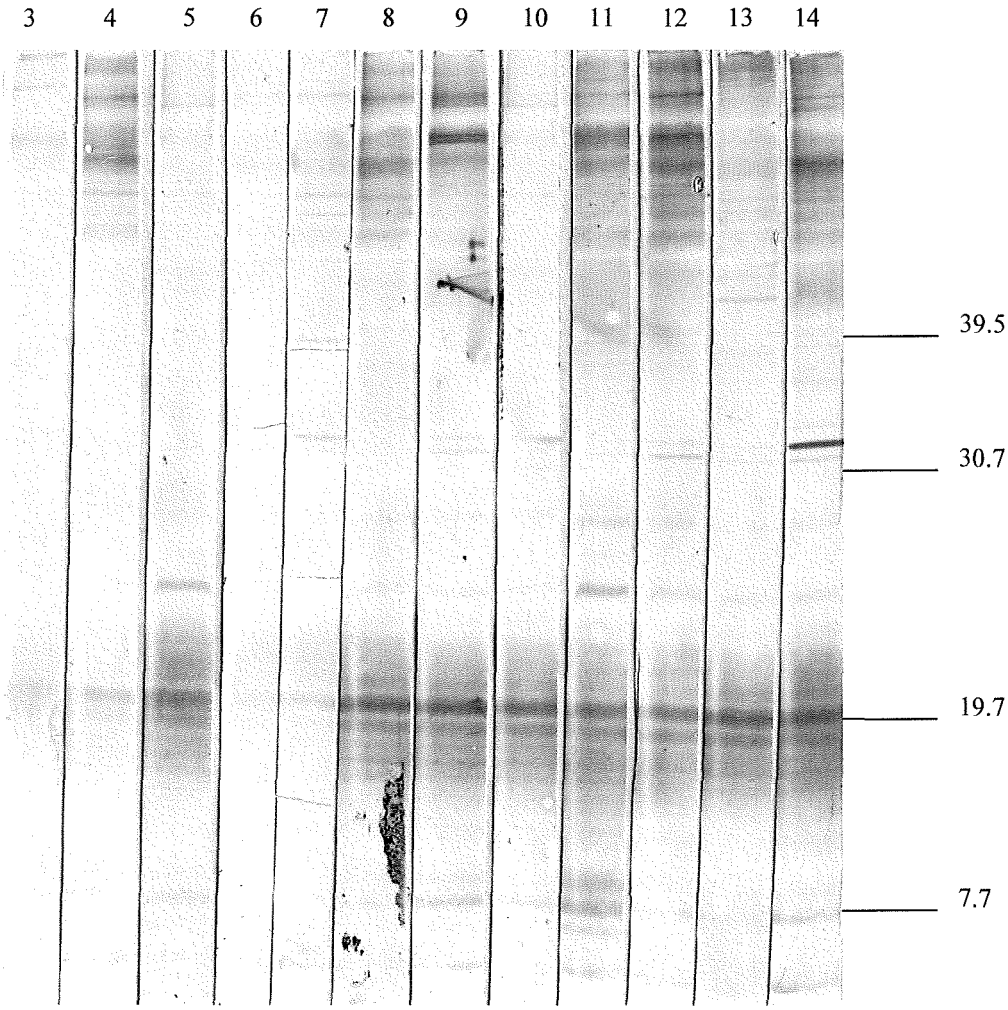



Optical Density Index
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Monoclonal Antibody Concentration (%) in H. contortus ELISA

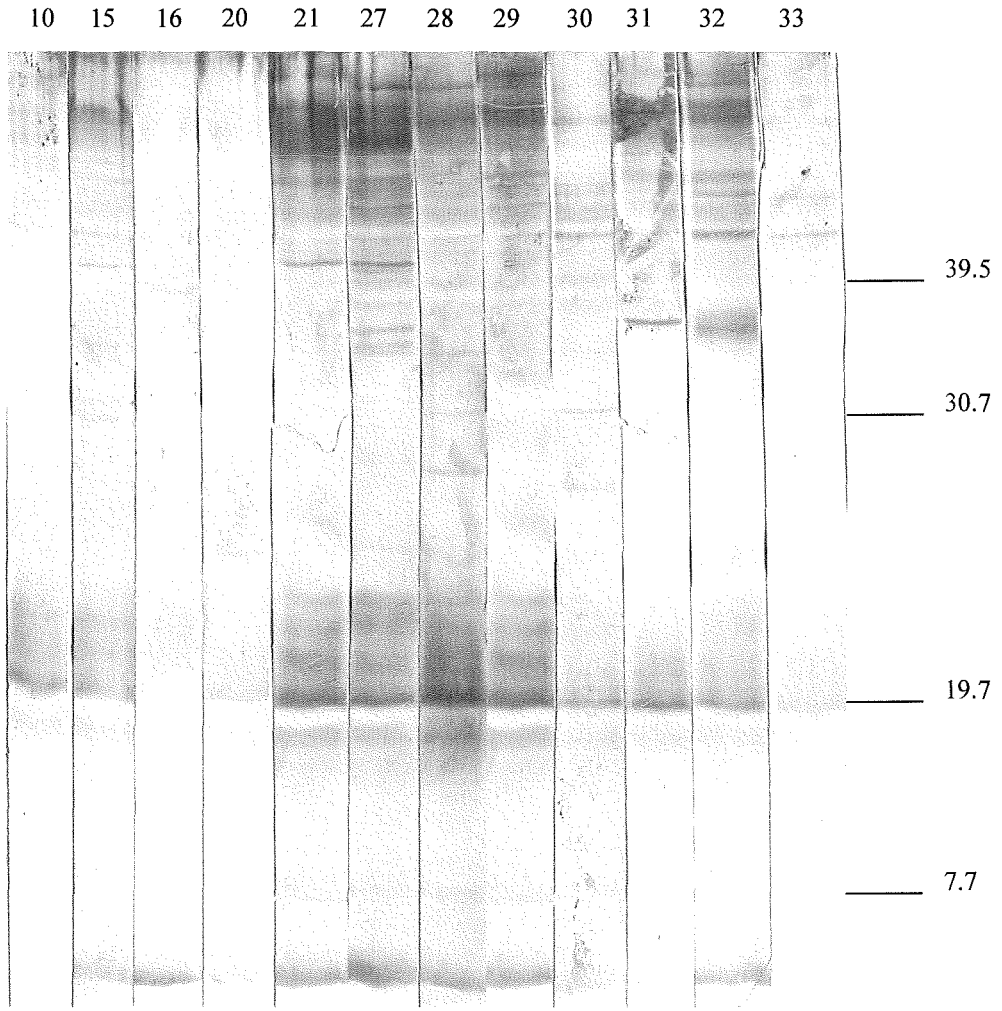
Appendix 7. Western Blots of *T. circumcincta.* Sheep sample numbers are shown along the top and molecular weight markers on the right hand scale (kDa).



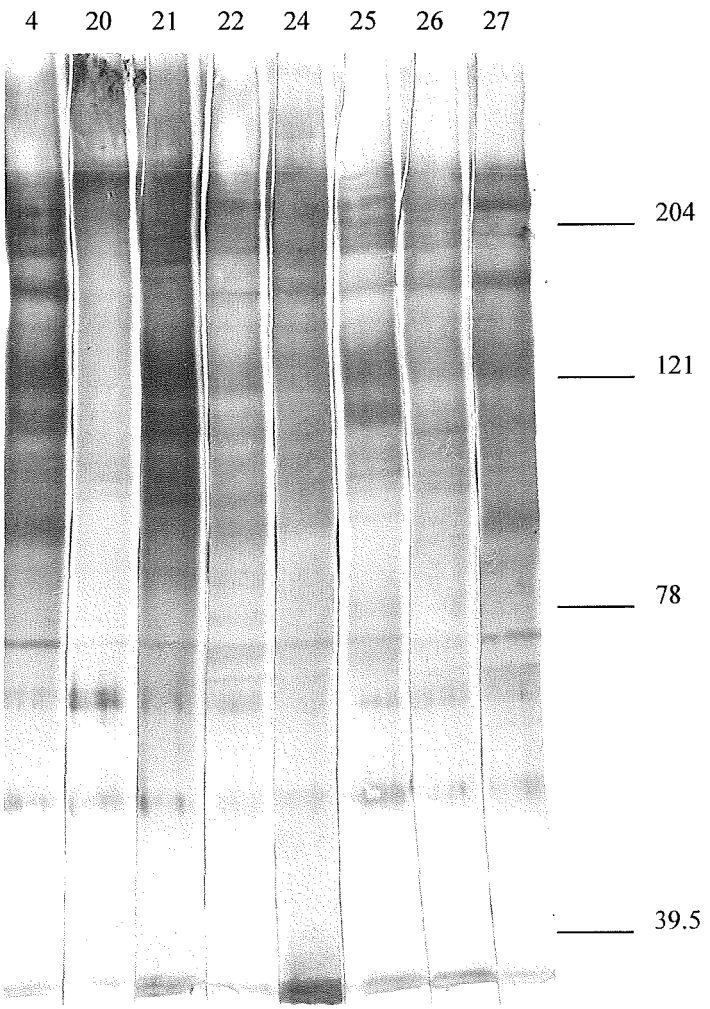
7.5% gel



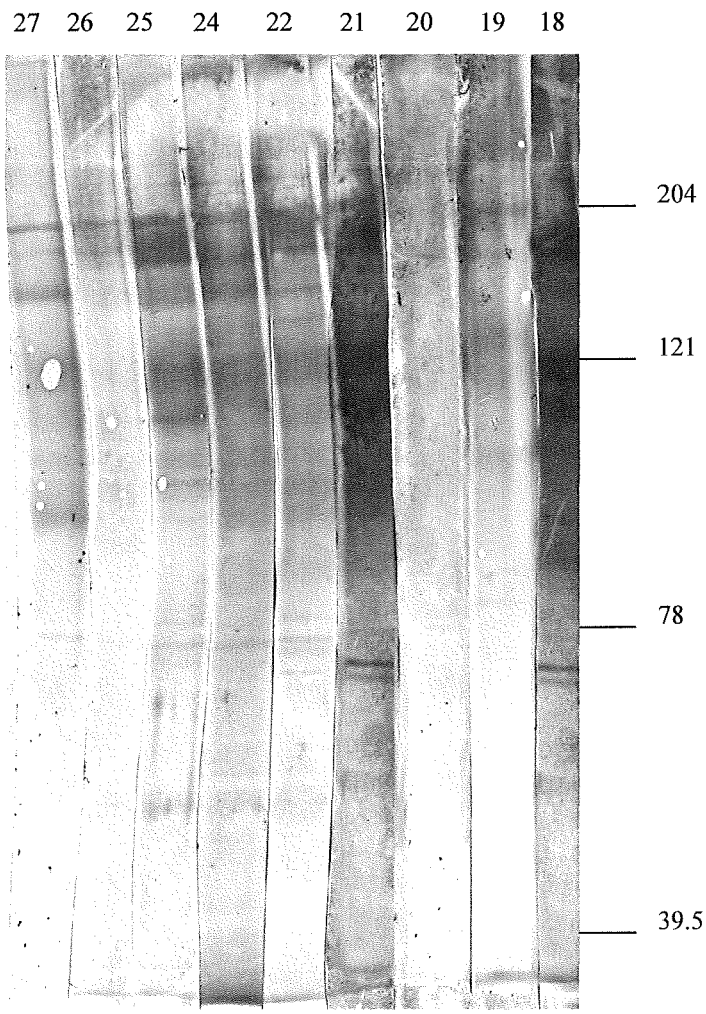
7.5% gel



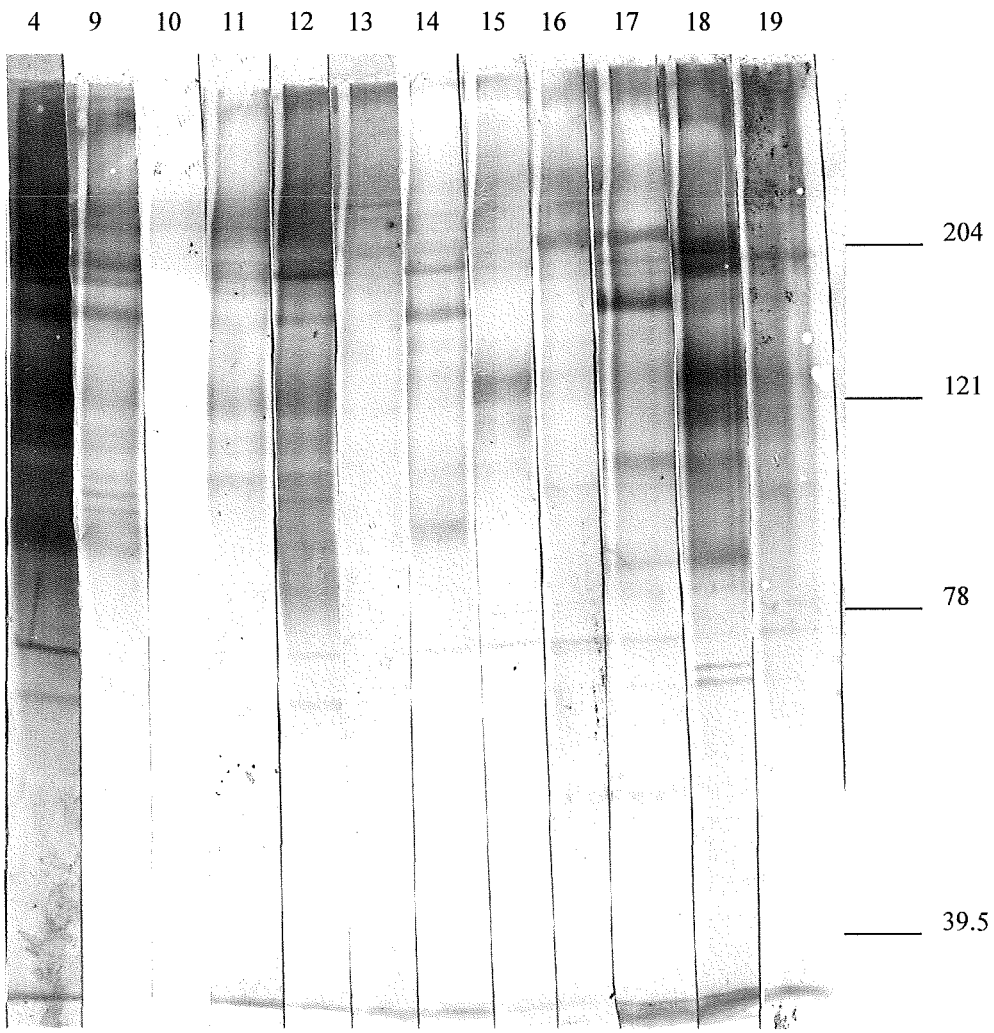
7.5% gel



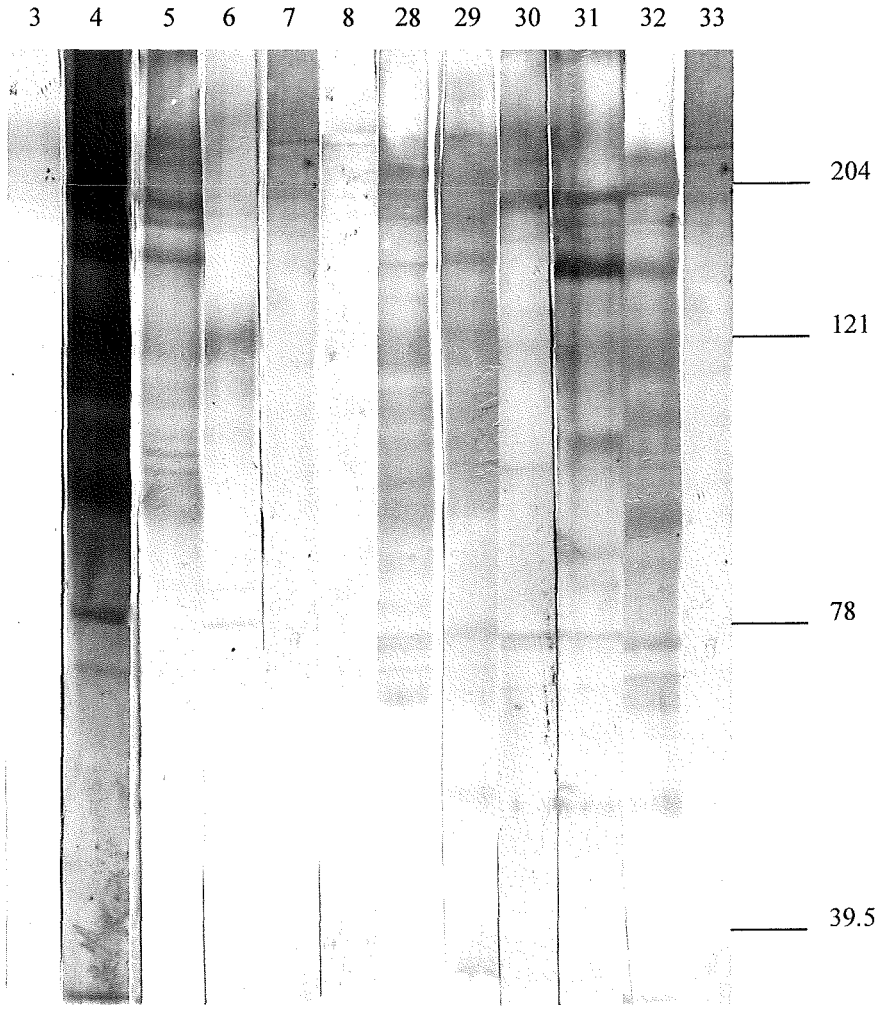
15% gel



15% gel



15% gel



15% gel

Appendix 8. Optical density indices for IgA directed against fourth-stage *H. contortus.*

Sample Number	Optical Density against L4	Worm Burden	Mean Adult Worm Length (mm)	Eggs per Gram of Faeces	Diet Group Basal diet (BD) Supplemented diet (SD)
14	0.272	2900	22.39	13900	SD
16	0.273	1250	23.18	29000	BD
17	0.226	1550	25.66	16300	BD
18	0.294	1900	25.02	27700	BD
20	0.197	2850	24.59	8500	SD
21	0.754	1300	23.57	2550	SD
25	0.379	800	22.94	1450	SD
26	0.191	1400	25.47	62600	BD
27	0.153	1600	25.07	16200	BD
28	0.236	0	-	50	SD
31	0.133	1150	24.16	14300	BD
32	0.156	400	21.07	200	SD
33	0.371	900	22.43	2250	SD
34	0.281	1950	24.62	30700	SD
35	0.242	750	24.62	18400	BD
36	0.219	1050	26.34	20600	BD
Negative Control	0.132				