Immunity to *Teladorsagia circumcincta* infection in Scottish Blackface sheep:

An investigation into the kinetics of the immune response, antigen recognition and the MHC

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2002

Abstract

Gastrointestinal parasitism is one of the greatest causes of disease and lost productivity in domestic animals and in addition has significant animal welfare implications. Anthelmintic treatment has been the preferred method of control for many years but with appearance of multi drug resistant strains of many of the major gastrointestinal parasites of sheep and cattle, other non-chemotherapeutic methods of control are being sought. Selective breeding of animals resistant to gastrointestinal parasitism is particularly attractive but identifying the phenotypic and genetic markers of resistance on which selection will be based is a major problem. Hence, this thesis has investigated the immune responses of Scottish Blackface sheep when infected with the gastrointestinal nematode *Teladorsagia circumcincta*, and how an understanding of these responses may facilitate the successful selection of resistant animals. The kinetics of the host's immune responses to challenge infection were studied and identified clear patterns in plasma IgA activity, peripheral eosinophil counts, faecal egg counts and plasma pepsinogen concentrations but not in plasma IgG activity. It was determined that when used in parallel and when tested at multiple time points, these parameters have much greater potential as markers of resistance than when used individually or more importantly if only assessed on a single occasion. Further work investigated the recognition of stage specific parasite antigens by host plasma IgA by Western blotting. After adjusting for differences in the activity of IgA in each plasma sample the work in this thesis identified that preferential recognition of a different set of antigens was associated with resistance in the group of experimentally challenged animals compared to previous publications. Additionally, and for the first time this investigation was also carried out on naturally infected animals. There was little correlation in the patterns of antigen recognition between the experimentally challenged and naturally infected animals. Finally, the role of the MHC was investigated and it was determined that MHC heterozygotes produced significantly more plasma IgA then MHC homozygotes but did not harbour significantly shorter worms. The analysis also confirmed in naturally infected sheep that there was no obvious relationship between MHC polymorphism and antigen recognition. The results suggested that resistance was due to the recognition of several molecules rather than a single molecule. The work detailed in this thesis has further increased our understanding of the complex host/parasite relationship and has confirmed that selective breeding using the various phenotypic and genetic markers studied is possible. However, this will only be viable if the tests involved in assessing these traits become cheaper and easier to perform, especially if they are to be carried out by the farmer, on the farm.

The research reported in this thesis is my own original work, except where otherwise stated, and has not been submitted for any other degree.

Acknowledgements

I would like to thank my supervisor Professor Mike Stear for giving me the opportunity to undertake the research presented in this thesis and for providing assistance and guidance, particularly in statistics, throughout my studies.

I am indebted to Stuart Mitchell for excellent technical assistance and for generally just being there when things went wrong. I also thank him for encouragement when I was down and for keeping me sane with tea and crosswords.

Thanks to everyone, past and present, in the Medicine Research Laboratory for providing a fun and stimulating working environment. Particular thanks go to Lizzie Gault for helping with cell culture.

Thanks must also go to my office mates from past and present, but particularly to Stuart Mitchell, Fiona Young and Andrew Kerr for putting up with the bad jokes and for helpful discussions.

I also acknowledge those people whose samples and data I have used in this study. The animal work and collection and processing of biological samples were conducted by members of this group employed on previous grants.

Funding for this work was provided by the Scottish Executive Environment and Rural Affairs Department through a LINK project (LK0610) entitled 'Genetic improvement of resistance to nematodes in terminal sire sheep'. This was a project incorporating the Roslin Institute, Glasgow University Veterinary School, the Meat and Livestock Commission (MLC), and Elite Texel Sires Ltd. I acknowledge of all these parties.

Thanks also to my family for constant support and encouragement. Far away but always close.

My greatest thanks and love go to Isla, my best friend and soulmate, my future wife, the best thing that has ever happened to me. Thanks for being there.

Finally, thanks to the girls, past and present, for furry fun and games

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Abbreviations

A. suum	*Ascaris suum*
A^2	Anderson-Darling test statistic
AIDS	acquired immunodeficiency syndrome
αβ T cell	alpha beta T cell
ALN	abomasal lymph node
APCs	antigen presenting cells
APS	ammonium persulphate
B. indicus	*Bos indicus*
B. taurus	*Bos taurus*
BCIP/NBT	bromo chloro indolyl phosphate/nitroblue tetrazolium
BSE	bovine spongiform encephalopathy
C. oncophora	*Cooperia oncophora*
C. punctata	*Cooperia punctata*
Ca	calcium
CCK	cholecystokinin
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
Cl	chloride
°C	degrees Celsius
cm	centimetre
CO_2	carbon dioxide
CP	crude protein
CRF	corticotrophin-releasing factor
CTMC	connective tissue mast cell
CTs	condensed tannins
Cu	copper
CVID	common variable immunodeficiency
D. flagrans	*Duddingtonia flagrans*
Da	Daltons
dH_20	distilled water
DM	dry matter
dpi	dots per inch
E. coli	*Escherichia coli*
ECP	eosinophil cationic protein
EDN	eosinophil derived neurotoxin
EDTA	disodium ethylene diamine tetracetic acid
EGTA	ethylene glycol tetracetic acid
ELISA	enzyme linked immunosorbent assay
EPF	eggs per female
EPO	eosinophil peroxidase
ES	excretory/secretory
F. hepatica	*Fasciola hepatica*
FCS	foetal calf serum
Fe	iron
FEC	faecal egg count
FET	Fisher's Exact test
FMD	foot and mouth disease
FW	formula weight
γd T cell	gamma delta T cell

g	grams
GL	globule leucocyte
GM-CSF	granulocyte macrophage colony stimulating factor
H. contortus	*Haemonchus contortus*
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HP	high protein
hr	hours
HTs	hydrolysable tannins
I	iodine
IFN-γ	interferon gamma
IgA, D, G, E, M	immunoglobulin, subclasses A, D, E, G and M
IL-1, -2 etc	interleukin-1, -2 etc
K	potassium
kDa	kilo Dalton
kg	kilograms
L. donovani	*Leishmania donovani*
L. monocytogenes	*Listeria monocytogenes*
L. major	*Leishmania major*
L_1	first stage larvae
L_2	second stage larvae
L_3	third stage larvae
L_4	fourth stage larvae
λ	lambda
ln	natural logarithm
log10	logarithm to the base 10
LP	low protein
M	molar
MAIDS	murine acquired immune deficiency syndrome
MALT	mucosal associated lymphoid tissue
MBP	major basic protein
Mg	magnesium
mg	milligrams
MHC	major histocompatibility complex
min	minutes
MIP-1α	macrophage inflammatory protein 1 alpha
μg	micrograms
μM	micromolar
μm	micrometre
ml	millilitres
mM	millimolar
MMC	mucosal mast cell
Mn	manganese
mRNA	messenger RNA
N. americanus	*Necatur americanus*
N. battus	*Nematodirus battus*
N. brasiliensis	*Nippostrongylus brasiliensis*
N. filicollis	*Nematodirus filicollis*
N. spathiger	*Nematodirus spathiger*
Na	sodium
NEM	N-ethylmaleimide
NO	nitric oxide

no.	number
NPY	neuropeptide Y
O. circumcincta	*Ostertagia circumcincta*
O. ostertagi	*Ostertagia ostertagi*
OD	optical density
OLA	ovine lymphocyte antigen
%	percent
P	phosphorous
P	probability
P. falciparum	*Plasmodium falciparum*
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	PBS + Tween 20
PBS-TSM	PBS + Tween 20 + skimmed milk powder
PC	personal computer
PCV	packed cell volume
p.i.	post infection
PMSF	phenyl methyl sulphonyl ketone
PPR	periparturient egg rise
r	correlation coefficient
R^2	coefficient of determination
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
S. japonicum	*Schistosoma japonicum*
S. mansoni	*Schistosoma mansoni*
S. stercoralis	*Strongyloides stercoralis*
SC	secretory component
SDS	sodium dodecylsulphate
Se	selenium
S.E.	standard error of the mean
s-IgA	secretory IgA
SIV	simian immunodeficiency virus
SMCP	sheep mast cell protease
spp.	species
SSA	sickle cell anaemia
T. axei	*Trichostrongylus axei*
T. circumcincta	*Teladorsagia circumcincta*
T. colubriformis	*Trichostrongylus colubriformis*
T. gondii	*Toxoplasma gondii*
T. trifurcata	*Teladorsagia trifurcata*
T. vitrinus	*Trichostrongylus vitrinus*
TC	transitional cell
TCR	T cell receptor
TGF-β	transforming growth factor beta
T_H1/T_H2	T helper cells, subtypes 1 and 2
TNF-α	tumour necrosis factor alpha
TPCK	N-tosylamide-L phenylalanine chloromethyl ketone
UK	United Kingdom
V	Volts
W. bancrofti	*Wuchereria bancrofti*
WL	worm length
Zn	zinc

Chapter 1

Introduction

1.1 General Introduction

World wide, parasitic infections are one of the greatest causes of disease and lost productivity in domestic animals (Vercruysse & Claerebout, 2001), and if left untreated would cause unacceptable welfare problems (Coles, 1998). Sales of antiparasitic drugs have grown more than in any other sector of the world wide animal health market in the last decade and now corner one quarter of the $18 billion market (Dalton & Mulcahy, 2001). Although parasites can infect and affect most internal and external niches of the body, nematode infections of the gastrointestinal tract of ruminants are particularly important. They affect productivity, cause animal welfare implications and seriously affect the economic viability of livestock farming if left untreated.

All the economically important gastrointestinal nematode parasites of ruminants belong to the Superfamily Trichostrongyloidea, Order Strongylida (the bursate nematodes) from the subclass Secernentea (the phasmid nematodes) (Table 1.1). They are called bursate nematodes as the males possess a copulatory 'bursa', a posterior umbrella-like cuticular expansion, which is absent from the males of all other orders of nematodes.

The genus Ostertagia is the major cause of parasitic gastritis in ruminants in temperate areas of the world (Urquhart *et al.*, 1996), and the sheep parasite, *Ostertagia circumcincta* (Stadelmann, 1894), or *Teladorsagia circumcincta* as it is now called is no exception. *Teladorsagia circumcincta* is the parasite of interest in this study.

This review chapter aims demonstrate our current understanding about the complex nature of this parasite and the interactions with its host, and how the work conducted in this study fits into what we know. The life cycle of the parasite, pathogenesis of infection and host factors affecting the establishment of infection are reviewed initially and in general while the role of the host immune system is reviewed in more detail. Current strategies for controlling these infetions are then considered and in particular breeding for resistance is reviewed in depth.

1.2 *Teladorsagia circumcincta* life cycle

Teladorsagia circumcincta has a direct life cycle with the definitive host being the sheep (*Ovis aries*) and goat (*Capra hircus*) (Fig 1.1; Egg, L_4 and adult pictures are

Genus	Species	Host	Site of infection	Geographical distribution
Ostertagia	*ostertagi*	cattle	abomasum	temperate areas globally although
	trifurcata	sheep + goats	"	in sub tropical areas when
	leptospicularis	sheep, goats, cattle + deer	"	associated with winter rainfall
(*Teladorsagia*)	*circumcincta*	sheep + goats	"	
Marshallagia	*marshalli*	small ruminants	"	tropics and subtropics
Haemonchus	*contortus*	sheep + goats	"	important in tropics and subtropics
	placei	cattle, sheep + others	"	although does occur elsewhere
Mecistocirrus	*digitatus*	buffalo, cattle	"	subtropics
Trichostrongylus	*axei*	most ruminants	"	very important in the tropics, but
	colubriformis	most ruminants	small intestine	still found in temperate regions
	vitrinus	most ruminants	duodenum	
Cooperia	*oncophora*	cattle	small intestine	can be important in subtropics and
	curticei	sheep + goats	" "	subtropics, has a secondary role in
	punctata	cattle + sheep	" "	temperate areas
	pectinata	cattle + sheep	" "	
Nematodirus	*battus*	sheep, goats + cattle	" "	found in temperate, cold, often
	filicollis	sheep + goats	" "	elevated environments
	helvetianus	cattle	" "	
	spathiger	ruminants	" "	

Table 1.1. Parasitic nematodes of the Trichostrongyloidea superfamily of major veterinary and economic importance. Table generated with information from Urquhart *et al.* (1996), Smyth (1994), Anderson (2000) and Balic *et al.* (2000a).

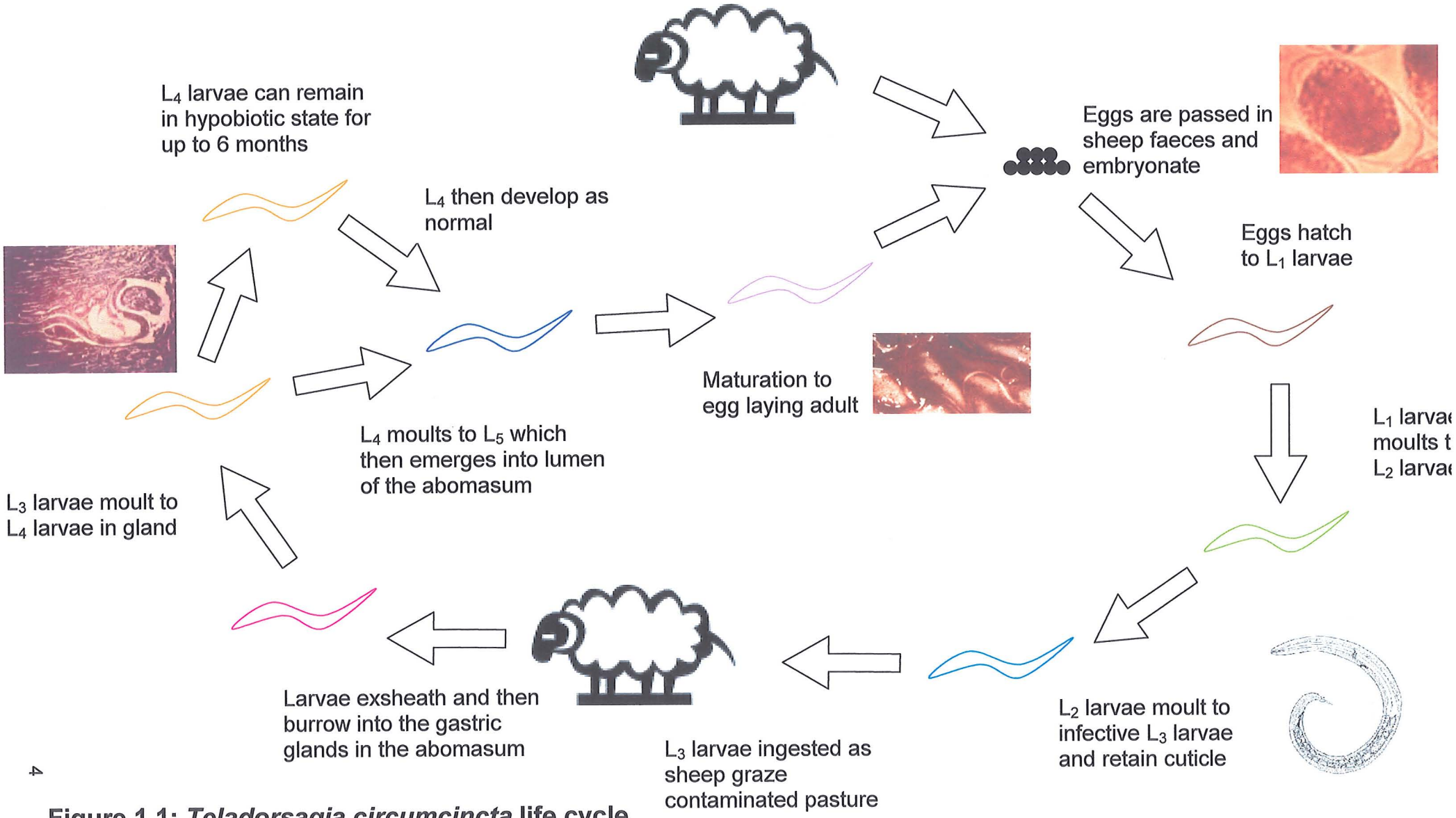

Figure 1.1: *Teladorsagia circumcincta* life cycle

© University of Missouri, L_3 picture © Animal and Natural Resources Institute, Beltsville, Maryland).

Eggs of *T. circumcincta* are deposited by gravid females into the lumen of the gut where they are passed with the host faeces. Eggs hatch to the L_1 larval stage where they feed on bacteria present within the faecal pat (Ciordia & Bizzell, 1963).

The first stage larvae moult into second stage (L_2) larvae and feed on bacteria within the faecal pat (Urquhart *et al.*, 1996). This larval stage begins to develop a cuticular sheath which is retained when the larvae moult to the infective L_3 stage. This sheath provides protection against environmental factors, particularly desiccation (Stromberg, 1997), but also means that the larvae cannot feed as they are completely enclosed and so are dependent on their stored reserves (Urquhart *et al.*, 1996).

1.2.1 Egg hatching

There has been a considerable amount of literature published describing the environmental conditions required for successful development from the egg to the infective L_3 stage.

Crofton (1965) reported a lower limit of 4°C for hatching of *O. circumcincta* eggs. Interestingly, Crofton & Whitlock (1965) report differences in the temperature ranges of required for hatching in geographically different *O. circumcincta* populations. *Ostertagia circumcincta* from Cornell, NY State, USA, hatched in temperatures ranging from 10-38°C while *O. circumcincta* from Bristol in England hatched at temperatures from 4-35°C.

Crofton (1965) also reported significant differences in the range of hatching temperatures and hatching times between eight different species of sheep nematode parasites. This contrasts with Ciordia & Bizzell (1963) who, working with nematodes of cattle, stated that no differences were observed in the rate of embryonation, hatching and larval development between the five nematodes studied (*C. punctata*, *C. oncophora*, *O. ostertagi*, *T. axei* and *T. colubriformis*). They reported that eggs of all of these species of nematode developed at temperatures as low as 6°C and as high as 35°C.

1.2.2 Development to L_3

Temperature also affects the rate of the developmental process to the infective L_3 stage and again there is a large degree of variation in the literature.

It is reported that eggs of *O. ostertagi* took up to 42 days to develop to L_3 at 5°C in artificial cultures (Enigk and Dey-Harza 1969 in Levine, 1980), although Ciordia & Bizzell (1963) could not demonstrate development to the infective stage at this temperature.

In Southern England, the minimum times to recover *O. circumcincta* L_3 larvae on herbage were 10, 8 and 2 weeks when the corresponding weekly mean maximum soil temperatures were approximately 7, 9 and 11°C respectively (Gibson & Everett, 1972). In Victoria, Australia, where soil temperatures did not fall below 10°C the mean minimum time to recover L_3 larvae on herbage was only 4-27 days (Callinan, 1978).

At the other extremes of temperature, Kates (1950) found that in Maryland, experiments which begun when weekly mean air temperatures were approximately 17, 21 and 26°C, L_3 larvae of *O. circumcincta* were first recovered from herbage at > 6, 4 and 1 weeks respectively. Corresponding temperatures in the Australian study (Callinan, 1978) were associated with yields of L_3 on herbage within two weeks if there was development at all.

Temperature also affects how many parasites actually develop to the infective L_3 stage. Ciordia & Bizzell (1963) observed that only 30% of *O. ostertagi* eggs developed to the infective L_3 stage at 25°C with even lower developmental success at higher (5.3% at 32°C) or lower (21.5% at 20°C) temperatures. The Australian field study of Callinan (1978) reported maximum yields of L_3 of *O. circumcincta* on herbage of between only 0-16%. Yields as high as 20% have been reported for *Trichostrongylus* spp. (Donald, 1968) although there have been several studies which have estimated that only a very small proportion (0-2%) of eggs ever become available as L_3 larvae on herbage for all the important species (Boag & Thomas, 1975; Niezen *et al.*, 1998).

Temperature may control the rate of the developmental process, but without moisture there is no development (Stromberg, 1997). Optimum conditions for transmission of *H.*

contortus and *Trichostrongylus* spp. were approximately 2.5cm or more of rain per month and temperatures ranging from 15-32 and 6-20°C respectively.

These reports suggest that temperature, moisture levels and the times reported for development from eggs into L_3 larvae vary greatly for the different species, and between different geographical regions, providing evidence that there has been natural selection in each ecological area (Crofton & Whitlock, 1965).

1.2.3 Migration of L_3 larvae from faecal pat

Infective L_3 larvae must migrate from the faeces onto the surrounding herbage in order to be ingested by the ruminant host. The degree and speed of migration is dependent on temperature (Krecek *et al.*, 1990), moisture (Bryan & Kerr, 1989), light intensity (Rees, 1950), relative humidity (Pandey *et al.*, 1993), herbage species (Niezen *et al.*, 1998) and herbage height and density (Silangwa & Todd, 1964).

Ostertagia ostertagi larvae can migrate laterally and vertically through the soil around a faecal pat (Krecek & Murrell, 1988). Early reports considered the soil as an insignificant source of L_3 larvae (Levine & Andersen, 1973), but more recent publications have demonstrated that the soil can act as an important reservoir for infective larvae (Krecek & Murrell, 1988), in particular providing an environment where larvae can survive over winter ready to emerge back onto pasture when climatic conditions become more favourable (Al Saqur *et al.*, 1982).

The area at the base of the herbage may be favourable for larval survival and may contain large numbers of larvae (Silangwa & Todd, 1964), perhaps acting as a safe environment like the soil. Under optimal conditions however, more larvae can be observed on the upper portion of the herbage (8-16cm) compared to the lower portion (soil to 8cm) (Williams & Bilkovich, 1973), presumably to increase the chance of ingestion by the ruminant host. Rees (1950) showed that there is daily cycle of vertical migration of infective larvae of *H. contortus* on experimental grass plots. Larval migration was assessed every two hours for 24 hours once each month for a year in the study of Rees (1950). The greatest number of larvae were found on the grass blades in the early morning and in the evening, with less being recovered at night and during the middle of the day. The time of the morning maximum became progressively earlier

passing from winter to summer, and the time of the evening maximum became progressively later. The reverse was true in the second half of the year. This has implications for parasite control. If farmers could limit their animals' grazing at these peak times then the associated risk of acquiring infective larvae would be considerably reduced.

The maximum distances to which larvae can migrate away from faecal matter are still a matter of debate. In Poland, Wertejuk (1959) in Williams & Bilkovich (1973) found that larvae of bovine nematodes migrated horizontally no more than 7cm in three days while *O. ostertagi* larvae in Austria migrated up to 30cm in seven to twelve days and *C. oncophora* migrated 15cm away from faeces in as little as 48 hours (Kutzer, 1967 in Williams & Bilkovich, 1973). Rose (1961) reported that in England most infective larvae of *O. ostertagi* migrated only approximately 5cm, with only a few migrating as far as 15cm. Williams & Bilkovich (1973) recorded that in Louisiana the majority of larvae of *O. ostertagi* at 2 to 15cm from the faecal pad but with 10-20% of larvae found in herbage in the 15 to 30 and 30 to 45cm ranges. Durie (1961) stated that larvae from cattle with mixed gastrointestinal nematode infections in Southern Queensland migrated up to 90cm, but usually not more than about 30cm, while Skinner & Todd, Jr., (1980), reported similar migration distances from *H. contortus* larvae from infected sheep in Illinois. In all of the cited examples given, maximum larval migration was critically dependent on suitable quantities of rainfall. Indeed, on the hottest, driest days larval migration may not occur at all (Skinner & Todd, Jr., 1980).

An interesting, but infrequently reported observation is the effect of rainfall on larval movement. As cow pats dry the number of active larvae available at the surface decreases, but if the pat is sprayed with water, the number of surface larvae increases (Grønvold, 1984; Grønvold & Hogh-Schmidt, 1989). By simulating rainfall these authors found that few larvae were dispersed when raindrops fell on pats with dry surfaces, however, when the surface had been watered, a significant number of larvae were transported away from the cow pat with the splash of a droplet. They observed that more than 90% of the larvae were transferred by splash droplets, as far as 90cm from the pat, while only a small proportion of larvae actively migrated in the water film on the herbage.

As well as rain splash dispersal of larvae it is conceivable that after heavy rainfall in which water might accumulate on the soil surface, larvae could be washed in currents over the soil surface to considerable distances from the faecal matter (Williams & Bilkovich, 1973). These authors also suggested that the wind, birds, arthropods and other invertebrates could be factors in the distribution of nematode infective larvae on soil and herbage and this was corroborated by the study of Skinner & Todd, Jr., (1980) who identified several species of arthropods associated with faeces, particularly beetles and ants. The explosive discharges of sporangia of the fungus *Pilobolus* spp. have also been implicated in the dissemination of infective larvae of trichostrongylids (Bizzell & Ciordia, 1965) as well as those of *Dictyocaulus viviparus*, the major cause of parasitic bronchitis in cattle (Eysker, 1991).

Migration of L_3 larvae from faeces can continue for as long as the faecal pellet persists (Callinan, 1978). Infective larvae can then survive on pasture for weeks to several months (Gibson & Everett, 1972), depending on parasite species and environmental conditions. Larvae struggle to survive in extremes of temperature, i.e. harsh dry summers (Williams & Bilkovich, 1973) or severe cold winters, although winter survival may be enhanced when larvae migrate into the soil or when larvae are protected by a blanket of snow (Stromberg *et al.*, 1991).

1.2.4 Development of parasite in the host

Infective *T. circumcincta* L_3 larvae are ingested by their ovine hosts when the sheep are feeding on pasture. Due to the very small size of infective larvae, there has been little work on the initial kinetics of arrival of larvae in the newly infected host. Threlkeld (1934) was one of the first authors to investigate the *T. circumcincta* life cycle but did not examine infected animals until 48 hr after initial infection. Similarly, the study of (Armour *et al.*, 1966) did not examine animals until four days post infection.

Ruminants have a single stomach with four compartments, the reticulum, rumen, omasum and the abomasum. Food first enters the reticulorumen where it is broken down by billions of micoorganisms. After fermentation in the reticulorumen, food passes to the omasum and then to the abomasum, the true stomach, which is the only site on the digestive tract that produces gastric juices (hydrochloric acid, pepsin and rennin). Ingesta only remain in the abomasum for 1-2 hours before passing to the small

and large intestines. Ruminants as the name suggests also ruminate, particularly when they are resting. Food is regurgitated from the reticulum then re-chewed and swallowed. How this may affect gastrointestinal larval infectivity has not been studied.

A paper by Dakkak *et al.* (1981) is the only report that describes the kinetics of the arrival of 3rd stage larvae into the abomasum. Although the study utilised infections with *H. contortus* larvae, the times at which larvae are seen in different parts of the alimentary tract would presumably be similar for most related gastrointestinal parasite species. Dakkak *et al.* (1981) enumerated the numbers of *H. contortus* passing through the omasoabomasal orifice during the first six hours after oral administration. Larvae moved from the reticulorumen to the abomasum more slowly than the liquid phase of the ruminal contents. Sheathed and unsheathed larvae were observed as soon as 10-20 minutes after initial oral administration passing to the abomasum. Only exsheathed larvae can develop and become adults. Large numbers of unsheathed larvae were observed passing to the abomasum thus it seems that exsheathment of most larvae in the reticulorumen occurs within the first hour of infection. The speed at which these larvae can pass through the alimentary tract might not allow sufficient time for establishment and could explain the sometimes small yields of parasite seen as adults.

Published values for the proportion of infective L_3 larvae which are able to develop in the host to the adult stage vary greatly according to the parasite species, the age of the host and infection regime. Kao *et al.* (2000) summarised the published information and showed that establishment rates for *T. circumcincta* range from only 1-14% in trickle infected 4 month old lambs to over 65% in 25 week old lambs given 2000 L_3 larvae each week for 5 weeks. The establishment rates of the other important parasite species are of a similar range but have been reported as high as 85% at 21 days in non-immune lambs given 5000 L_3 larvae of *H. contortus* (Adams, 1982).

Teladorsagia circumcincta like the other gastrointestinal nematodes exsheath in the reticulorumen and pass to the abomasum over a matter of days. Where they burrow into the mucosa (Threlkeld, 1934). Third stage larvae then moult to the fourth stage over the next three days (Armour *et al.*, 1966). Fourth stage larvae then moult into 5th stage or adult worms which emerge from the mucosa to live in the lumen of the abomasum or can remain as fourth stage larvae and remain in the mucosa. Threlkeld (1934) observed adult worms as soon as nine days after infection.

Arrested larval development is a phenomenon in which larvae are able to temporarily suspend their own development at the L_4 stage. There are four stimuli which can trigger arrested development; seasonal effects, density dependence, parasite genetics and host immunity. The environmental trigger is thought to be received by the free living infective stages prior to ingestion by the host (Urquhart *et al.*, 1996). Arrested larval development often coincides with extremes of climatic conditions so that the progeny of arrested larvae will have a better chance of survival when more favourable conditions prevail. It is not clear what triggers the signal for larvae to arrest their development or resume the maturation process or how the signal is transmitted but it may be analogous to the pheromone signalling system believed to control dauer larva development in *Caenorhabditis elegans* (Ren *et al.*, 1996). This signalling system involving chemosensory neurones has also been implicated in the arrested larval development of *H. contortus* (Ashton *et al.*, 1999). Arrested larval development may not manifest in deliberate infections if environmental conditions are strictly controlled and may explain why arrested development was not observed in the study by Threlkeld (1934).

Adult worms continue to develop before they become sexually mature. Female worms are significantly longer than male worms (Stear *et al.*, 1995c; Threlkeld, 1934). It is not clear but this appears to be a morphological feature common to all adult nematode species (Smyth, 1994). Sexually active adults mate with females which lay eggs that pass out with the host faeces. There is no information available regarding the mating behaviour of the worms, i.e. does a female worm mate with one male, lay eggs, mate with a different male and lay more eggs, or the female may mate with more than one male before eggs are laid and/or may retain the sperm of a single or multiple males for subsequent fertilisation.

Eggs could be seen in female worms at day 16 post infection although faecal egg counts were still negative in the study of (Armour *et al.*, 1966). By 21 days, faecal egg counts were positive (Armour *et al.*, 1966), indicating that eggs had first been laid between 16 and 21 days post infection. Eggs have been observed in faeces as early 14 days post infection (Stear *et al.*, 1995a). Worm populations had not altered markedly by 28 and 38 days post infection compared to that at 21 days, although a greater proportion of worms were in the adult stage and the number of arrested larvae had decreased (Armour *et al.*, 1966). By the 60^{th} day of infection few worms of any description were found and faecal egg counts were negative once more. The prepatent period, the time taken for

development from infection until mature adults produce eggs, for *T. circumcincta* is traditionally considered to be 21 days (Urquhart *et al.*, 1996). However, Armour *et al.* (1966) reported the first eggs in faeces at 21 days post infection, but observed gravid females at day 16, while Threlkeld (1934) reported some gravid adults at day 15 with the first appearance of eggs in faeces at 17 days and Stear *et al.* (1995a) found eggs in faeces at 14 days. Stear *et al.* (1995d) stated that the prepatent period of *T. circumcincta* in lambs receiving their second deliberate infection varied from 20 to 59 days. These animals will already have developed some kind of immunity to infection so that worms may take longer to develop. It is likely that the prepatent period for *T. circumcincta* is 14 or more days in parasite naïve animals and the differences that are observed in these studies are due to experimental differences or are due to differences in the ages of animals used.

1.2.5 Sex ratios of adult worms

Threlkeld (1934) did not enumerate the numbers of male and female adult worms in his study on *T. circumcincta* in sheep. However, in his later study, Threlkeld (1946) reported a male to female sex ratio of 0.875 in the 300 randomly selected adult worms he counted in deliberate infections of cattle with *O. ostertagi*. There were no significant differences detected in the sex ratio of adult worms in the study by Armour *et al.* (1966), at any period of the experiment. This is in contrast with other studies that have found considerable adult worm sex differences. Stear *et al.* (1998) stated that Scottish Blackface sheep naturally infected with predominantly *T. circumcincta* had male to female adult sex ratios ranging from 0.7 to 0.76 in four consecutive years on a commercial farm. Infections of lambs with *N. battus* gave male to female sex ratios of 0.63 in previously exposed then challenged lambs compared to ratios of 0.9 in challenge only controls (Israf *et al.*, 1997). These authors explained their observations by suggesting that preferential rejection of male worms by immune lambs was occurring but did not suggest the mechanisms by which this may have proceeded.

The opposite is true in *Nipppostrongylus brasiliensis* infections in rats but is dependent on the stage of the infection. Female worms are more numerous than male worms for about the first two weeks of infection; at 10 days the male to female ratio was 0.93 in the study of Haley (1961). Males then became more frequent with female worms being lost more rapidly than male worms. Haley (1961) reported the male to female sex ratio

at 20 and 30 days post infection as 1.53 and 2.06 respectively. The author also reported that the greater loss of female worms was independent of the size of the initial challenge infection given. This author also gave no indication for possible mechanisms for preferential male worm survival.

There is also a male-biased sex ratio in adult worms of *Schistosoma mansoni* (Boissier & Mone, 2000). These authors showed that in experimental unisexual infections, male cercariae are more infective than females, but in bisexual infections the infectivity of the two sexes is similar, irrespective of the cercarial sex ratio. Boissier & Mone (2000) suggested that the differences in sex ratios observed in natural infections may be attributed to behavioural and/or biochemical male-female interactions.

Explanations for the sex ratio differences are difficult to find. Some authors have suggested that differences in sex ratios may be due to worm burdens (Haukisalmi *et al.*, 1996); Stear, unpublished observations), while others report that sex ratios of worms are independent of worm burden (Anderson & Schad, 1985). It may be that female third stage larvae of the major gastrointestinal nematode species survive better on pasture so that more female larvae are actually ingested. If markers could be used which differentiated between male and female larvae but did not affect their infectivity then this could be investigated. Additionally, it is unclear at what stage the sex of the worms is determined, it may be that the sex of the third stage larvae is not predetermined and that some environmental or host cue determines the sexual outcome of the worms. This remains an area requiring considerable further investigation.

1.3 Host factors affecting the establishment of infection

1.3.1 Age of host

The young are more susceptible than their older counterparts in a wide range of parasitic infections such as *Plasmodium falciparum* malaria (Sabatinelli *et al.*, 1996), schistosomiasis (Anderson, 1993) as well as human whipworm caused by *Trichuris muris* (Bundy *et al.*, 1987) and hookworm infections caused by *Necatur americanus* (Quinnell *et al.*, 2001). Young goats, up to 12 months of age, are more susceptible to

infection with *Strongyloides papillosus*, a common threadworm of ruminants (Pienaar *et al.*, 1999) while the same is true for ovine infections with *Cryptosporidium parvum* (Ortega-Mora & Wright, 1994).

This phenomenon, called unresponsiveness or hyporesponsiveness (Schallig, 2000), is well-described in gastrointestinal nematode infections of ruminants but is not very well understood. Manton *et al.* (1962) gave one of the first accounts of age associated unresponsiveness. These authors reported that lambs aged 6 months or younger which had been immunised with *H. contortus* larvae were not resistant to homologous challenge, where as the same immunising procedure in older sheep consistently produced a high degree of protection. Unresponsiveness in young animals has also been confirmed in infections with irradiated *H. contortus* larvae (Benitez-Usher *et al.*, 1977), irradiated *T. colubriformis* larvae (Dineen *et al.*, 1978) and *T. circumcincta* larvae (Smith *et al.*, 1985a). Young animals become more resistant as they grow older (Gibson & Parfitt, 1972; Stear *et al.*, 2000b).

The immune system of sheep does not appear to be fully operational when animals are very young. Although the manifestation of immunological hyporesponsiveness in young animals may, in part, be due to their not having had sufficient exposure to pathogens and antigens to develop active immunity (Watson *et al.*, 1994) or due to the effects of stress during weaning (Watson & Gill, 1991a). It is simply the immaturity of the animals' immune system that appears to be most important.

However, Gregg *et al.* (1978) reported that in experimental infections of merino sheep with irradiated *T. colubriformis* larvae, serum levels of antibody reflected the extent of antigen exposure and there was no evidence that the unresponsiveness of lambs was due to a deficiency in antibody production. They also stated that unresponsiveness was not associated with the numbers of circulating lymphocytes, monocytes or granulocytes, or with the number of mast cells, eosinophils and neutrophils at the site of infection. It is perhaps not that a young animal's immune system/response in inadequate but rather it is inappropriate and so is not suitable to combat these infections.

Lambs have significantly lower proportions of $CD4^+$ and $CD8^+$ lymphocytes than mature sheep (Colditz *et al.*, 1996). Blood lymphocytes from young sheep produce less interferon gamma (IFN-γ) *in vitro* (Colditz *et al.*, 1996) and, in general, young animals

mount smaller antibody responses than older sheep (Watson & Gill, 1991a). Young naturally infected grazing lambs have significantly fewer globule leucocytes/mucosal mast cells than older animals (Douch & Morum, 1993). Peripheral eosinophil and mast cell counts were significantly higher in 9 month old sheep compared to young lambs following vaccination with an experimental adult excretory/secretory (ES) vaccine and subsequent challenge with *H. contortus* (Schallig *et al.*, 1997).

These observations taken together suggest that young animals may be lacking a T_H2 type response. There is some evidence to suggest a typical T_H1 response may be induced in young lambs which may not be sufficient to protect against gastrointestinal nematode infections (Schallig, 2000).

1.3.2 Sex of the host

Possible relationships between host gender and host susceptibility to parasites have been widely studied for human, rodent and ruminant infections.

Human infections with the protozoan parasite *Entamoeba histolytica* give rise to equal numbers of male and female asymtomatic cases, while the frequency of invasive amebiasis, the acute form of the disease, is significantly more prevalent in males than in females (Acuna-Soto *et al.*, 2000). Male subjects carry significantly heavier infections of the human hookworm *N. americanus* than females (Behnke *et al.*, 2000) while the intensity of infection with *Wuchereria bancrofti*, the cause of human filariasis, is reduced in females compared to males (Alexander & Grenfell, 1999).

The situation in rodents does not appear to be as clear. Mock & Nacy (1988) reported that in three different strains of mice infected with *Leishmania major*, males had significantly greater liver parasite burdens than females. This was corroborated by Anuradha *et al.* (1990) who found that male hamsters were more susceptible than females to infections with *Leishmania donovani*. Other parasitic infections in mice exhibit other sex differences. Female mice infected with *Toxoplasma gondii* are considerably more susceptible than their male counterparts (Walker *et al.*, 1997). The immunological basis for these observed differences was explained by the fact that the male mice rapidly produced high levels of interleukin (IL)-12, interferon (IFN)-γ and tumour necrosis factor (TNF)-α compared to the female mice. Eloi-Santos *et al.* (1992)

stated that in three different strains of mice, females were more susceptible to *S. mansoni* infections than males. Mortality of female worms was higher and schistosomula were significantly more successful in developing into adult worms in female mice. Interestingly, there were no gender differences observed in the susceptibility of infection with *Taenia taeniaeformis*, a tapeworm of wild deermice (Theis & Schwab, 1992).

A number of studies in ruminants have identified differences in immunity between the sexes, but others have not. Females were more heavily infected than males in goats traditionally reared in Spain and naturally infected with a range of gastrointestinal nematode, mainly comprising *T. circumcincta* and *T. trifurcata* but also *T. vitrinus*, *N. filicollis* and *Trichostrongylus capricola* (Valcarcel & Garcia, 1999). Albers *et al.* (1987) found no differences in susceptibility between the sexes in *H. contortus* infected merino lambs.

There is however a larger body of evidence that states that female sheep are significantly more resistant to infections with gastrointestinal nematodes than are males. This has been demonstrated in deliberate infections with *H. contortus* (Adams, 1989), *T. colubriformis* (Windon & Dineen, 1981) and *T. circumcincta* (Gruner *et al.*, 1994), as well as natural mixed predominantly *T. circumcincta* infections (Gulland & Fox, 1992; Stear *et al.*, 1995b).

Some of these studies cited, in both mice and sheep, consider the role of host hormones as being particularly important in susceptibility, and this will be addressed in the next section. It must be noted though that the majority of male sheep in the farming industry are castrated and so the differences seen between males and females cannot wholly be caused by male sex hormone differences.

1.3.3 Reproductive status of the host

As stated above the sex of a host is important in determining susceptibility but the reproductive status of that host is possibly of even greater importance. Most studies have concentrated on the role of female hormones, and particularly those during pregnancy in disease susceptibility with only a few studies investigating how male hormones affect the outcome of infection with parasites.

Male and female hamsters receiving testosterone exogenously had significantly enhanced *L. donovani* parasite counts compared to their respective controls (Anuradha *et al.*, 1990). Administration of estradiol, a direct testosterone antagonist, suppressed parasite infection in both male and female hamsters while male castration lowered parasite intake and female ovarectomy promoted infection. Similar findings were published by Mock & Nacy (1988), who showed that castration of male mice resulted in a 20% reduction in the number of *L. major* liver parasites while exogenous administration of testosterone to female mice resulted in an 88% increase in parasite numbers.

Although these, and many other studies have identified testosterone as being a major determining factor in susceptibility, a recent paper by Ganley & Rajan (2001), investigating filarial worm infections in mice suggested that high endogenous testosterone did not necessarily decrease the ability of mice to resist nematode infections. Results obtained from administration of exogenous testosterone should be interpreted with caution.

The role of female sex hormones, and in particular during pregnancy and lactation, as factors involved in susceptibility to infection have been widely studied in humans, mice and domestic animals. Pregnant mice are more susceptible to infection with *T. gondii* than are non-pregnant females (Thouvenin *et al.*, 1997). Spleen cells from these infected pregnant mice produced more IFN-γ (a type 1 cytokine) and more nitric oxide (NO) than non-pregnant mice, and the type 2 response (IL-4 and IL-10) was much weaker. This supports the hypothesis cited previously (Schallig, 2000), that animals lack a T_H2 response during infection and that an increased T_H1 response is not sufficient to protect against parasitic infestation.

Pregnancy in humans is associated with a greater susceptibility to many disease causing pathogens including *P. falciparum* (malaria) (Schleiermacher *et al.*, 2001), the hepatitis E virus (hepatitis E), *Coxiella burnetii* (Q fever or Rickettsia), *Listeria monocytogenes* (listeria) and *T. gondii* (toxoplasmosis) (J.L. Smith, 1999) but interestingly the intensity of infection of bancroftian filariasis, caused by *W. bancrofti*, is significantly reduced in pregnant women (Alexander & Grenfell, 1999).

The importance of reproductive status in ruminants in determining susceptibility to gastrointestinal nematode infection has been particularly widely studied. Pregnant and lactating ruminants show a marked increase in susceptibility to gastrointestinal nematode infection (Dunsmore, 1965). It is generally held that this change in disease susceptibility is caused by impaired immune status (O'Sullivan & Donald, 1970) and manifests in the form of the periparturient egg rise (PPR) or 'spring rise' in faecal egg counts from infected individuals. While the name spring rise is still commonly used, the term PPR is more accurate as egg count rises are observed in autumn lambing ewes (Dunsmore, 1965). The importance of the PPR is that it occurs at the time when the numbers of new susceptible hosts are increasing and so ensures the survival and propagation of the worm species (Urquhart *et al.*, 1996).

While the PPR may begin in late pregnancy, it is mainly associated with lactation. Romjali *et al.* (1997) and Gruner *et al.* (1992) showed that litter size affected post parturition faecal egg counts. Bigger litters were associated with higher lactation intensities resulting in higher levels of stress for lactating ewes which led to increased immune suppression.

Although factors such as nutrition and stress, as described and a lack of antigenic stimulation can be apportioned some of the blame for the PPR, hormonal suppression of immunity might be the most important. All of the parasitological manifestations of acquired immunity to nematodes (resistance to establishment, arrested development, reduced worm fecundity, expulsion of established worms) appear to be compromised giving rise to the increase in worm numbers and egg output (Barger, 1993). The reduction in parasite specific immune responses occurs synchronously with significant increases in the circulating levels of the pituitary hormone prolactin, whose main function is the initiation and maintenance of lactation. Immune integrity is restored when prolactin levels drop at the end of lactation or when suckling lambs are removed from lactating ewes (Urquhart *et al.*, 1996).

The role of prolactin has been considered to be of major importance for many years and was originally reviewed by (Dunsmore, 1965). However, more recent work has cast doubt on the principal role of prolactin in the PPR. Jeffcoate *et al.* (1990) used a prolactin antagonist which significantly reduced prolactin levels but had no effect on the PPR of infected lactating ewes. Now that prolactin appears to have been eliminated as

the major inducer of immune suppression a significant amount of research needs to be conducted in order to determine the important hormones involved. Given the strong association between prolactin and immune suppression it seems likely that another neuroendocrine or hormonal chemical compound closely associated with prolactin could be involved in the suppression of the immune competency resulting in the PPR.

1.3.4 Host Nutrition

The importance of nutrition in gastrointestinal nematode infections has been recognised for many decades by veterinarians and health workers who have observed that malnutrition and intestinal parasitism share a similar geographical distribution, with the same individuals experiencing both disease states simultaneously (Koski & Scott, 2001). It has been demonstrated on numerous occasions that infection leads to malnutrition and alternatively malnutrition increases susceptibility to infection (van Houtert & Sykes, 1996).

1.3.4.1 Influence of infection on the nutritional status of the host

Gastrointestinal parasitism in ruminants leads to impairment of live-weight gain, soft tissue deposition, skeletal growth, and milk and wool production (Coop & Holmes, 1996). Parasite reduced reduction of host voluntary feed intake, or anorexia, is the major consequence of infection with *T. circumcincta* (Sykes & Coop, 1977). In infections with *T. colubriformis* the physiological responses listed above tend to be caused more by a reduction in the efficiency of food utilisation (Coop & Kyriazakis, 1999).

Experiments have shown a key feature of host dysfunction to be the increased loss of endogenous protein into the gastrointestinal tract, partly from leakage of plasma protein, but also from increased exfoliation of gut epithelial cells and mucoprotein secretion (Coop & Kyriazakis, 1999).

In abomasal infections such as those with *T. circumcincta* most of the protein passing into the lumen of the gastrointestinal tract will be reabsorbed, although they may be absorbed as non-protein nitrogen. This is likely to be impaired however in animals

which have concurrent infections with small intestinal parasite species of *Cooperia*, *Trichostrongylus* and *Nematodirus*. Overall, there is a net movement of protein from productive processes such as meat, bone, milk and wool production into the synthesis of plasma proteins and repair of the gastrointestinal tract and mucus secretion.

1.3.4.2 Influence of nutrition on parasite establishment (resistance)

The general consensus is that protein intake does not appear to influence initial parasite establishment in naïve sheep, although the pathophysiological consequences are usually more severe in animals on lower planes of protein intake (Coop & Kyriazakis, 1999).

An early study by Bawden (1969) reported no differences in initial parasite establishment in sheep fed either a low protein (60g crude protein (CP)/kg dry matter (DM) or high protein (190g CP/kg DM) when infected with *Oesophagostomum columbianum*. The authors did report though that the number of adult parasites counted at slaughter at 56 days after infection was higher in the lambs fed the low protein diet.

Worm free female Scottish Blackface lambs were fed on low protein (LP; 88g CP/kg DM) or high protein (HP; 170g CP/kg DM) and infected with *H. contortus* larvae in the study by (Abbott *et al.*, 1985). The authors reported that faecal egg counts (LP 12000; HP 15000) and worm burdens (LP 550; HP 816) were actually higher at six and five weeks respectively in the animals fed the higher protein diet although the differences did not attain statistical significance. The same experimental protocol carried out on Finn-Dorset lambs again showed no significant differences in faecal egg counts or worm burdens between animals fed high or low protein diets, although adverse clinical signs were observed more frequently in the lambs on the poorer plane of nutrition (Abbott *et al.*, 1986).

1.3.4.3 Influence of nutrition on established infections

Determining the effects of nutrition in deliberate trickle or natural infections is much more difficult as it is difficult to say if differences in nutritional plane are causing the host to act differently against the new larvae that are being ingested or if they are directed against the infections that are probably already present.

Datta *et al.* (1999) reported that cross bred lambs which had short-term (9 weeks) provision of protein enriched diets prior to being put out to pasture had consistently higher live-weight gain and wool production, higher antibody responses to both *H. contortus* and *T. colubriformis*, and lower faecal egg counts than did lambs previously provided with low protein diets. These authors postulated that short periods of enhanced post-weaning nutrition could have long term and perhaps life long effects on production. This confirmed the results of a previous study which had demonstrated that that protein supplementation enhanced immunity to *T. circumcincta* in lambs trickle infected then challenged with the parasite (Coop *et al.*, 1995).

Stear *et al.* (2000a) investigated the effects of urea supplementation on resistance during a deliberate infection over winter with *T. circumcincta* in seven month old lambs which had spent the previous grazing season on naturally infected pasture. Animals were split into relatively resistant and susceptible groups based on faecal egg counts during the natural infection. Relatively resistant animals on a urea supplemented diet allowed fewer larvae to establish. These animals also had higher fructosamine concentrations, higher albumin concentrations and decreased pepsinogen responses suggesting that there was less mucosal damage in these animals. The authors suggest that a combination of relatively resistant sheep plus nutritional supplementation appears most efficient at controlling infection. Urea supplementation has also been shown to counteract the effects of infection with *Haemonchus* and *Trichostrongylus* spp. (Knox & Steel, 1996).

In general, the improvements in resilience caused by dietary supplementation with protein are greatest in young, naïve animals, in which pathophysiological disturbances to the gastrointestinal tract are most common (Coop & Kyriazakis, 2001). Recent evidence has also shown improved resilience in breeding ewes supplemented with protein. Pregnant ewes parasitised with *T. circumcincta* and fed at 130% of their protein requirement gained more weight, produced heavier lambs and had a higher calculated milk production than comparable ewes fed at 85% of their protein requirement (Houdijk *et al.*, 2000). This was confirmed by Donaldson *et al.* (2001) who demonstrated an inverse relationship between worm burdens and protein concentration in periparturient ewes protein supplemented with fish meal and trickle infected with *T. circumcincta* and *T. colubriformis*. Growth rates of lambs in the latter study were significantly higher for ewes fed the highest protein diet (1.25 times estimated requirement) compared to ewes

fed the lowest protein diet (0.8 times estimated requirement). There was also no effect of nutritional plane on worm lengths or fecundities of either parasite species.

1.3.4.4 Effect of dietary energy rather than protein

While most research has concentrated on the effects of protein supplementation on resistance to infection, there has been little work on the effects of dietary energy requirements or supplementation on parasite resistance. While a positive effect of energy supplementation on faecal egg output has been recorded in some instances (Ferre *et al.*, 1995; Garcia-Perez 1994 in Valderrabano *et al.*, 2002), no response was observed in others (Donaldson *et al.*, 1998). Also, energy-restricted Soay sheep naturally infected with gastrointestinal nematodes on St Kilda show increased mortality (Gulland, 1992).

There have also been a few studies that have directly examined the effects of energy restriction independent of protein deficiency in laboratory models and the results strongly suggest that the effects of energy restriction during gastrointestinal nematode infections are independent of protein restriction (Koski & Scott, 2001). Prolonged *N. brasiliensis* survival occurred as a result of energy restriction in rats (Lunn & Northrop-Clewes, 1993), while a 20-25% reduction in energy intake, independent of any alteration in protein status, exerted dramatic effects on survival of *Heligmosomoides polygyrus* in mice (Koski *et al.*, 1999).

A recent study by Valderrabano *et al.* (2002) attempted to clarify the effects of energy supplementation on gastrointestinal parasite resistance. Four month old female lambs were infected with *T. circumcincta* larvae. Lambs were split into two groups, the first was fed a normal diet while the other was fed 60% of the amount of food eaten by the other group, hence energy restricted. Both diets contained adequate levels of protein. While plane of nutrition did not show a significant effect on faecal egg counts or on worm burdens, both female worm size and their fecundity decreased significantly with the increased level of nutrition. This response was accompanied by a significant increase in the concentration of circulating eosinophils suggesting that the immune response of lambs consuming high levels of energy was enhanced compared to that of lambs kept on a restricted diet. The authors claimed that in young female lambs fed on adequate levels of protein, an improvement in energy supply does not only improve

carcass characteristics but clearly enhances the development of resistance to gastrointestinal nematode infections and that this may have decisive management implications for the control of parasitic infections in sustainable production systems.

1.3.4.5 Macro and micro-nutrients

While most of the research has been directed to the effects of protein supplementation there are only a few studies which have shown evidence that both macro-minerals and trace elements can influence the host-parasite relationship (Coop & Holmes, 1996). The requirement for macro-minerals such as calcium (Ca), phosphorus (P), magnesium (Mg), potassium (K), and sodium (Na) and chloride (Cl) and for trace elements such as selenium (Se), copper (Cu), zinc (Zn), iodine (I), manganese (Mn) and iron (Fe) is widely known (Bell, 1997). The effects of deficiency or supplementation of these minerals and elements on parasite resistance is however less well understood.

Intestinal parasitism can impair the absorption of P from the intestine and result in reduced mineralisation of the skeleton (Sykes *et al.*, 1975). These parameters were unaffected by infection with *T. circumcincta*, an abomasal nematode in the study by Wilson & Field (1983). Coop & Field (1983) were able to demonstrate that P supplementation increased the weight gain of lambs trickle infected with *T. vitrinus* compared to diet controls. The faecal egg counts and total worm burdens were higher (10950 worms) in lambs receiving the low P diet compared to those on the high P diet (1240 worms), suggesting that decreased P levels may impair the development of resistance to continuous infection.

Calcium absorption is also impaired in intestinal infections (Wilson & Field, 1983), while Mg absorption appeared to be unaffected (Bown *et al.*, 1989). Mice infected with *Ascaris suum* and supplemented with dietary Mg had lower lung larval burdens than animals on lower Mg dietary planes (Laubach, 1989).

Administration of copper oxide wire particles (COWP), which release soluble Cu in the abomasum, 5 days prior to infection with gastrointestinal nematodes reduced the establishment of *H. contortus* and *T. circumcincta* in treated lambs by 96% and 56% respectively (Bang *et al.*, 1990). There was no significant effect of COWP treatment on the establishment of *T. colubriformis*, an intestinal parasite.

Molybdenum supplementation reduced worm populations of *T. vitrinus* and *H. contortus* by 23% and 78% respectively in trickle infected sheep (Suttle *et al.*, 1992a; Suttle *et al.*, 1992b). Selenium deficiency does not appear to affect resistance to nematode infection (McDonald *et al.*, 1989) while Co deficiency can induce higher faecal egg counts (FEC) and increased pepsinogen levels in lambs experimentally infected with *T. circumcincta* (Ferguson *et al.*, 1989).

Most of the research on trace elements and gastrointestinal nematode infections in humans and laboratory models has focussed on deficiencies of zinc, vitamin A and iron which are amongst the most pervasive nutritional deficiencies in humans worldwide although there is no evidence for their importance in infections of ruminants (Koski & Scott, 2001). Zinc deficiency may be particularly important in gastrointestinal infections as it has been linked with IgA deficiency (Cunningham-Rundles, 2001). In support of this Beach *et al.* (1983) reported that the offspring of mice moderately deprived of zinc during gestation showed an aberrant pattern of development of serum IgA despite complete nutritional rehabilitation beginning at birth. It was only by six months of age that serum IgA concentrations were similar to the offspring of control dams. Cross-fostering of zinc-deprived offspring to dams adequately nourished during pregnancy did little to ameliorate their aberrant pattern of serum immunoglobulin development. The alterations of immune ontogenesis in these mice could not be attributed to the persistence of abnormal plasma zinc levels, as these were within normal ranges. These findings could help to explain the variation among young lambs in antibody responses in response to parasite challenge in the first 6 months after birth.

1.3.4.6 Diet derived anti-parasitic compounds

There are various secondary plant metabolites that are thought to have antiparasitic properties such as phenolic metabolites, nitrogen containing metabolites and terpenoids (Seigler, 1979). Most research has concentrated on the tannins, a class of phenolic secondary metabolites. Tannins play a role in plant defences, protecting them from microbial and parasitic infestation (Swain, 1979). Tannins are commonly divided into hydrolysable and condensed tannins (HTs and CTs), and as CTs are relatively stable in the digestive tract of animals and rarely have toxic effects, research has focussed on the potential use of plants high in CTs to control gastrointestinal parasitism in herbivorous hosts (Coop & Kyriazakis, 2001).

Lambs artificially infected with *T. colubriformis* had greater daily live weight gains, lower nematode FEC and lower worm burdens when grazing the perennial Mediterranean legume *Hedysarum coronarium* (sulla; ~12% CTs) than when grazing *Medicago sativa* (lucerne), which does not contain CTs (Niezen *et al.*, 1995). Niezen *et al.* (2002a) however found that in animals fed the same diet, there was no effect on *T. colubriformis* numbers by feeding on CTs while *T. circumcincta* numbers and FEC were significantly reduced in animals feeding on CT containing fodder.

Recent experiments have shown evidence for a long-term effect of Quebracho CTs, derived from a woody perennial dicotyledon (*Schinopsis* spp.), during both the initial establishment and on the established *T. colubriformis* population in sheep (Athanasiadou *et al.*, 2000). These authors suggested that the effect observed was a direct anthelmintic effect of the condensed tannins included in the sheep diets. Egg hatching and larval development in faeces can also be reduced by feeding infected lambs with various CT containing diets (Niezen *et al.*, 2002b).

While host nutrition is clearly critical in disease outcome and severity, definitive information about the effects of protein, energy and mineral deficiency/supplementation remains unclear. Along with the large number of medicinal plants with possible anthelmintic properties this will remain an area, and deservedly so, receiving considerable further research and resources.

1.3.5 Influence of host behaviour

As stated previously, in order for animals to become infected they must ingest larvae while feeding on pasture. Although cattle and sheep avoid grazing near faeces of their own and other species (Forbes & Hodgson, 1985), their parasitic and nutritional status may override their faecal avoidance behaviour.

Hutchings *et al.* (1998) investigated the grazing strategies of parasitised and unparasitised lambs. They found that increasing amounts of faeces on experimental grass sward trays was associated with a reduced proportion of bites taken from each sward, and reduced bite depth and mass. They also found that the fresher faecal samples were, the less sheep would graze the sward. This presented an interesting point to note in that grass contaminated with fresh faeces poses the least risk of infection, since larvae

must migrate from the faeces to the grass, yet fresh faeces presented the strongest stimulus for rejection of the grass sward. These authors suggested that odour plays an important role in faeces avoidance in that fresh, presumably strongly smelling faeces will be avoided over older faeces, even if the risk of infection in the latter is increased.

Parasitised animals also had significantly reduced bite rates compared to uninfected animals (Hutchings *et al.*, 1998), while parasitic status did not affect bite depth or mass. This study also showed that given a choice of swards contaminated with different amounts of faeces, parasitised animals took significantly more bites from swards contaminated with the least amount of faeces compared with their unparasitised counterparts. Infected animals also took more bites from control swards or swards with the oldest faecal contamination compared to uninfected control animals.

As a consequence of the concentrating effects of digestion and defecation there tend to be high levels of nutrients, namely nitrogen (N), phosphorus (P) and potassium (K), in areas of faecal deposits ((Hutchings *et al.*, 2000). Thus, animals are faced with trade-offs between foraging nutrient-enriched tall herbage around faecal deposits with the increased risk of parasite ingestion which occurs in these areas.

Given the choice, all animals, independent of parasitic or nutritional status, choose high N containing grass over low N containing grass (Hutchings *et al.*, 1999). These authors also showed that nutrient deficient animals took more parasitic risks, i.e. feeding closer to faeces, than non-nutrient deficient animals. Subclinical parasitism resulted in increased rejection of high N content faecal contaminated pasture, reduced bite rates and reduced bite depth compared to uninfected animals.

Given the limited published material, it does appear that sheep can, and do, attempt to limit their risk of parasite ingestion by avoiding grazing near faecal material. Parasitised animals reduce their risk of further infection by reducing the amount that they eat and by increasing their avoidance of faeces, and even nutritionally deficient animals will not significantly alter their behaviour if the risk of parasitic infection is too great.

1.3.6 Breed

There is considerable evidence which shows differences in susceptibility between different breeds for a range of diseases. For example, *Bos taurus* breeds of cattle in West and Central Africa, namely N'Dama and West African Shorthorn are relatively resistant to trypanosome infection, while *Bos indicus* breeds of European origin are generally very susceptible to infection (Murray *et al.*, 1991). Pena *et al.* (2000) showed that Brangus (*B. indicus* crossbreed) cattle were more resistant to nematode infection then Angus (*B. taurus*) cattle.

Some breeds of sheep are more resistant than others to nematode infection and this is true for all the major nematode species described previously. For example Scottish Blackface sheep are more resistant than Finn-Dorset sheep to *H. contortus* infection (Altaif & Dargie, 1978a) while Romanov sheep are more susceptible then Lacaune sheep to infections with *N. spathiger* and *T. circumcincta* (Gruner *et al.*, 1986). There have been many other studies identifying breed differences and these are summarised in (Gray, 1991).

Gray (1991) suggests that the usefulness of resistance genes present between breeds depends very much on the breed of sheep currently being used and the nature of the product. It would make sense for a meat producer to change breeds, for example, if Dorsets were shown to be more resistant then Border Leicesters. But some of the differences between breeds have been found in comparisons between breeds which have very different production characteristics. For example, Red Masai, Florida Native, St Croix and Barbados Blackbelly are breeds with coarse coloured wool and have all been shown to be highly resistant to *H. contortus* in comparison to European breeds and to Rambouillets or Merinos, yet it is highly unlikely that a producer of high quality wool would undertake the lengthy process of interbreeding to acquire only the genetic characteristics of parasite resistance (Gray, 1991).

1.3.7 Time of weaning

While the intake of solid food is necessary to promote normal ruminal development (Baldwin, 2000), there is evidence to suggest that weaning too early can have detrimental effects on a young animals ability to withstand gastrointestinal nematode

infection. Watson & Gill (1991b) investigated the effect of weaning on antibody responses and nematode parasitism in Merino lambs. Lambs weaned at eight weeks old were compared with control lambs which remained with their dams; both groups grazed the same pasture. Weaning significantly reduced growth rate, control lambs being, on average, 6kg heavier than weaned lambs at 15 weeks old. When lambs were experimentally infected with 5000 *H. contortus* and 10,000 *T. colubriformis* larvae at eight weeks old, the mean faecal egg count for weaned lambs was twice that for controls at 12 weeks old. Serum antibody responses to *H. contortus* and *T. colubriformis* differed significantly between the two groups, with controls responding earlier and more strongly than weaned lambs. The authors suggest that the practical significance of their findings is that up to three months old, suckled lambs, when faced with a substantial parasite challenge, have much better prospects than weaned lambs.

1.4 Pathogenesis of *T. circumcincta* infection

1.4.1 Feed Intake

The majority of gastrointestinal nematodes cause a reduction in voluntary food intake or anorexia, the degree of inappetance being dependent on the level of infection (Symons, 1985). Reduced food intake is also seen in many other parasitic diseases, in various host species, such as those caused by *Eimeria* spp., *Fasciola hepatica*, *S. mansoni* and *A. suum* (Crompton, 1984).

Pair-feeding studies (Sykes & Coop, 1977) revealed that inappetance may account for over 60% of the difference in weight gain between *Ostertagia*- infected and *ad libitum* fed controls. The mechanisms leading to anorexia in *T. circumcincta* infections is still unclear, although there have been several hypotheses suggested. It has been proposed that pain may be important but this is difficult to assess (Holmes, 1987).

Preliminary studies established an association between elevated blood gastrin levels and depressed food intake in *Ostertagia*-infected calves (Fox *et al.*, 1989a). The same authors later demonstrated a 40% depression in appetite in worm free animals when endogenous blood gastrin concentrations were raised indirectly by a human gastric acid secretion inhibitor (Fox *et al.*, 1989b).

More recently, research has concentrated on the role of host hormones and neuropeptides as mediators of inappetance following infection. Administration to rats of cholecystokinin (CCK), a hormone isolated originally from porcine gastrointestinal tract, induced a reduction in both solid and liquid food (Gibbs *et al.*, 1973). Ovington *et al.* (1985) reported that rats with the heaviest doses of *N. brasiliensis*, which expressed the most severe food intake reduction, showed significantly reduced plasma CCK levels, while *T. colubriformis* infections in sheep induced significantly increased plasma CCK concentrations (Symons & Hennessy, 1981). Administration of CCK receptor antagonists increased short term feeding in *T. colubriformis* infected and control sheep while administration of a CCK agonist depressed short term feed intake only in infected animals (Dynes *et al.*, 1998). Total daily feed consumption was not influenced by any of the experimental treatments. The response was greater in infected animals but the stimulation occurred in both groups, so the authors suggest an involvement of central CCK in feed intake in general, rather than a role specific to the parasite-induced disturbance. The importance of CCK is therefore still unclear and may require further investigation.

The role of a further satiety signal, corticotrophin-releasing factor (CRF) as well as two other neuropeptides which promote food intake, neuropeptide Y (NPY) and galanin, have also been investigated in *N. brasiliensis* infections in rats (Fox, 1997). Horbury *et al.* (1995) observed a significant relationship between the degree of *N. brasiliensis* induced anorexia in rats and induction of NPY mRNA. The authors found that NPY gene expression was greatest in animals with the largest reductions in feed intake and suggested that the NPY feeding stimulus was being overridden by other neural or endocrine signals. While these neuropeptides are known to have similar functions in sheep their importance as mediators of parasite induced anorexia remains uninvestigated.

1.4.2 Gastrointestinal function

While infection causes inappetance, there are other consequences such as changes in gut motility, gastrointestinal secretions and in digestion and absorption.

1.4.2.1 Gastrointestinal motility

While one of the more important actions of gastrin is to stimulate acid and pepsin secretion by the abomasum, the peptide also affects the contractility of smooth muscle, inhibiting reticulo-ruminal motility and slowing down gastric emptying (Fox, 1993). This would result in a stasis of ingesta in the reticulo-rumen and abomasum and lead to a reduction in feed intake. Reductions in the rate of passage of ingesta have been reported for infections with *O. ostertagi*, *T. axei* and *H. contortus* (Fox, 1997).

1.4.2.2 Gastrointestinal secretions

It is widely accepted that there are significant changes in gastrointestinal secretions in *T. circumcincta* infections and these include a reduction in gastric acid, and an increase in circulating pepsinogen and gastrin levels.

Developing larvae cause a reduction in the functional gastric gland mass responsible for the production of the highly acidic proteolytic gastric juice; in particular, the parietal cells, which produce hydrochloric acid, are replaced by rapidly dividing, undifferentiated non-acid secreting cells (Urquhart *et al.*, 1996). This leads to a reduction in the acidity of the abomasal fluid, the pH increasing from 2 to 7. This results in the failure to activate pepsinogen to pepsin and so denature proteins. There is also an enhanced permeability of the abomasal epithelium to macromolecules such as pepsinogen and plasma proteins. This leads to a leakage of pepsinogen into the circulation leading to significantly elevated concentrations of plasma pepsinogen following infection and the loss of plasma proteins (plasma and red cells, exfoliated epithelial cells and mucus (Holmes, 1987)) into the gut lumen eventually leading to hypoalbuminaemia (Urquhart *et al.*, 1996).

While an increase in abomasal pH and increased epithelial permeability are important factors in increased gastrin and pepsinogen secretions, they do not appear to be the only factors. It has been suggested that direct physical stimulation of gastrin secreting cells by the parasites may stimulate gastrin secretion or that stimulation of gastrin secreting cells and zymogenic cells could be attributed to chemical stimulation by a parasite derived secretion (McKellar, 1993). *In vitro* studies showed that pepsinogen release (and smooth muscle contraction) could be stimulated by *T. circumcincta* ES antigens

(Scott & McKellar, 1998) while adult *T. circumcincta* prevented from physical contact with the gastric mucosa by restraint in porous bags were able to raise the abomasal pH (Simpson *et al.*, 1999).

1.4.3 Protein metabolism

Although reduced feed consumption and diarrhoea affect liveweight gain they do not wholly account for the loss in production seen following infection. Current evidence suggests that this is primarily because of the substantial leakage of endogenous protein into the gastrointestinal tract.

Despite some reabsorption the protein leakage leads to reduced nitrogen retention and subsequently to reduced growth rates and productivity. In the majority of studies this has been associated with increased urinary nitrogen loss (Holmes, 1987). Sykes & Coop (1977) demonstrated a significant reduction in deposition of fat and protein in infected animals compared to pair-fed controls. There was a reduction in muscle and kidney cortex protein synthesis in infected compared to pair fed animals in the study of Jones & Symons (1982), while the synthesis of liver proteins increased in infected animals. Symons & Jones (1975) showed that protein synthesis by homegenates of wool follicles of sheep with trichostrongylosis was depressed by over 50%.

As a result of such studies Symons (1985) concluded that, due to inappetance, gastrointestinal losses of protein and increased rates of gastrointestinal tissue protein metabolism, there is a net movement of amino acid nitrogen from muscle and skin to the liver and gastrointestinal tract which decreases the availability for growth and milk and wool production.

1.5 The host immune system

Any immune response involves, firstly, recognition of the pathogen or other foreign material, and secondly a reaction to eliminate it. Broadly, the different types of immune response fall into two categories: the innate (or non-adaptive) immune responses and the acquired (or adaptive) immune responses. The important difference between these is that the acquired immune response is highly specific for a particular pathogen. Additionally, although the innate response does not alter on repeated exposure to the

same pathogen, the adaptive response improves with each successive encounter with the same pathogen, in other words the acquired immune response has immunological memory. The cells and processes involved in these immune responses will be described and in particular those involved in the control of parasitic and specifically gastrointestinal infections will be considered.

1.5.1 Effector cells

Immune responses are produced primarily by cells called leucocytes which can be further subdivided to groups of cells called the phagocytes (mononuclear phagocytes, neutrophils, eosinophils) and lymphocytes (B cells, T cells and large granular lymphocytes). There are also another group of auxiliary cells (basophils, mast cells and platelets) that play a role in the immune system.

1.5.1.1 Monocytes/macrophages

The mononuclear phagocyte system has two main functions, which result from the activities of two different types of bone marrow-derived cells. The 'professional' phagocytic macrophages whose main role is to remove particulate antigens, and the antigen presenting cells (APCs), whose role is to take up, process and present antigenic peptides to T cells (Roitt *et al.*, 2001). This group of cells makes up approximately 1-6% of the leukocytes in peripheral blood and 5-20% of the leukocytes in peripheral lymph in sheep (Young, 1998).

Myeloid progenitors in the bone marrow differentiate into promonocytes and then into circulating monocytes which migrate into the various organs to become macrophages (Roitt *et al.*, 2001). The monocytes contain intracytoplasmic lysosomes which contain peroxidase and several acid hydrolases which are important for intracellular killing of microorganisms. Monocytes/macrophages actively phagocytose organisms or even tumour cells *in vitro* by adherence through specialised receptors. These cells also have receptors for IgG and complement with which microorganisms may be coated (Roitt *et al.*, 2001).

The antigen presenting cells are a group of leucocytes with very efficient immunostimulatory capacity. Some have a pivotal role in the induction of T-helper (T_H)

cells and so are seen as the interface between the innate and adaptive immune responses. APCs are found primarily in the skin, lymph nodes, spleen, within or underneath most mucosal epithelia and in the thymus. APCs in the gastrointestinal epithelia (mucosal associated lymphoid tissue: MALT) are termed as follicular dendritic cells and present antigen to B cells. They lack class II (major histocompatibility complex) MHC molecules but bind antigen via complement receptors. Both macrophages and classical B cells are rich in class II MHC membrane molecules and so also have APC capacity (Roitt *et al.*, 2001).

1.5.1.2 Polymorphonuclear granulocytes

The polymorphonuclear granulocytes are a group of short lived cells which mainly consist of the neutrophils. The leukocytes in sheep peripheral blood generally constitute 10-50% neutrophils, 0-15% eosinophils and 0-3% basophils (Young, 1998). These cells do not show any inherent specificity for antigens, but play an important phagocytic role in the acute inflammation usually synergizing with antibodies and complement.

1.5.1.2.1 Neutrophils

Neutrophils are short lived phagocytic cells which are attracted to sites of inflammation by protein fragments released when complement is activated and by the products of other leukocytes and platelets (Roitt *et al.*, 2001). Neutrophils contain antibiotic proteins stored in two types of granules. The primary (azurophilic) granules contain acid hydrolases, myeloperoxidase and lysozyme while the secondary (specific) granules contain lactoferrin and lysozyme. Ingested organisms are contained within vacuoles termed phagosomes which fuse with the lysosomes to form phagolysosomes.

1.5.1.2.2 Eosinophils

Eosinophils are not present in high numbers in the peripheral blood, but larger numbers are found in the tissues, and in particular in the gastrointestinal tract. After a helminth infection eosinophil numbers can increase dramatically (= eosinophilia) in both blood and tissues. There is also a very directional migration of eosinophils towards a parasite target. The granules of eosinophils contain amongst other things eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN). Eosinphils can also produce a range of cytokines (IL-

1,2,3,4,5,6,8,10, granulocyte macrophage colony stimulating factor (GM-CSF), TNF-α, transforming growth factor beta (TGF)-β and IFN-γ) and chemokines (macrophage inflammatory protein (MIP)-1α and eotaxin) (Balic *et al.*, 2000a).

Interleukin-5 (IL-5) is the key cytokine responsible for the dramatic, T-cell dependent eosinophilia in blood and tissues following helminth infection (Roitt *et al.*, 2001). Doligalska *et al.* (1999) showed that in sheep naturally infected with gastrointestinal nematodes, animals with higher concentrations of IL-5 had greater peripheral eosinophilia and those animals with higher numbers of eosinophils had lower faecal egg counts.

Eosinophil degranulation is generally initiated by cross linking of surface receptors. They have receptors for various complement components and immunoglobulin isotypes, including IgG and IgA, but have low affinity for IgE at least in sheep (Balic *et al.*, 2000a). Eosinophils also express receptors for the secretory component (SC) of secretory IgA, and binding of SC provides the most potent stimulus for eosinophil degranulation (Lamkhioued *et al.*, 1995). Specific binding and degranulation of eosinophils on the surface of nematode larvae *in vitro*, mediated by antibody and complement and resulting in killing of the parasite has been demonstrated for human, murine and ovine eosinophils (Balic *et al.*, 2000a). While the main function of eosinophils during nematodes infections is cytotoxicity, in particular through the release of granule proteins and production of superoxide anions, leucotrienes synthesised by eosinophils after activation can mediate vasodilation, smooth muscle contraction and mucus secretion.

1.5.1.2.3 Mast cells and basophils

Mast cells and basophils are very similar in structure and function, although they are completely distinct cell types and derive from the same haemopoietic stem cell (Balic *et al.*, 2000a). Basophils circulate as mature cells in the blood stream from where they can be quickly mobilised to inflamed tissue sites while mast cells reside and mature in the tissues and are not found in the circulation. The major similarity between basophils and mast cells is the cross-linking of high affinity receptors for IgE expressed on the cell surface. There are two different kinds of mast cells; the mucosal mast cell (MMC) associated with the mucosa, and the connective tissue mast cell (CTMC) (Roitt *et al.*,

2001). The expansion of these mast cells in parasitised animals is dependent on cytokines produced by activated T-cells, and in particular IL-3 (Madden *et al.*, 1991). Mast cell numbers in tissues can increase through recruitment and maturation of precursors from the circulation, through local proliferation or more rapidly through migration of mature mast cells from other tissue sites.

Basophils and mast cells contain an array of potent biological mediators such as histamine, leukotrienes and platelet activating factor. They also produce chemotactic factors for neutrophils and eosinophils as well as a range of cytokines and chemokines. As well as IgE-dependent activation, mast cells can be activated and triggered by direct contact with a pathogen or after contact with soluble molecules derived from the pathogen (Balic *et al.*, 2000a).

1.5.1.2.4 Globule leucocytes

The globule leucocyte (GL) is a cell that has been found in the mucosa of many vertebrates (Akpavie & Pirie, 1989). The cell was first described by Weill in 1919 and it was postulated in the 1960s that the GL was an end stage cell resulting from degranulation of MMC (Murray *et al.*, 1968). Kamiya *et al.* (1983) showed that mast cell deficient mice completely lacked GL. Huntley *et al.* (1984) proved that the GL was derived from the MMC, through a transitional cell (TC) stage. Apart from the gradation of granular size from the small granules of the MMC through the mixed large and small granules of the TC to the very large granules of the GL, all three cell types contain proteoglycan, serine esterase, dopamine and surface as well as intracellular immunoglobulin.

1.5.1.3 Lymphocytes

Lymphocytes make up the majority of leukocytes constituting 40-75% of the cells in peripheral blood and over 90% of the leukocytes in lymph (Young, 1998). They are split into B cells and T cells of which there are several types.

1.5.1.3.1 T cells

T cells can develop into effector cells that kill other cells that are infected with intracelular pathogens or that activate other cells of the immune system including

macrphages and B cells. T cell subsets can be distinguished by their different antigen receptors. The definitive lineage marker is the T-cell antigen receptor (TCR) of which there are two defined types: the αβ T cell and the γδ T cell. In most mammals, approximately 90-95% of blood T cells are αβ T cells and the remaining 5-10% are γδ T cells, but in sheep up to 60% of blood T cells can be γδ T cells particularly in perinatal and young animals (Hein & Peterhans, 1998). TCR $\gamma\delta^+$ T cells are also prominent, but do not form a majority, in other recirculating lymphocyte compartments such as those in afferent and efferent lymph. TCR $\gamma\delta^+$ T cells are relatively frequent in mucosal epithelia where they are believed to play an important protective role. Some $\gamma\delta^+$ T cells may be able to recognise antigens directly, with no need for APCs (Roitt *et al.*, 2001).

The αβ T cells are further subdivided into two distinct non-overlapping populations; a subset which carries the CD4 cell surface marker and mainly 'helps' or 'induces' immune responses (T_H), and a subset which carries the CD8 marker and is predominantly cytotoxic (T_C) (Roitt *et al.*, 2001). $CD4^+$ T cells recognise their specific antigens in association with MHC class II molecules, whereas $CD8^+$ T cells recognise antigens in association with MHC class I molecules. Thus, the presence of CD4 or CD8 restricts the types of host cell with which the T cell can interact. There are twice as many $CD4^+$ as $CD8^+$ T cells in peripheral blood, and three times as many in the central and peripheral lymph in sheep (Young, 1998).

The $CD4^+$ T cells can be further classified based on their cytokine production. The T_H1 subset secretes IL-2 and IFN-γ and mediate several functions associated with cytotoxicity and local inflammatory reactions and so these cells are important for combating pathogens including viruses, bacteria and intracellular parasites. The T_H2 subset of T cells produces IL-4, IL-5, IL-6 and IL-10 and are more effective at stimulating B cells to proliferate and produce antibodies, and therefore function primarily to protect against extracellular microorganisms (Roitt *et al.*, 2001).

1.5.1.3.2 B cells

B cells constitute approximately one fifth of the lymphocytes in peripheral blood in sheep and are defined by the presence of surface immunoglobulin. Naïve mature B cells exit the bone marrow and enter the periphery. If these cells do not encounter antigen,

they soon die within a few weeks by apoptosis. If however, these cells encounter specific antigen, they undergo activation, proliferation and differentiation leading to the generation of plasma cells and memory B cells (Roitt *et al.*, 2001). The immune response to most antigens depends on both T cells and B cells recognising the antigen (T-dependent) but some antigens are capable of activating B cells without MHC class II restricted T cell help (T-independent). The T-independent antigens tend to be bacterial cell wall components and induce poor immunological memory.

For B cells to be stimulated they must bind antigen through B cell immunoglobulin (Ig) receptors. They must also receive signals from the T_H cells that respond to processed antigen bound to MHC class II molecules on the surface of the B cell. The most important signal that the B cell receives from the T_H cell is through activation of CD40, a member of the tumour necrosis factor (TNF) receptor family. Upon activation, T cells transiently express a ligand, CD40L, which reacts with CD40 and helps to drive the B cell into cell division. Following activation, antigen specific B cells can follow either of two developmental pathways. The first path involves proliferation and differentiation into antibody forming cells (plasma cells). The function of these cells is to rapidly clear antigen but these cells die within two weeks. In the second pathway the B cells differentiate into memory B cells. These cells have the ability to remember that an antigen has been encountered in the past and are able to quickly differentiate into antigen specific antibody producing plasma cells if the same antigen is produced in the future. This is the basis of the adaptive immune response (Roitt *et al.*, 2001).

1.5.2 Antibody

Antibodies or immunoglobulins are a group of glycoproteins present in the serum and tissue fluids of all higher vertebrates, and on the surface of B cells, where they recognise antigen. There are five distinct classes of immunoglobulin, namely IgG, IgA, IgM, IgD and IgE although there is no evidence that sheep express IgD (Hein, 1998). The different immunoglobulins differ in size, charge, amino acid composition and carbohydrate content. In addition to the differences between classes, the immunoglobulins within each class are also very heterogeneous. Each immunoglobulin molecule is bifunctional, one region of the molecule is concerned with binding to antigen while a different region mediates effector functions such as binding of the

immunoglobulin to host tissues, to various cells of the immune system and to phagocytic cells (Roitt *et al.*, 2001).

The basic structure of all immunoglobulin molecules is a unit consisting of two identical light polypeptide chains and two identical heavy polypeptide chains which are linked together with disulphide bonds in classical 'Y' shape. The class and subclass of the immunoglobulins are determined by their heavy chain type. In humans there are four classes of IgG, unsurprisingly named, IgG1 to IgG4. There are also two classes of human IgA but as yet only one class each of IgM, IgD and IgE has been identified. Sheep have only two classes of IgG; IgG1 and IgG2, but these are not directly comparable to the IgG classes of other species (Hein, 1998). Sheep also have one class each of IgA, IgM and IgE (Hein, 1998). In general the IgG subclasses are the most prominent isotypes in serum, while IgA is most prominent at mucosal surfaces such as the lungs and gastrointestinal tract, but in ruminants there is a significant amount of IgG1 found in the mucosa.

Serum IgA mostly occurs as a polymeric dimer of the basic four-chain unit. In the mucosa the secretory IgA (s-IgA) form of the protein is, in a functional sense, the most important (Roitt *et al.*, 2001). IgA dimers secreted into the intestinal lamina propria by plasma cells bind to poly-Ig receptors on the internal (basolateral) surface of the epithelial cells. The s-IgA-receptor complex is then endocytosed and transported across the cell while still bound to the membrane of the transport vesicles. These vesicles fuse with the plasma membrane at the luminal surface. The poly-Ig receptor is then cleaved by a membrane protease to release the complex. The immunoglobulin thus comes away from the cell with part of the poly-Ig receptor (secretory component) still attached. The secretory component remains with the secreted immunoglobulin and is thought to help protect it from degradation by proteolytic enzymes (Janeway & Travers, 1994).

Following primary antigenic challenge, there is an initial lag phase when no antibody can be detected. This is followed by phases in which the antibody titre increases logarithmically to a plateau and then declines. The decline occurs because the antibodies are either naturally catabolised or bind to the antigen and are cleared from the circulation. The antibody response following secondary antigenic challenge follows a similar pattern but with some distinct differences. The secondary response has a shorter lag time and an extended plateau and decline. The antibody titre is also

significantly greater in secondary responses, typically 10-fold or more than plateau levels in the primary response. There is also a significant change in the class of antibodies released, from IgM in the primary response to IgG in the secondary response. The affinity of the antibodies in the secondary response is also usually much higher, and this is referred to as 'affinity maturation' (Roitt *et al.*, 2001).

1.5.3 The role of the MHC

The major histocompatibility complex (MHC) encodes two sets of highly polymorphic cell-surface molecules, termed MHC class I and MHC class II. The αβ TCR recognises processed antigen as peptide fragments bound to MHC class I or class II molecules. The MHC gene complex was first identified by Gorer in the 1930s when it was observed that histocompatibility, i.e. the ability to accept grafts from another strain, depended on the donor and recipient sharing the same MHC haplotype. A peptide binding cleft is formed by the folding of the MHC molecule. This cleft accommodates peptides that have been processed by the cell, to be presented to T cells. The cleft on class II molecules is more open than that of class I so that longer peptides can be accommodated. Genes of the MHC are among the most polymorphic loci known for vertebrates (Bodmer *et al.*, 1990). Sheep MHC class II contains several different (*Ovar*) genes with additional genes expected to be identified and there are several different alleles at each of these genes. The binding pockets within the clefts are able to accommodate different peptides depending on the haplotype, and because the MHC molecules are so polymorphic, and because cells can express several different MHC molecules, cells can present many different antigenic peptides to a T cell.

1.5.4 Cytokines

Cytokines are small protein signalling molecules which are part of an extracellular signalling network that controls every function of the innate and adaptive immune responses. There are no distinct groups of cytokines rather the nomenclature is based on the originally identified function of the cytokines. The main groups are the interleukins, interferons, tumour necrosis factors, growth factors, colony stimulating factors and chemokines.

Most cell types release one kind of cytokine or another which then react with other cell types. Some cytokines have specific functions but others have many functions. For example, TNF-α induces cells to produce other cytokines, acts as a growth factor, regulates haematopoiesis and lymphocyte development and plays an important role in the regulation of macrophage activation. As mentioned previously $CD4^+$ T_H cells can be identified based on the profile of their cytokine secretion. The T_H1 cytokines include IFN-γ and IL-2 and are associated with cell-mediated inflammatory reactions. The major T_H2 cytokines are IL-4, IL-5, IL-9, IL-10 and IL-13. They are associated with strong antibody and allergic responses. Cytokines from T_H1 cells inhibit the actions of T_H2 and vice versa. Thus, an immune response tends to polarise into a T_H1 or a T_H2 type of response (Roitt *et al.*, 2001).

Cytokines have also been recently shown to control antibody isotype switching. This hypothesis was proposed after it was shown that T cells in mucosal sites in mice were shown to stimulate IgA production (Roitt *et al.*, 2001). IL-4 preferentially switches activated B cells to the IgG1 or IgE isotype with concomitant suppression of the other isotypes in mice. Similarly, IL-5 induces a 5- to 10-fold increase in IgA production with no change in the other isotypes. In mice TGF-β induces the switch to IgA or IgG2b but in humans it only induces IgA production (Xu *et al.*, 2000). Although there are no published articles on the specific actions of cytokines on antibody isotype switching in sheep it is likely their effects will be similar to those in other species. Incidentally, TGF-β2 has also been shown to be positively regulated by estrogen and prolactin (Schneider *et al.*, 1996) and has also been implicated in the reactivation of tissue-arrested *Anyclostoma caninum* hookworm larvae in dogs (Arasu & Kwak, 1999) making it a candidate for influencing arrested development in *T. circumcincta* infections.

1.5.5 Specific immune responses in gastrointestinal nematode infections in sheep

The immune responses to gastrointestinal nematode infections are not fully understood as yet, but there are a series of common immune events which have been elucidated. Mucosal mast cell hyperplasia, the appearance of globule leucocytes, eosinophilia, increased mucous production and the production of specific antibodies define the immune response following gastrointestinal nematode infection.

1.5.5.1 Mast cell hyperplasia and appearance of globule leucocytes

Mucosal mast cell hyperplasia and the appearance of globule leucocytes in the mucosal tissue are well recognised phenomena in both rodent and ruminant gastrointestinal nematode infections (Miller, 1984). Studies have shown that mast cell hyperplasia in the mucosal tissue is predominantly associated with the presence of adult parasites. After a primary infection of lambs with *H. contortus*, an increase in MMC numbers can be observed at 14-15 days post infection (Salman & Duncan, 1984). A small increase in MMC and GL numbers was observed in lambs at five days post infection with 50,000 L_3 *H. contortus* but higher numbers of MMC were detected in lambs bearing adult nematode burdens derived from an infection of 10,000 L_3 (Balic *et al.*, 2000a). This agrees with the studies of Pfeffer *et al.* (1996) and Winter *et al.* (1997) who showed that MMC numbers were only increased at 11-14 days and after 18 days following deliberate infections with *T. axei* and *N. battus* larvae respectively.

Higher numbers of MMC are found in animals which have received multiple or natural infections. The study of Pfeffer *et al.* (1996) showed that in sheep infected weekly with *T. axei*, increasing numbers of MMC were noted for the first five weeks of infection, and numbers then plateaued at levels five times those seen in uninfected sheep. MMC were present after four weeks of weekly infection of sheep with *T. circumcincta* while GL were not found until after eight weeks of continuous dosing (Seaton *et al.*, 1989). The delayed appearance of GL in the mucosal tissue in comparison to MMC is consistent with the GL being derived from the mast cell (Huntley *et al.*, 1984).

The association between resistance to gastrointestinal nematode infection is stronger for GL than for MMC. In sheep grazing contaminated pasture there was a significant negative correlation between adult worm burdens and the number of GL but not with the number of MMC (Douch *et al.*, 1986). A similar finding was reported by Stear *et al.* 1995c) in sensitised lambs given challenge doses of *T. circumcincta.* Degranulation of MMC and GL, assessed by increases in the amount of sheep mast cell protease (SMCP), can occur within two hours after challenge and is associated with rapid expulsion of challenge larvae in previously immunised animals (Huntley *et al.*, 1987). Other mast cell derived mediators such as histamine, leucotrienes, thromboxins and prostaglandins are released during degranulation after challenge with gastrointestinal nematodes (Jones

et al., 1994). Rothwell (1989) suggested that these products of the MMC/GL degranulation make an environment hostile to the nematode due to the inflammation-associated changes in the gastrointestinal mucosa and that they may also directly effect nematode survival.

1.5.5.2 Eosinophilia

There is considerable evidence for a significant role for eosinophils in resistance to at least some gastrointestinal parasites. Increased numbers of eosinophils are seen during primary infection with *H. contortus* (Salman & Duncan, 1984). The number of eosinophils in the abomasal tissue peaked at five days post-infection in the study of Balic *et al.* (2000b), but after which time eosinophil numbers declined but were still significantly higher than in uninfected controls at 27-36 days post infection. Abomasal eosinophil numbers were also slightly increased at 10 and 21 days post infection of sheep with 50 000 L_3 of *T. circumcincta* in the study of (Stevenson *et al.*, 1994). In the same study a biphasic peripheral blood eosinophil response was observed with an initial moderate peak at 7-9 days post-infection, followed by a decline at 14 days, and with a further slightly higher increase until slaughter at 21 days. These results suggest that moderate eosinophilia occurs during primary infection, first in the tissue and then in the peripheral blood and is mainly associated with the larval infection stages rather than the adults (Balic *et al.*, 2000a). The moderate eosinophil response seen in a primary infections seems to have little impact on nematode establishment in the tissues and it is likely that eosinophils in natural host/parasite systems are not sufficiently activated and/or equipped with specific antibodies during primary infections to be effective killers of infective larvae (Balic *et al.*, 2000a).

Eosinophil numbers, both in the mucosa and in the peripheral blood, are significantly higher after repeated or challenge infections (Balic *et al.*, 2000a). Lambs repeatedly infected with *T. circumcincta* L_3 larvae for seven weeks, then drenched and challenged with 50 000 L_3 ten days later, showed increased abomasal tissue eosinophil numbers ten days post-challenge, which were double those observed in lambs which had received only a primary infection (Stevenson *et al.*, 1994). Similarly, Stear *et al.* (1995d) reported a peak at 14 days post challenge of peripheral eosinophils in sheep previously naturally infected and then receiving their second challenge infection of *T. circumcincta.*

The increased numbers of eosinophils in the mucosa and periphery have unsurprisingly been shown to be associated with resistance to infection. Among sheep deliberately infected with *T. circumcincta* local and peripheral eosinophilia (Stear *et al.*, 1995c; Stear *et al.*, 2002) as well as eosinophil-related responses (Stevenson *et al.*, 1994) are associated with resistance to infection as assessed by reduced FEC. In addition, peripheral eosinophilia is often but not always higher in sheep that are resistant to the nematodes *T. colubriformis* (Kimambo *et al.*, 1988) and *H. contortus* (Gill, 1991) as well as sheep selected for resistance to natural infection in New Zealand (Douch *et al.*, 1996a).

1.5.5.3 Lymphocytosis

There are significant changes in the numbers and specificities of lymphocytes following both primary and challenge infections with gastrointestinal nematodes. Almeria *et al.* (1997) reported significant increases in lymphocyte numbers at four days post infection in the abomasal lamina propria and abomasal lymph node of calves after primary infection with *O. ostertagi*. Cell numbers however dropped to control levels by day 8 when the adult worm burden was greatest. Similar findings were reported in primary infections with *H. contortus* (Balic *et al.*, 2000b) and *T. colubriformis* (McClure *et al.*, 1992). In all cases it was the increase in the numbers of γδ T cells, B cells and $CD4^+$ T cells but not $CD8^+$ T cells which accounted for the increase in lymphocyte numbers. As described previously sheep have an unusually large proportion of γδ T cells and the common observation in most infection models of an increase in γδ T cell numbers during primary infection agrees with the increasing role attributed to γδ T cells in directing the immune response profiles during primary infections (Ferrick *et al.*, 1995).

The profile of cellular changes is very similar following challenge infection except that the responses are more intense. The changes are manifested by an increase by an increase in γδ T cells, B cells and CD4 T cells expressing activation markers MHC class II and CD25 (Balic *et al.*, 2002), while CD8 cells generally remain unaltered or decrease (Balic *et al.*, 2000a). This agrees with the general lack of a functional role for CD8 cells in murine helminth infections (Finkelman *et al.*, 1997), and the crucial role of CD4 T cells in immunity to ruminant gastrointestinal nematode infections established by CD4 depletion studies (Gill *et al.*, 1993a).

1.5.5.4 Antibody responses

As for the other components of the immune response, the antibody response is significantly stronger, and is initiated more quickly during challenge compared to primary infections. There is however confounding evidence of which antibody isotypes predominate and are most important in resistance. For example, Gill *et al.* (1993a) reported that increased serum IgG1 and IgA responses were negatively correlated with faecal egg counts in *H. contortus* infected sheep. In contrast was the study of Sinski *et al.* (1995) who reported that in *T. circumcincta* challenge infections IgA and IgG1 levels were negatively correlated, and only increased IgA responses were associated with resistance. A close temporal relationship was observed between the rise in local anti-worm IgA antibodies and the self-cure reaction in *H. contortus* infected sheep in the study of Charley-Poulain *et al.* (1984), but no mention was made of the importance of any other antibody isotype. Sinski *et al.* (1995) demonstrated specifically that an increased IgA response against fourth stage larvae was associated with decreased adult female worm lengths which in turn led to decreases in FEC. There was no correlation between the level of IgA and worm burdens in the study of (Sinski *et al.* (1995). The significant role of IgA in resistance to *T. circumcincta* has also been confirmed in natural infection conditions (Stear *et al.*, 1999b). A significant role for IgA has also been reported for human *Strongyloides stercoralis* infections (Atkins *et al.*, 1999).

Recently, with the production of new monoclonal antibodies against ovine IgE, there have been several studies investigating the protective role of IgE in gastrointestinal nematode infections of sheep. Huntley *et al.* (1998a) stated that there were significant IgE responses to third stage larvae but not to adult worms in sheep challenged with *T. circumcincta*. Subsequently Huntley *et al.* (2001) has shown that increased IgE responses are associated with reduced faecal egg counts. However, these results have not been repeatable in a different population of sheep (M.J. Stear, personal communication). Baker & Gershwin (1993) also demonstrated a negative relationship between IgE and worm burdens in *O. ostertagi* infected calves. A role for IgE in resistance to gastrointestinal nematodes is consistent with the need for IgE in MMC degranulation as described previously.

1.5.6 Interactions and summary

From the current information available it appears that there may be two major mechanisms which attempt to control these gastrointestinal infections in sheep. Firstly, an IgE mediated hypersensitivity reaction involving large numbers of infiltrating MMC and GL is directed against incoming third stage larvae and may operate by controlling worm numbers, particularly in secondary infections. Also, an IgA mediated response targeted against developing fourth stage larvae and adult worms could control worm growth and hence fecundity. The IgA may, or may not interact with eosinophils and as such is an area requiring further work.

1.6 Control Strategies

1.6.1 Anthelmintics and anthelmintic resistance

There are a wide range of chemical anthelmintics available for the treatment and prevention of gastrointestinal nematodes in sheep. Boersema (1985) lists 26 anthelmintics which are split into 6 different classes which are effective against various species of abomasal and intestinal parasites. The different classes are based on the mode of action of the anthelmintics.

Three of the anthelmintic classes make up the majority of commercially used treatments; these are the benzimidazoles, the imidazothiazoles and the avermectins/milbemycins. The benzimidazoles and the imidazothiazoles are effective against virtually all species of gastrointestinal nematodes of sheep and goats as well as cattle. The avermectins/milbemycins are effective against the three abomasal species and some intestinal species of gastrointestinal nematode but are not effective against *Strongyloides* in sheep (Boersema, 1985).

Although these anthelmintics can be very successful in eliminating and preventing gastrointestinal parasitism, particularly when used in conjunction with planned rotational grazing regimes (see later in this section), the emergence of nematodes which are resistant to anthelmintic treatment is giving great cause for concern.

Resistance to benzimidazole (*H. contortus*) was first described by Drudge et al. in 1964 in Australia and New Zealand where animals tended to be drenched more frequently throughout the year than in the UK. The first case of benzimidazole resistance (*T. circumcincta*) in British sheep was recorded in Cheshire (Britt, 1982) about 20 years after thiabendazole was first introduced into the UK (Jackson & Coop, 2000). Since this first report nematode populations resistant to ivermectin (Jackson *et al.*, 1992) and to levamisole (Hong *et al.*, 1996) have also been reported. The incidence of triple resistant nematode species, that is worms that are resistant to the three main classes of anthelmintics described, is also now on the increase in the UK (Coles *et al.*, 1996).

The use and development of new anthelmintics is not sustainable in the long term, as resistance to any new drugs is likely to develop relatively quickly. Also, reports of the advent of genetically modified food products, of disease outbreaks (*E. coli*, BSE, FMD) and of chemical and antibiotic residues in food have heightened consumer concerns about what they are eating (Dalton & Mulcahy, 2001). There are also concerns about the undesirable environmental effects of chemicals. With the rapid emergence of multi-resistant strains of parasite, finding non-anthelmintic methods of parasite control is paramount. Therefore there are a variety of non-chemotherapeutic methods of control currently under investigation and partially in use and these are described in the rest of this section.

1.6.2 Vaccination

Vaccines are safe, leave no chemical residues (and therefore there are no with-holding periods for animals), are environmentally friendly and will be acceptable to consumers and users alike who are already familiar with the concept of vaccination in human medicine (Dalton & Mulcahy, 2001). While there are some vaccines available against such things as toxoplasmosis in sheep and tick borne diseases caused by the tick *Boophilus microplus*, with the exception of that for the bovine lungworm, *Dictyocaulus viviparus*, there are no commercially available vaccines for the control of helminth infections in ruminants (W.D. Smith, 1999).

It is unlikely that antiparasite vaccines will attain the almost 100% efficacy associated with new anthelmintics and bacterial/viral vaccines, but evidence obtained by using computer models of population dynamics of host-parasite interactions indicate that

adequate control can be achieved with vaccine efficacies well below 100% (Knox, 2000). Modelling has demonstrated that a vaccine giving 50% protection at weaning to all sheep was about as effective as a strategic drenching program at controlling worms (Barger, 1996). With a conventional vaccine, excellent control of *T. colubriformis* was achieved with 60% efficacy in 80% of the flock. And with a novel antigen vaccine, results were better than strategic drenching programs if there was better than 80% efficacy or 80% of the flock responded with 60% protection or better (Barnes *et al.*, 1995).

Attempts have been made to immunise both young and older animals with vaccines made from a variety of parasite antigen preparations. Whole and gamma irradiated larval preparations have successfully been used to immunise mature (> 6 months), but not young animals (Knox, 2000). Recent work has concentrated on isolating and identifying specific immunogenic parasite proteins, both somatic and excretory/secretory, which might act as suitable vaccine agents. Antigens which are preferentially recognised by resistant animals could also provide additional markers for the breeding of more resistant lines of animals and this is described later.

McGillivery *et al.* (1989) extracted and isolated a 31 kDa glycoprotein antigen from the infective L_3 stage of *O. circumcincta* which was recognised by total antibody in sera of infected sheep as early as 3 weeks after experimental infection. The same group then reported that the purified 31 kDa antigen had been used to successfully immunise lambs against challenge infections with *O. circumcincta* (McGillivery *et al.*, 1992), but this could not be confirmed in three subsequent trials using the same 31 kDa antigen as an immunising agent (Morton *et al.*, 1995). Newton *et al.* (1997) later reported that this molecule was likely to be a beta- galactoside-binding lectin-like protein (galectin). While these authors did not attempt to test the galectin proteins as vaccine candidates, a galectin (Hco-gal-2) characterised from the gut of *H. contortus* larvae did not confer any protection against infection with *H. contortus* as judged by FEC and worm counts (Newlands *et al.*, 1999).

Surface antigens of *O. circumcincta* L_3 larvae eliciting strong bile and serum IgA responses (Wedrychowicz *et al.*, 1992) were used to vaccinate Finn-Dorset lambs. Worm burdens of immunised and challenged lambs were significantly lower than those in challenged control animals (Wedrychowicz *et al.*, 1995). Mucosal and bile IgM

antibodies recognising the L_3 surface antigens were more prevalent in the vaccinated lambs while there were no differences between groups in the levels of mucosal and bile IgA.

There have been many other studies recently that have used components from larval excretions/secretions and particularly gut and surface derived proteins to immunise animals prior to challenge. Vaccines targeted against gut antigens are particularly appealing since it has been shown that species of *Haemonchus* and *Ostertagia* appeared to ingest host antibody (Murray & Smith, 1994). The majority of these studies have been conducted for *H. contortus* (see Coyne & Brake (2001) for details), and have demonstrated levels of protection ranging from 17 to 95%.

In 1996 Barger (1996) wrote that "the most probable time to commercial availability of a vaccine against *H. contortus* based on concealed antigen was judged to be 5 years, and in excess of 10 years for vaccines against other nematode parasites". Five years has now come and gone and there are no vaccines commercially available. Although a considerable amount of research has been conducted since 1996, it is highly unlikely that a vaccine will be available any time soon. Until a vaccine can be demonstrated to produce adequate protection especially in young vulnerable animals, the necessity to use other methods of control, in particular anthelmintics, will remain.

1.6.3 Nutrition

The importance of nutrition in gastrointestinal infection has been described previously. The use of different protein/energy supplements and plant derived anthelmintics as methods of parasite control has also been discussed.

1.6.4 Grazing management

Grazing management techniques can offer relatively simple and rapid solutions for improving helminth control and reducing anthelmintic usage (Barger, 1997). Alternate grazing of pastures by sheep and cattle is a way of preparing less contaminated pastures; for young sheep, by pre-grazing with cattle or for young cattle, by pre-grazing with sheep (Barger, 1996). Moving newly weaned lambs on to clean pasture, i.e. pasture not grazed by sheep in the previous season, before the expected mid-summer rise in herbage

infection has also been shown to be effective in reducing parasite loads and improving production (Githigia *et al.*, 2001). The same authors also reported that, contrary to some other reports, the benefits of moving lambs to clean pasture could be achieved whether the move was accompanied by anthelmintic treatment or not.

Mixed grazing of cattle and pigs also favours the reduction of *O. ostertagi* larval levels on pasture (Fernández *et al.*, 2001). The reduction in larval numbers is mainly due to the grazing behaviour of the pigs, which by grazing up to the very edge of the cattle faeces, will either expose the larvae in faeces to adverse environmental summer conditions or ingest the cattle larvae, or both. Bird *et al.* (2001) reported a study which undertook to replace a community of sheep endoparasites that had been classified as resistant to levamisole and albendazole with a community of more susceptible parasites using a dilution approach that could be integrated into the management of a commercial flock. Strategically timed anthelmintic treatments combined with pasture management reduced to non-detectable levels the endemic community of anthelmintic resistant parasites in the flock.

The major problems associated with this method of parasite control are the levels of planning required and the fact that pastures cannot be used for grazing for extended periods thereby possibly limiting the numbers of animals a rotational grazing scheme could sustain. This obviously introduces economics into the equation. However, this method of control may be more cost effective than treating higher numbers of animals with anthelmintics. Given the current opinion, grazing management is definitely a viable alternative to anthelmintic treatment although further research would still be welcomed.

1.6.5 Biological control

A further area of non-chemotherapeutic research receiving considerable attention in recent years is that of biological control. Biological control is operationally defined as the action of natural enemies which maintain a host population at levels lower than would occur in the absence of the enemies (Waller & Faedo, 1996). A biological control agent does not eliminate the target organism, but significantly reduces the pest population growth. A large range of organisms have been identified that are capable of parasite egg and/or larval destruction. These organisms exert their effects indirectly, by

habitat (dung) destruction, or directly by using the free-living parasite stages as a food source.

1.6.5.1 Indirect biological control candidates

Earthworms play an important role in the structural decomposition and disappearance of dung (Holter, 1979). Earthworm activity significantly reduced the number of infective larvae on pasture in the study of Grønvold (1987), while experimental plots which had earthworms present had significantly reduced (~63%) infective larval counts compared to plots without earthworms (Waghorn *et al.*, 2002).

Dung beetles also play an important role in dung disposal with each beetle eating more than its own body weight in dung each day (Holter, 1979). Dung beetle activity significantly reduces the number of infective gastrointestinal parasite larvae on pasture (Fincher, 1973).

Recent research has shown that residues of ruminant drug treatments had significant effects on dung beetle mortality which was greatest when drug treatment coincided with the emergence new generations of beetles in the spring (Errouissi *et al.*, 2001). The long term of effects chemical residues on insect ecology and subsequent faecal decomposition remains unclear.

1.6.5.2 Direct biological control candidates

A variety of animal species from different orders have been shown to have direct deleterious effects on parasite larvae and/or eggs. These include microarthropods such as Macrochelid mites, certain protozoa, other predacious nematodes, viruses and bacteria (see Waller & Faedo (1996) for a review). Most of the research however has concentrated on the role of fungi in controlling parasite numbers. The nematode destroying fungi belong to a heterogeneous group of micro fungi that utilise nematodes either as the main source of nutrients or supplementary to a saprophytic existence (Barron, 1977 in Larsen, 2000).

Larsen (2000) lists at least 10 species of fungi which have been shown to have deleterious effects on a range of animal nematode larvae. *Duddingtonia flagrans* has been demonstrated to significantly reduce faecal egg counts and increase liveweight

gain in young sheep on pasture receiving a mixed infection and fed on barley grains on which *D. flagrans* had been cultured (Knox & Faedo, 2001) and in calves under similar conditions (Šarkunas *et al.*, 2000). Fungal spores present in faecal matter have been demonstrated to significantly reduce the number of eggs and larvae of *H. contortus* (Pena *et al.*, 2002), *O. circumcincta* (Githigia *et al.*, 1997) and *T. colubriformis* (Faedo *et al.*, 1997) from sheep, as well as major parasites from cattle, horses and pigs (Larsen, 2000).

The use of *D. flagrans* or other biological methods, alone or in conjunction with other control methods may be an alternative to the chemical control of infection, particularly given the increase in anthelmintic resistance. Additional research must be conducted to identify the most appropriate method of administration of the biological control agents. Any possible implications to the host and subsequently those consuming or using the animals in question must also be investigated.

1.6.6 Breeding for resistance

Domestic animals have been selectively bred for increased production traits since the agricultural revolution. Selective breeding of domestic livestock for enhanced disease resistance is now becoming more common throughout the world. Resistance to mastitis is a component of the selection objective for cattle in Scandinavia (Heringstad *et al.*, 2000), while selective breeding of pigs in Canada for enhanced immune responsiveness shows commercial promise (Mallard *et al.*, 1998). And for many years chickens have been bred for improved resistance to avian lymphoid leucosis complex and Marek's disease (Cole, 1968). Recently, sheep breeders in Australia, New Zealand and the UK have started to breed for resistance to gastrointestinal nematode infections (Bisset & Morris, 1996; Woolaston & Baker, 1996).

To increase productivity, reduce animal suffering and to reduce overall herd infection breeders want to select animals with fewer and/or shorter worms. But to measure these parameters animals would need to be killed, which makes breeding from them difficult. Selective breeding for disease resistance must therefore use markers which are associated with resistance. There are now several phenotypic markers and more recently a few genetic markers that have been associated with resistance.

Breeding from animals that can maintain good productivity (weight gain) in the face of infection is perhaps the easiest marker to monitor.

1.6.6.1 FEC

Selection for reduced faecal egg counts is relatively easy to assess and is now part of breeding programmes. Breeding for reduced FEC also has the added benefit in that pasture contamination will be reduced in subsequent generations. However, there remains some disagreement about the effectiveness of using FEC as a marker since they have shown unfavourable associations with production traits. For example, (Bisset & Morris, 1996) reported that although genetically low FEC Romney lambs had significantly reduced burdens of the most important nematode species, they had no significant production advantages over their higher FEC counterparts when grazed together under the same larval challenge. Indeed some results have indicated a slightly unfavourable association between FEC and wool production and growth rate in lambs (Mcewan *et al.*, 1995). Other limitations to the usefulness of FEC include potential losses of production while drench is withheld for FEC testing, the inability to store samples for long periods and the labour-intensive nature of the method making automation unlikely (Douch *et al.*, 1996a). Bisset & Morris (1996) suggested that the main benefits of selectively breeding for low FEC are likely to be derived indirectly as a result of reduced pasture contamination as described previously.

1.6.6.2 Anaemia

An important clinical sign of parasitic infection for some gastrointestinal nematodes such as *H. contortus* is the degree of anaemia, which may be measured by estimating packed cell volume (PCV) in a haematocrit. Anaemia can also be assessed readily by the sheep breeder by examination of the colour of the ocular mucous membranes since mucous membrane pallor is readily evident when the PCV is lower than 12%. The system, based on the use of a colour chart (FAMACHA© [FAffa Malan Chart] Bath *et al.*, 1997) was launched in 2001 and is now in use by resource-poor goat farmers in South Africa (Vatta *et al.*, 2002). While PCV is highly correlated with *H. contortus* worm burdens (Albers *et al.*, 1987), it is nevertheless a non-specific sign of anaemia and only useful for blood sucking worms such as *H. contortus* (Beh & Maddox, 1996).

There is also the possibility of confounding results from trypanosomiasis which also causes anaemia (Katunguka-Rwakishaya *et al.*, 1992).

1.6.6.3 Total antibody

Douch *et al.* (1995) measured total antibody following natural pasture challenge in Romney sheep in New Zealand. Total serum antibody levels were correlated with FEC (r = 0.56) and heritabilities were moderate in 8-month-old lambs (0.29), and it was concluded that selection based on progeny serum total antibody levels in 8-month-old lambs would result in around 60% of the genetic gain in reduction of FEC as would be obtained by selecting on FEC directly. Much more research has been conducted on the role of specific antibody isotypes on resistance as described previously.

1.6.6.4 IgA

Immunoglobulin A appears to be the most important factor in regulating *T. circumcincta* worm fecundity in Scottish Blackface lambs (Stear *et al.*, 1997). In the same study the heritability of the parasite-specific IgA responses was 0.6 (Stear *et al.*, 2001a). This suggests that parasite specific IgA responses are a possible selection criterion, at least for predominantly *T. circumcincta* infections in Scottish Blackface sheep. For example, Douch *et al.* (1994) investigating *T. colubriformis* infections in Romney lambs, reported strong L_3 specific IgG1 responses but very low L_3 specific IgA responses. Immunoglobulin G1 responses were not correlated with resistance in the study of Stear *et al.* (1995c).

1.6.6.5 IgE

Baker & Gershwin (1993) reported that lymph IgE responses from *O. ostertagi* infected cattle were inversely correlated with worm burdens, but serum IgE levels were significantly lower and so were unreliable for predicting worm burdens. Similarly Huntley *et al.* (1998b) found that IgE concentrations in lymph were 4-fold higher than in serum in Greyface x Suffolk lambs challenged with *T. circumcincta*. Huntley *et al.* (2001) later showed significantly higher *T. circumcincta* L_3 specific lymph IgE antibody in a small number of lambs with low accumulative FEC compared to animals with high accumulative FEC. There was no indication in the latter study as to whether serum IgE

responses were correlated with resistance. While IgE appears to be a useful marker for identifying resistant animals, it is not practicable to routinely take gastric lymph samples for IgE determination. Until serum IgE concentrations are shown to correlate with resistance and so make identification of suitably resistant animals possible, IgE will not be used as a trait as part of any selection scheme.

1.6.6.6 Eosinophilia

Blood and mucosal tissue eosinophilia is a commonly observed feature of the immune response to infection as described previously. However, there are still conflicting reports concerning the relationship between parasitic infections in sheep and peripheral eosinophil counts, therefore questioning the reliability of this trait as a selection criterion. Dawkins *et al.* (1989) showed that the level of peripheral eosinophilia following vaccination and challenge infection was associated with resistance to *T. colubriformis* in selected high responder lambs. Buddle *et al.* (1992) found that the level of eosinophilia was inversely correlated to FEC following both artificial and natural infestation with *T. colubriformis* in New Zealand Romneys, and similar results were obtained for *T. circumcincta* in Scottish Blackface sheep (Stear *et al.*, 1995d). Doligalska *et al.* (1999) reported that although significant, less than 3% of the variation among lambs in faecal egg counts could be attributed to variation in peripheral eosinophil numbers in a two year study of natural mixed gastrointestinal nematode infections in Wrzosowka sheep in Poland.

In contrast to the selected articles described above there are as many which argue the opposite. While tissue eosinophilia was associated with increased resistance to *T. colubriformis* and *H. contortus* in lines of Australian Merinos and New Zealand Romneys selected for resistance on the basis of low FEC in the study of (Hohenhaus & Outteridge, 1995), blood eosinophilia was distinct from eosinophil infiltration of the gut which was poorly associated with resistance. Gill (1991) found no significant differences in circulating eosinophil numbers between resistant and susceptible *H. contortus* lambs while Pernthaner *et al.* (1995) found no significant correlation between FEC and eosinophil counts in resistant Romney lambs. No significant increase in peripheral eosinophil numbers was observed in Romney rams challenged with both *O. circumcincta* and *O. ostertagi* larvae. Woolaston *et al.* (1996) found considerable inconsistency between FEC and circulating eosinophil counts in three different sheep

flocks naturally infected or challenged with *T. colubriformis*. The latter authors suggested that as peripheral eosinophilia appears to be less heritable than FEC, is only informative under certain conditions and is no simpler to measure, and concluded that peripheral eosinophilia offered no advantage over FEC as a selection criterion for resistance. The inconsistent results show that peripheral eosinophilia is not a reliable indicator of parasite burden in sheep and as such the reliability of this trait as a selection criterion remains unclear.

Following natural, predominantly *T. circumcincta* infection, peripheral eosinophil concentrations were higher in animals with lower egg counts but only in lambs that were at least three months of age (Stear *et al.*, 2002). The reduced egg counts were due to lowered parasite fecundity rather than reduced parasite burdens. The authors suggested that eosinophil concentrations might be a useful indicator of resistance to nematode infection but only in older lambs that have been continually exposed to infection.

1.6.6.7 Plasma pepsinogen

Plasma pepsinogen levels correlate significantly with FEC (Stear *et al.*, 1995d). More recently, Stear *et al.* (1999a) demonstrated that there was a very strong correlation between *T. circumcincta* adult female worm lengths and plasma pepsinogen concentrations but very little evidence for an association with worm numbers.

1.6.6.8 Fructosamine

Fructosamines are stable covalent ketoamines formed by the nonenzymatic reaction of glucose with amino acid groups on proteins (Bernstein, 1987). Fructosamine concentration reflects average glucose and protein concentrations as well as rates of protein turnover. Protein turnover increases with nematode infection, and Heath & Connan (1991) have shown that fructosamine concentrations decrease following deliberate gastrointestinal infection. Stear *et al.* (2001b), investigating natural infections on a commercial Scottish Blackface farm showed that fructosamine concentration was a heritable trait and that lambs with increased fructosamine concentrations had fewer worms but the mean length of adult female *T. circumcincta* was longer. Fructosamine concentrations were also significantly associated with variation in the subsequent

acquisition of nematodes. The authors suggested that fructosamine concentrations were potentially useful indicators of the severity of nematode infection and may predict the magnitude of subsequent infection.

1.6.6.9 Mast cell proteinases

Proteinases are released from mast cells during gastrointestinal infection and can be detected in serum (Huntley *et al.*, 1987). Douch *et al.* (1996b) has shown that in *T. colubriformis* challenge infections, serum levels of SMCP were significantly positively correlated with specific anti- *T. colubriformis* L_3 antibody in serum, blood eosinophils and negatively with FEC. These results suggested the potential use of SMCP as a non-invasive indicator of gastrointestinal mast cell responses to nematode infection.

1.6.6.10 Antigen recognition

As all animals may not respond adequately to a vaccine it may be possible to selectively breed animals that preferentially recognise parasite molecules that produce protective immune responses.

Huntley *et al.* (2001) demonstrated that in Greyface x Suffolk lambs experimentally infected with *T. circumcincta*, relatively high IgE recognition of a broad *T. circumcincta* L_3 140-150 kDa band (high molecular weight allergen for *T. circumcincta*; HMW*Tc*) was associated with low FEC. This could not be confirmed in a different population of sheep (M.J. Stear, personal communication).

McCririe *et al.* (1997) investigated the recognition of third stage and adult *T. circumcincta* antigens by plasma IgA from experimentally challenged Scottish Blackface sheep. These authors found that preferential recognition of three third stage antigens was associated with significant differences in worm burdens. Animals which preferentially recognised the L_3 bands at approximately 22, 31 and 48 kDa had worm burdens which were 5, 5, and 7 times greater respectively than animals that did not recognise these bands. Additionally, animals which recognised the adult stage antigens at 28 and 37 kDa had worms which were significantly shorter and significantly longer respectively than animals that did not recognise these bands.

Strain & Stear (1999) studied the same deliberately infected animals as the study of McCririe *et al.* (1997) but investigated the recognition of fourth stage *T. circumcincta* antigens. The authors concluded that recognition of two fourth stage antigens (87 and 129 kDa) was associated with significant reductions in adult female worm lengths and hence fecundity.

1.6.6.11 Genetic markers

The problem with all of the phenotypic markers described is the necessity for an infection to establish, at least in part, before the trait can be measured and superior animals identified. It is desirable that selection criteria be developed that will be informative in the healthy animal and this has become a reality with the use of modern technologies utilising genetic markers.

1.6.6.11.1 Haemoglobin

Sheep have two major alleles (A and B) for haemoglobin. Several studies have suggested that animals with haemoglobin type AA (HbAA) are more resistant then HbAB, which in turn are more resistant than HbBB, to infection with *H. contortus* (Preston & Allonby, 1979) and *O. circumcincta* (Altaif & Dargie, 1978b). However, other workers have been unable to confirm this association (Kassai *et al.*, 1990). Therefore, no general conclusion can be drawn concerning the usefulness of haemoglobin type as a predictive marker for resistance.

1.6.6.11.2 Interferon gamma (IFN-γ)

Crawford (1998) in Coltman *et al.* (2001) suggested there was an association between an allele at a microsatellite locus located in the first intron of the interferon gamma gene and resistance to gastrointestinal parasitism. This has been recently verified by Coltman *et al.* (2001) who reported reduced FEC were associated with differences at the IFN-γ microsatellite allele in free living Soay sheep infected predominantly with *T. circumcincta*. The same allele was also associated with increased *T. circumcincta*-specific IgA levels. These studies are consistent with the idea that a functional polymorphism leading to reduced expression or efficacy of IFN-γ could enhance the

immune response to gastrointestinal nematodes by favouring the activity of the T_H2 cell subset and antibody associated immune mechanisms.

1.6.6.11.3 MHC

The major histocompatibility complex (MHC) plays a vital role in host defence against infection as described previously. The class II genes are among the most polymorphic genes in humans (Bodmer *et al.*, 1990), and assuming the same is true in sheep makes the MHC an ideal candidate for selection. Associations between the MHC and nematode infection have been reported in several species (Stear *et al.*, 1990).

Douch & Outteridge (1989) investigated the relationship between ovine lymphocyte antigens (MHC class I antigens) and parasitological parameters in two flocks of Romney sheep in New Zealand. The study showed that animals that possessed the OLA combination SY 1a + 1b had significantly lower FEC than animals that did not possess this combination. SY 6 occurred significantly more frequently in above average FEC sheep and was associated with significantly higher FEC during secondary challenge infection. Interestingly, OLA SY 6 is also associated with resistance to footrot (Outteridge *et al.*, 1989), and this may be one reason why this method is not yet in use as a selection strategy.

Much more encouraging was the report of Schwaiger *et al.* (1995) which investigated the expression of MHC class II genes in Scottish Blackface lambs naturally infected, predominantly with *O. circumcincta*. This study demonstrated that substitution of the most common allele (I) with allele G2 resulted in a 58-fold reduction in FEC in 6 month old lambs and a 22-fold reduction in 5 month old lambs. The MHC class I antigen G13br was associated with reduced FEC in the same flock of lambs (Stear *et al.*, 1996a). Additionally, there was a significant correlation between presence of the G13br class I antigen and *DRB1* G2 allele, or in other words the alleles are in linkage disequilibrium. Similar associations between alleles at the *DRB1* locus and FEC have been found in Soay sheep (Paterson *et al.*, 1998).

The *DRB1* locus within the MHC in the study of Schwaiger *et al.* (1995) accounted for approximately 10% of the total variation in FEC, and with the addition of sex, sire and dam, the model accounted for 81% of the variation in FEC in 6 month old lambs.

The direct involvement of *DR* molecules in regulating resistance against gastrointestinal nematodes is likely because of their central role in antigen presentation and antibody responsiveness (Schwaiger *et al.*, 1995). The substitution of the I for the G2 allele by selective breeding should produce animals with superior resistance to *T. circumcincta.* However, associations with other economically important traits must first be checked in case breeding for the G2 allele selects animals with poorer productivity and/or increased susceptibility to other pathogens.

1.6.6.12 Selective breeding: conclusions

If genetic markers, or any markers for that matter, for particular traits are identified, the question remains under what circumstances should that marker be included as an additional selection criterion in a selection program for that trait? The answer as always is an economic one; a marker should be used in practice when the financial benefit from the extra response obtained from using the marker is greater than the additional cost associated with the marker (Gray, 1997). The range of markers of resistance is now quite large, and will continue to grow as more markers are identified. If the production and economic gains from selecting for a trait based on a range of markers outweighs the costs of testing then selective breeding, in conjunction with appropriate nutritional enhancement, grazing management and possibly vaccination, appears to be the most suitable method of reducing infection, reducing animal suffering and increasing animal production.

1.7 Objectives

There have been a considerable number of studies which have investigated the immune responses of sheep to infection with gastrointestinal nematodes but gaps in our knowledge remain. In particular there is still some doubt about how useful these measurements of the immune response are as markers of resistance for selective breeding. This thesis aims to determine and attempt to clarify the usefulness of, in particular, the plasma IgA and eosiniophil responses of the infected host as selection markers and subsequently goes on to examine if the MHC is also a suitbable selection candidate.

The initial aim of this thesis was to investigate the kinetics of the host immune response during an artificial challenge infection of Scottish Blackface lambs with *T. circumcincta*. Previous investigations have tended to use immunological and parasitological data collected at or near the time of slaughter to determine resistant animals. While this information is still useful, it is still unclear what is happening as an infection proceeds and how these changes might determine subsequent resistant status. Therefore, the kinetic analyses, of plasma IgA and eosinophilia in particular, detailed in this thesis has attempted to determine whether measurements taken throughout the infection rather than just at the end might be more informative and more accurate in determining relatively resistant from relatively susceptible animals.

The author is aware that using peripheral immune measurements as markers of resistance is not as useful or as informative as those measurements taken locally at the site of infection. However, for a trait to be used as a selection indicator it must be cost effective as well as informative. There would be a significant cost deficit rather than cost benefit in trying to measure local immune responses as animals would require surgery to have cannulae fitted, and this would be impossible for the farmer to do this on site on his own.

Although there are limitations to using peripheral immune measurements, anything is better than nothing. If the cost effective, on-site measurement of these indicator traits is to become a reality for the farmer, peripheral measurements will have to suffice, subsequently, the data reported in this thesis might be of some benefit.

To confirm and extend previous reports which have described associations between recognition of specific parasite larval antigens and resistance, Western blotting assays were conducted utilising standardised plasma IgA samples from the same artificial challenge infection described previously to ascertain whether preferential recognition of specific parasite antigens conferred resistance or susceptibility.

The earlier investigations used plasma samples which were all diluted at the same level and so contained different amounts of IgA. Preliminary investigations suggested that these differences in antibody quantity might confound recognition of parasite antigens therefore all samples were standardised for IgA quantity prior to use in the Western blotting assays. The samples being tested had been examined previously therefore one of the aims of this thesis was to determine whether standardising the IgA concentrations

changed the outcome of the Western blotting recognition assays and if they were in turn more useful and or accurate.

Additionally a much larger group of naturally, predominantly *T. circumcincta* infected sheep were also assessed for plasma IgA specific antigen recognition to determine if naturally infected sheep demonstrated the same associations with resistance as did there deliberately infected counterparts. Animals from this part of the study came from a commercial sheep farm where normal animal husbandary methods were in operation. An investigation of this type is as near to natural as one can get therefore the results gained from this part of the study should be of particular relevance to the sheep breeding industry. An important question of this part of the study was whether resistance appeared to be associated with recognition of a single or of a group of larval antigens.

The MHC is critically important in antigen recognition and the immune system, therefore the final chapter of this thesis aims to establish whether there are any specific associations between MHC polymorphism and plasma IgA recognition of specific antigens and in both deliberate and naturally infected sheep. A similar experiment has been conducted on the experimentally infected animals which looked at total IgG recognition of parasite antigens but this has not been done for IgA. Additionally this is the first time this kind of examination has been conducted on a large number of naturally infected animals. The results of this part of the thesis might have implications for our understanding of the role of the MHC in antigen recognition.

As described earlier in this chapter there is confounding evidence about the impact of MHC heterzygosity and homozygosity on susceptibility to disease and infection. In an attempt to clarify the situation one of the major objectives of this part of the study was to ascertain if MHC homozygotic sheep were more susceptible to infection with single and multiple species of gastrointestinal nematodes as has been demonstrated for several disease conditions of other host species. This has been conducted on the deliberately infected animals and of more relevance, the larger number of naturally infected lambs on the commercial farm, the latter again for the first time.

There is evidence that certain alleles confer resistance and others susceptibility. Intensive selection for the advantage alleles might lead to a situation of increasing homozygosity therefore the inforamtion gained from the work detailed in this section of

the thesis will ascertain whether such a situation would subsequently have a detrimental effect if homozygotes do tend to be more susceptible to infection.

The overall objective of the work detailed in this thesis is to identify markers of resistance and susceptibility which will facilitate the improvement of selection schemes in an efficient and importantly cost effective manner. While the use of peripheral immunological measurements may not be ideal it is better than having no viable measurement at all and when used in conjunction with for example MHC data might significantly assist the sheep farmer in making a more informed decision about which animals should be used for breeding.

Chapter 2

Materials and Methods

In this chapter are detailed the general materials and methods used in this study. Details of specific assays and any minor changes to these methods are detailed in the relevant chapters. Methods which were not carried out in person are acknowledged in the acknowledgement section. The animal and parasitological work described in this thesis was performed prior to the author commencing study. The subsequent work detailed in the thesis (ELISAs, Western blots etc) were performed by the author on stored biological samples taken during the animal experiments. However, the present author has had experience of most of the experimental techniques described on projects which will be published at a later date. Recipes for solutions are given in appendix A. All chemicals are from Sigma unless otherwise stated.

2.1 Parasitological methods

2.1.1 Female worm lengths and number of eggs *in utero*

Adult female worms were selected at random from the abomasal contents and examined microscopically. The number of eggs in each female worm was counted. Worms were measured by image analysis (PC-Image, Foster Findlay Associates Ltd) to determine mean worm length.

2.1.2 Faecal egg counts

The concentration of nematode eggs in faeces was estimated with a modified McMaster technique (Gordon & Whitlock, 1939). Eggs were counted from 3g of faeces taken directly from the rectum of each animal and each egg counted represented 50 eggs per gram of faeces. To reduce measurement variation, initially two then subsequently four replicate counts were made for each sample. With four replicates, each egg counted represented 12.5 eggs per gram of faeces.

2.1.3 Necropsy and total worm counts

Standard parasitological procedures were used to identify and count all nematodes in the gastrointestinal tract (Armour *et al.*, 1966). Specific details are given the relevant chapters.

2.1.4 Preparation of parasite somatic extracts

The strain of *T. circumcincta* used was originally a gift from the Moredun Research Institute and has been subsequently maintained by passage through parasite-naïve sheep at Glasgow University Veterinary School.

2.1.4.1 Third stage larvae

Third-stage larvae were collected from faecal cultures of eggs from monospecifically infected sheep and exsheathed in 1% Milton (Milton 2, Richardson-Vicks Ltd) in phosphate buffered saline (PBS) pH 7.4, for 10 min at 37°C. Exsheathed larvae were then resuspended in 50ml PBS and centrifuged at 1000rpm for 10min. The supernatant was removed and the wash was repeated twice. The larval pellet was then washed once in PBS containing 100IU ml^{-1} penicillin, 0.1mg ml^{-1} streptomycin, 2.5μg ml^{-1} amphotericin B and 0.05mg ml^{-1} gentamicin. Larvae were then given a final wash in 50ml of 10mM Tris buffer pH 8.3, containing 1mM disodium ethylene diamine tetracetic acid (EDTA), 1mM ethylene glycol bis (2-amino ethyl ether)-N,N,N',N'-tetracetic acid (EGTA), 1mM N-ethylmaleimide (NEM), 0.1μM pepstatin, 1mM phenyl methyl sulphonyl fluoride (PMSF), and 0.1mM N-tosylamide-L-phenylalanine chlorormethyl ketone (TPCK) as protease inhibitors (Protease inhibitor solution) ((Maizels *et al.*, 1991)). The larval pellet was then resuspended in an equal volume of protease-inhibitor solution containing 1% sodium deoxycholate and homogenised using a handheld electric homogeniser (Janke & Kunkel IKA Labortechnik, Staufen, Germany) on ice. When larvae were completely homogenised, they were centrifuged at 2000rpm for 20min and the soluble extract was filtered through a sterile 0.2μm syringe filter. The extract was again spun at 2000rpm for 20min and the supernatant aliquoted and stored at –80°C for subsequent use.

2.1.4.2 Fourth stage larvae

Fourth stage larvae were obtained from the abomasum of lambs four days after infection of parasite naïve lambs with 75 000 *T. circumcincta* infective larvae. The abomasum was removed and opened along the greater curvature, the contents were washed under a slow running tap. The abomasum was cut into strips and suspended from a stick in PBS in a Baermann funnel at 37°C. The larvae migrated into the PBS and were collected at

10min intervals from the bottom of the funnel until migration ended. The larvae were then rebaermanised into PBS through surgical gauze suspended in 50ml tubes to remove any gross abomasal debris. Soluble protein extract of fourth-stage larvae was then prepared in the same way as for third-stage larvae except there was no exsheathment.

The protein content of each antigen preparation was determined using a commercial kit (BCA protein assay kit, Pierce, Rockford, Illinois).

2.2 Serological methods

2.2.1 Blood Sampling

Blood samples were collected by jugular venepuncture into evacuated glass tubes containing 20mM disodium EDTA (Becton Dickinson UK Ltd, Oxford, UK) as an anticoagulant. Plasma and buffy coats (the leucocyte rich region of whole blood) were obtained by centrifugation at 1000g for 30 minutes and stored at –20°C until required.

2.2.2 Peripheral blood eosinophil concentration

Ten µl of whole EDTA blood was added to 90 µl of eosinophil diluting fluid (50% propane1,2 diol, 0.1% phloxine B) to give a 1:10 dilution, left for five minutes and duplicate samples counted on a haemocytometer (Dacie & Lewis, 1979). Each cell counted represented 5.6 cells per µl of whole blood.

2.2.3 Plasma pepsinogen concentration

Plasma pepsinogen concentrations were initially determined by the method of (Edwards *et al.*, 1960) for challenge infection part of this study. Subsequently, an improved method, an adaptation of Paynter (1992), was used for subsequent determinations. Details of the methods used are given in the relevant sections.

2.3 Cell culture of rat anti-sheep IgA monoclonal

The cell line (M1521) was originally obtained from Dr. S. Hobbes, Dr. P. Bird and Professor I. McConnell has been successfully maintained by the following culture protocol.

A vial of frozen cells was removed from liquid nitrogen, thawed quickly in water at 37°C and suspended in 10ml of culture media (90% RPMI 1640 (+2mM L-glutamine), 10% Foetal calf serum (FCS), 50μg/ml Gentamicin (Gibco)). Cells were pelleted by centrifugation at 1000rpm for 5 min. Cell viability was checked my mixing 100μl cell suspension with 100μl 0.4% Trypan Blue stain and counting the number of cells on a haemocytometer. Cells were diluted accordingly into culture media to give a cell concentration of $3x10^5$ cells per ml. Cultures were incubated at 37°C in 5% CO_2 and examined on a daily basis.

After approximately 4 days, when cells were confluent, cultures were centrifuged for 10 min at 1000rpm. The supernatant was removed and stored at –20°C in 1ml aliquots to be used as the anti-IgA antibody in the ELISA and Western assays. Cells were then either re-seeded to produce more antibody supernatant or frozen for subsequent culture as follows. Cells were resuspended in 10ml culture media, counted as described previously, then resuspended at a concentration of $5x10^6$/ml in freezing medium (10% dimethyl sulphoxide, 20% FCS, 70% RPMI 1640 (+2mM L-glutamine)). Cell suspensions were then frozen in a "Mr Frosty" cell cryopreservation container (Nalgene), at – 80°C for 24 hours, which allowed for a cooling rate of –1°C/min. Vials were then transferred to liquid nitrogen for long term storage.

2.4 ELISA assays

2.4.1 Enzyme-linked immunosorbent assay (ELISA) for detection of parasite specific ovine IgA

Each well of a 96-well flat-bottomed microtitre plate (Nunc) was coated with 100μl of larval antigen preparation (L_3 or L_4) at 5μg ml^{-1} in 0.06M sodium carbonate buffer pH9.6 overnight at 4°C. The plates were washed five times with 0.01M PBS containing

0.05% Tween 20 (PBS-T). In order to minimise non-specific binding of antibody the wells were incubated with 200µl per well of PBS-T containing 4% skimmed milk powder (PBS-TSM) for two hours at 37°C. The plates were then washed five times in PBS-T. The plates were then incubated with 100µl per well, in triplicate, of individual sheep plasma samples diluted 1:10 in PBS-TSM for 30min at 37°C. Positive and negative controls were run in triplicate on each plate to minimise the effect of variation between plates on different days. After another five washes in PBS-T plates were incubated with 100µl per well of a monoclonal rat IgG anti-sheep IgA diluted in PBS-TSM for 30min at 37°C. After five washes in PBS-T the plates were incubated with 100µl per well of a mouse anti-rat IgG alkaline phosphatase antibody conjugate (Sigma) diluted in PBS-TSM for 30min at 37°C. After a final five washes in PBS-T the plates were incubated in 100µl per well with Bluephos Phosphatase Microwell Substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) at 37°C. The optical density of each well was then read at 635nm with a multichannel spectrophotometer (Titertek Multiscan MC, Labsystems, Oy, Finland or Dynex MRX, Dynex Technologies, Ashford, UK) at five minute intervals, until the positive control optical density reading was between 1.5-2. Different batches of monoclonal and secondary antibody were titrated before use to acquire an appropriate level of dilution.

2.4.2 Enzyme-linked immunosorbent assay (ELISA) for detection of parasite specific ovine IgG

Each well of a 96-well flat-bottomed microtitre plate (Nunc) was coated with 100µl of larval antigen preparation (L_3 or L_4) at 5µg ml^{-1} in 0.06M sodium carbonate buffer pH9.6 overnight at 4°C. The plates were washed five times with PBS-T. In order to minimise non-specific binding of antibody the wells were incubated with 200µl per well of PBS-TSM for two hours at 37°C (Venkatesan and Wakelin, 1993). The plates were then washed five times in PBS-T. The plates were then incubated with 100µl per well, in triplicate, of individual sheep plasma samples diluted 1:1000 in PBS-TSM for 30min at 37°C. Positive and negative controls were run in triplicate on each plate to minimise the effect of variation between plates on different days. After another five washes in PBS-T plates were incubated with 100µl per well of a donkey anti-sheep IgG (whole molecule) alkaline phosphatase antibody conjugate (Sigma, #A-5187) diluted in PBS-TSM for 30min at 37°C. After a final five washes in PBS-T the plates were incubated in

100µl per well with Bluephos Phosphatase Microwell Substrate at 37°C. The optical density of each well was then read at 635nm with a multichannel spectrophotometer at five minute intervals, until the positive control optical density reading was between 1.5-2.

2.4.3 Optical density indices

Positive and negative controls in each ELISA assay were pooled plasma samples from individual animals which had given either very strong (positive control) or very weak (negative control) optical densities in preliminary studies (Sinski *et al.*, 1995).

To minimise the variation between results obtained on different days and between plates, optical densities for each sample were transformed into an optical density (OD) index using Equation 2.1 (Sinski *et al.*, 1995):

$$OD\ Index = \frac{OD\ of\ test\ sample - OD\ of\ negative\ control}{OD\ of\ positive\ control - OD\ of\ negative\ control}$$

Equation 2.1: Equation for transforming the optical density of test samples into optical density indices.

2.5 SDS PAGE and Western blotting

2.5.1 Protein fractionation

Protein fractionation was performed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), using the Biorad Protein II xi electrophoresis system. Preliminary assays employed either 7.5% or 12.5% polyacrylamide gels and details are given in the specific sections. Subsequent assays used linear 5 - 20% gradient gels and are described here.

Gels were cast according to the manufacturer's instructions (Hoefer Scientific Instruments, 1994) both for single % and gradient gels (Appendix A). Gradient gels were formed with a Pharmacia gradient mixer and an LKB Bromma 2132 Microperpex peristaltic pump which was set at '56' so that the gel was cast in approximately 10 minutes.

Gradient gels were run with 20μg protein per lane for both L_3 and L_4 somatic parasite extracts. Protein size markers (Biorad precision protein markers or Biorad broad range markers) were always run in unison. Lanes which were not used for samples or markers were always run with sample loading buffer (Hoefer Scientific Instruments, 1994). This ensured that each well ran the same during separation as samples may have partially diffused horizontally during electrophoresis if adjacent wells had been left empty. Gels were run at a constant 200V with cooling until the dye front reached the bottom of the gel, this was approximately 7 hours for large gradient gels. Proteins were then transferred overnight to nitrocellulose membranes with a pore size of 0.45μm (Biorad) at 60V with cooling using the Biorad Trans-Blot system following the method of (Towbin *et al.*, 1979).

Alternatively, gels were stained with Coomassie Brilliant Blue (Appendix A) at room temperature on a rotating table for at least four hours but usually overnight. The gel was then destained for several hours with multiple changes of destain until the background staining of the gel had been removed. Several pieces of tissue paper were placed in the destaining solution as destaining proceeded as they absorbed the stain as it leached from the gel and so accelerated the destaining process. Gels were then scanned or photographed and either stored in water/alcohol or dried.

2.5.2 Ponceau staining

Following transfer, protein marker lanes were removed. The membrane was then incubated in an excess of Ponceau S stain for 20 minutes on a rocking table to visualise the protein bands. Excess stain was washed off with frequent changes in dH_2O and membranes were then cut into ~5mm wide strips for further processing.

All further steps were carried out in Biorad mini incubation trays at room temperature on a rocking table with a ten second cycle.

2.5.3 Western blotting

To avoid non-specific binding of proteins, membranes were incubated with 1.5ml PBS-TSM for 90min. Following three five minute washes with PBS-T, membrane strips were incubated in 1.5ml of sheep plasma samples diluted in PBS-TSM for 2hr. Following a further three five minute washes in PBS-T, strips were incubated with 1.5ml of rat IgG anti-sheep IgA monoclonal antibody diluted 1:40 in PBS-TSM for 1hr. Strips were washed three times for five minutes and then incubated with 1.5ml of mouse anti-rat IgG alkaline phosphatase conjugate diluted 1:1000 in PBS-TSM for a further hour. Following a final three five minute washes in PBS-T strips were incubated with 1.5ml of BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium) phosphatase membrane substrate (KPL) for approximately 20min to visualise bands. Developed strips were then subjected to two quick washes in dH_20 and then dried in the dark on plastic coated paper overnight.

Dried strips were glued to plastic coated paper, aligned and mounted with previously removed marker lanes for further analysis.

2.5.4 Analysis of Westerns

2.5.4.1 Image capture

Mounted membrane strips were scanned into a PC with an Agfa 1212p document scanner (Agfa-Gevaert Ltd, Brentford, UK) utilising the Agfa Scanwise v1.00 scanner software at a resolution of 200 dots per inch (dpi) to produce a preview image. The original material type was set to "photo matt". The scale was set to 100%, image and scanning resolutions set to 600 dpi, and image control set to greyscale (black and white).

2.5.4.2 Image cleaning

Scanned and saved images were then imported into the Adobe Photoshop 5.5 software package (Adobe Systems UK, Uxbridge, UK) in order to clean up the scanned images. Because of the nature of the assay, small bits of protein sometimes precipitated out of solution, reacted with the substrate and so appeared as black spots on the developed

strips. The TotalLab software used for subsequent analysis of bands often wrongly selected these spots of background "noise" as bands on the blot, therefore they were removed from images.

The image was viewed at 200% size, and using the eyedropper tool, the colours round the spot of "noise" were selected. These selected colours were then used to carefully paint over the spot of noise. Figure 2.1 demonstrates that the cleaning process only eliminated the background spots from the images and did not alter or enhance the banding pattern in any way.

2.5.4.3 Molecular weight determination

Cleaned images were then imported into the TotalLab (v1.11) (Phoretix, Newcastle upon Tyne, UK) software suite for analysis. Although the software can perform the analyses automatically, they were always done manually to assure consistency between separate gel analyses.

The lane creation tool was used to define the positions and areas occupied by the lanes of interest on the Western image. Lane boundaries were specified and adjusted carefully so that background noise at the edge of strips would not be identified incorrectly as bands.

Background subtraction was performed to take account of the background intensity of the membrane material on the image, in effect, the colour of the membrane. This was done so that the calculated band volumes represented the volume of actual material in the band, rather than the volume of all material including the membrane, at each particular position. The rolling disc option was used for background subtraction with a radius set at 100. This option calculates the background as if a disc, with the specified radius, were rolling underneath the lane profile. The larger the radius of the disc, the less the background rises with the profile.

Band detection was then performed. This was done automatically using the following parameters and then checked manually. The minimum slope figure represents how pronounced the band must be from its surrounding area in the lane and was set at 30. The noise reduction figure represents the degree to which small local peaks should be ignored on the profile and was set at 3. The percentage maximum peak is a threshold

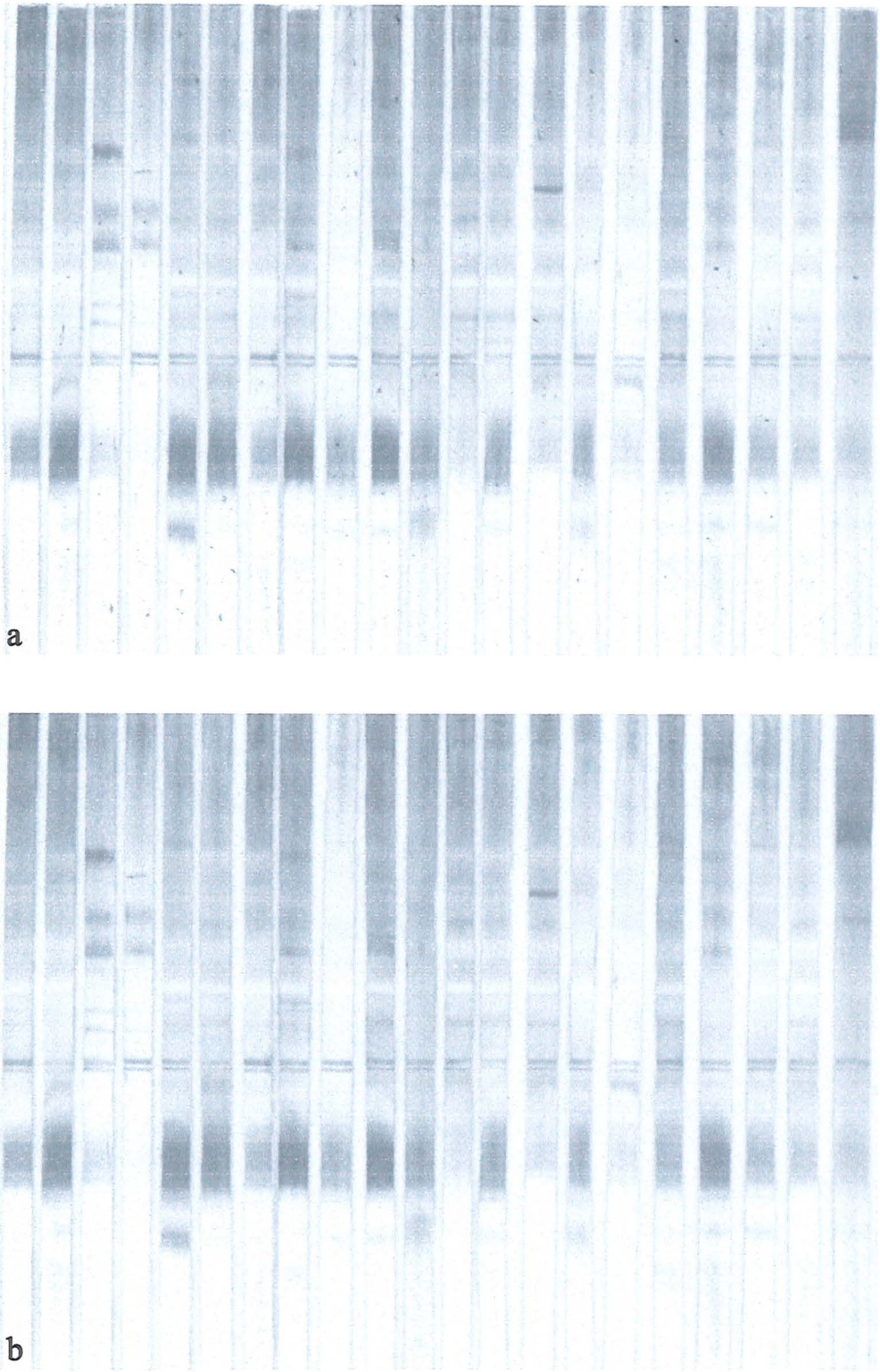

Figure 2.1: Example of background noise removal from Western blot. (a) before and (b) after cleaning process. Note that the cleaning process only eliminated the dark spots of noise and did not interfere with the banding patterns on the blot.

parameter which discards peaks of under a certain size in relation to the highest peak and was set at zero. Despite the cleaning of images to remove background noise the sensitivity of the software still sometimes suggested that bands were present when in fact it was just background noise. Therefore, all membranes were examined carefully by eye and compared to those identified by the software in order to confirm whether bands were present or not. Only bands which both the software and user could identify were used.

The molecular weight of unknown bands was calculated by assigning known molecular weights to marker lanes on each gel. A log curve of the marker lane was plotted by the software, from which it extrapolated sizes for each of the unknown bands detected. For each calibration the software gave an R^2 value. R^2 or the coefficient of determination is the proportion of the variability in the response variable (y) that can be accounted for by the predictor variable (x). R^2 values were consistently above 0.9 suggesting that the marker lanes very accurately fitted the regression model calculated and could be used to assign sizes to unknown bands with confidence.

TotalLab produced a table of results for each analysis which contained values for estimated molecular weight of each band, band volume and band intensity. The software assigned a reference number to each of the approximately 70 bands recognised across each gel. Because of the sensitivity of the curve fitting algorithm used by the software, bands assigned the same reference number in different lanes could have small differences in molecular weight. Bands were scored visually as being the same or different, across and between gels. The size of identical bands was then averaged across all gels to give an approximate band size for each specific band. Molecular weights of bands were also rounded to the nearest 1000, or 500 Da where necessary.

Two matrices for band recognition were then produced. The first used peak responses for positive bands while those which were not recognised were given a value of zero. The second matrix simply used ones and zeroes for positive and negative recognition of bands respectively and did not take into account the strength of the antibody response to a particular parasite antigen. Subsequent analyses of band recognition data specify whether the peak value or positive/negative matrices were used where necessary.

2.6 Adjusting antibody concentration for ELISAs and Westerns

A titration of the positive control used in the ELISA assay was performed varying its concentration. Doubling dilutions from 1:2.5 to 1:5120 in PBS-T were tested and used to construct a calibration curve from which dilutions of test samples could be estimated. Details of each assay are described in specific chapters.

2.7 *MHC DRB1* typing

Polymorphism at the class II DRB1 locus was defined with a DNA-based procedure which defined alleles on the basis of variation in the length of a microsatellite within the intron lying between exons 2 and 3 and on hybridisation of the coding region with specific oligonucleotides (Schwaiger *et al.*, 1995). The MHC typing data used in the present study were generated from the natural and deliberate infection studies of Schwaiger *et al.* (1995) and McCririe *et al.* (1997) respectively.

2.8 Test for the homogeneity of correlation coefficients

The "test for the homogeneity of correlation coefficients" (Gomez & Gomez, 1984; Sokal & Rohlf, 1995) was used to check that the correlations between ELISA and Western IgA responses at distinct time points could be considered as samples from a population exhibiting a common correlation among the variables. The Costat v6.003 computer package was used after downloading from www.cohort.com (CoHort Software, Monterey, CA, USA).

The software package conducted the test as follows:

Step 1: The correlation coefficients were calculated for the k sets of data. Each correlation coefficient was denoted r1, r2,..., rk.

Step 2: Each computed r value was transformed to a value of z according Fisher's z-transformation:

$$z_i = 0.5 \ln \frac{1+r}{1-r}$$ where ln is the natural logarithm.

Step 3: A weighted value of z was calculated and the following table of data produced:

(1) n_i	(2) n_i-3	(3) r_i	(4) z_i	(5) weighted $z_i=(n_i-3)z_i$
5	2	0.9409	1.7460	3.4920
5	2	0.3448	0.3595	0.7191
5	2	0.9415	1.7515	3.5029
5	2	0.9613	1.9634	3.9267
5	2	0.9153	1.5593	3.1187
5	2	0.8896	1.4199	2.8399
5	2	0.9333	1.6830	3.3661
35	14		10.4826	20.9652

Step 4: The weighted mean of the z values were then calculated as:

$$\overline{z}_w = \frac{\sum_{i=1}^{k}(n_i-3)z_i}{\sum_{i=1}^{k}(n_i-3)} = \frac{\text{sum of column (5)}}{\text{sum of column (2)}} = \frac{20.9652}{14} = 1.4975$$

where n_i (i =1,...,k) is the number of paired observations in the ith set of data for the computation of the r_i value in step one. When n_i is the same for all sets of data (as is the case here), the z value is simply the standard arithmetic mean of the sum of the z_i values (column 4).

Step 5: The χ^2 value (chi-square) was then calculated as:

$$\chi^2 = \sum_{i=1}^{k} (n_i - 3)(z_i - \bar{z}_w)^2$$

In the example here this equates to:

$\chi^2 = 2(1.7460 - 1.4975)^2 + 2(0.3595 - 1.4975)^2 + 2(1.7515 - 1.4975)^2 + 2(1.9634 - 1.4975)^2 + 2(1.5593 - 1.4975)^2 + 2(1.4199 - 1.4975)^2 + 2(1.6830 - 1.4975)^2 = 3.3669$

Step 6: The computed χ^2 value was compared to the tabular χ^2 values with 6 (k-1) degrees of freedom. The hypothesis of homogeneity of the linear correlation coefficients would be rejected if the computed χ^2 value was greater than the corresponding tabular χ^2 value at the prescribed level of significance. The tabular χ^2 values with 6 degrees for freedom are 12.592 and 16.812 at the 5% and 1% levels of significance respectively. These were higher than the computed χ^2 value, therefore, the test was not significant and the hypothesis of homogeneity could not be rejected.

Step 6: As the χ^2 test was not significant, a value for the pooled linear correlation coefficient could be computed according to:

$$r_p = \frac{e^{2\bar{z}} - 1}{e^{2\bar{z}} + 1} = \frac{e^{2(1.4975)} - 1}{e^{2(1.4975)} + 1} = 0.905$$

Thus, the simple linear correlations between the plasma IgA responses measured by ELISA and Western assays can be measured with a single coefficient of 0.905. This value is also higher than the simple arithmetic mean of the seven individual correlations (0.847).

Chapter 3

Kinetics of the immune response of Scottish Blackface sheep to a challenge infection with *Teladorsagia circumcincta*

3.1 Introduction

Due to the emergence of anthelmintic resistance in parasite populations, non-chemotherapeutic methods are being investigated to control *T. circumcincta* infection (Beh & Maddox, 1996). Breeding for resistance to infection remains one of the major foci of research. A detailed understanding of the immune mechanisms involved in the resistance to infection will in general lead to better more sustainable methods of control and in particular will aid in the identification of resistant animals.

The roles of host antibodies, effector cells such as mast cells, basophils, globule leucocytes and eosinophils, as well as cytokines in relation to immune responses to parasitic infections, particularly of ruminants has been described in chapter one.

Increased IgA concentrations in mucus and plasma are negatively associated with adult female worm lengths and subsequently worm fecundity in *T. circumcincta* infections (Stear *et al.*, 1995c). Immunoglobulin E is also well recognised as an important antibody isotype during infections of many parasite species such as *T. colubriformis* (Shaw *et al.*, 1998), *O. ostertagi* (Baker & Gershwin, 1993), and *T. circumcincta* (Huntley *et al.*, 1998b). While IgG1 and IgG2 are the predominant antibody isotypes in ovine serum, IgA predominates in mucosal secretions. Although IgG1 and IgG2 are found in the mucosa only the IgG1 subclass is found at significant concentrations and even then only at levels which are at the lower end of the range of concentrations for IgA (Lascelles *et al.*, 1985; Smith *et al.*, 1975). The IgG2 found in the mucosa could be caused by leakage from the blood. Therefore, the importance of the IgG subclasses as mediators of gastrointestinal parasitic infections at the local level remains unclear.

An increase in the number of eosinophils (eosinophilia) is a distinctive feature of helminth infections (Behm & Ovington, 2000; Meeusen & Balic, 2000). Eosinophil degranulation is generally initiated by cross-linking of surface receptors. They have receptors for various complement components and immunoglobulin isotypes, including IgG and IgA and low-affinity for IgE at least in sheep (Balic *et al.*, 2000a). Eosinophils also express receptors for the secretory component (SC) of secretory IgA, and binding of SC is a potent stimulus for eosinophil degranulation (Lamkhioued *et al.*, 1995).

This intrinsic association between IgA and the eosinophil suggests that they may play an important complementary role in combating gastrointestinal parasitic infections. Stevenson *et al.* (1994) studied the kinetics of the eosinophil response following *T. circumcincta* infection, while the kinetics of the host IgA response have also been described (Smith *et al.*, 1985a and 1985b; Wedrychowicz *et al.*, 1992). However, there remains a gap in our knowledge about the joint kinetics of the host antibody and eosinophil responses and how these relate to resistance.

There is also a need to ascertain how some of the other indicator traits change as an infection proceeds and how these relate to resistance. For example, plasma pepsinogen concentrations rise following abomasal nematode infections in cattle and sheep, although the precise mechanisms of its release in to the periphery are not understood (Fox, 1997). Therefore plasma pepsinogen concentration can be used as a marker for diagnosis of infection but how it relates to the other markers across time and how in turn they relate to infection remains unclear. In addition, how the kinetics of the egg output proceeds following infection in relation to the other variables needs consideration.

Previous studies have tended to use data from post-mortem examinations to determine the relative resistance of infected individuals, i.e. the IgA and peripheral eosinophil response at slaughter (Sinski *et al.*, 1995; Stear *et al.*, 1995c). An increased knowledge of the kinetics of the immune response following infection may simplify identification of resistant animals.

This portion of the study therefore has two aims. Firstly, to investigate the kinetics of various components of the host's response to challenge infections with *T. circumcincta* in Scottish Blackface lambs. Additionally, it also considers how the measurement of indicator traits during an infection correspond to subsequent measures of resistance and whether they can enhance existing methods of identifying resistant animals.

3.2 Materials and methods

3.2.1 Animals and Experimental Design

Thirty female Scottish Blackface lambs were selected at random from a flock of 200 lambs on a commercial farm in Southwest Strathclyde. The lambs were born within a two-week period in the second half of April 1991, and were kept together on the same field after weaning at three months until purchase at six months of age. All lambs had had been exposed to natural, mixed, predominantly *T. circumcincta* on the farm before selection.

At six months old the thirty lambs were transferred to Glasgow Veterinary School and housed in one group, indoors, on straw-covered concrete floors under conditions designed to prevent accidental re-infection with nematode parasites. At nine months of age, when egg counts were low, but not zero, 24 of the 30 sheep were each given a single oral dose of 50 000 infective, third-stage (L_3) larvae of *T. circumcincta*. (This strain originally came from the Moredun Institute, Edinburgh, and had successfully undergone passage through parasite free sheep at least 15 times). After eight weeks 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 dose rates recommended by the manufacturers, which were 7.5mg kg^{-1} and 5mg kg^{-1} respectively. After a further four weeks, when all lambs consistently had egg counts of zero, the same 24 lambs were reinfected with 50 000 infective *T. circumcincta* L_3 larvae. A further three lambs were also infected at the second date only while the remaining three lambs served as experimentally uninfected controls. All animals were monitored for a further 61 days and were then necropsied at the local abattoir. All assays performed in the present study relate to the period of the second challenge infection just before slaughter.

Although this experimental design is very artificial, animals ingest small numbers of larvae over a long period of time, it was chosen so that so that the immune response kinetics of a single larval challenge could be monitored. It was hypothesised at the time of the experimental work that the immune response might be magnified by using a single large larval challenge and therefore make identification of marker traits more obvious.

3.2.2 Parasitological methods

3.2.2.1 Necropsy and total worm counts

The gastrointestinal tract was removed and examined for parasites. There were no nematodes or cestodes present on microscopic or macroscopic examination of the small intestinal contents or on macroscopic examination of the large intestine. The abomasum was removed and opened along the greater curvature and the inner surface washed with tap water under moderate pressure. The contents and washings were made up to 2L from which 10 separate 2ml aliquots from a 200ml subsample were examined to estimate the size of the adult nematode population (Armour *et al.*, 1966).

One half of the abomasum was used to collect mucus and mucosal tissues for antibody detection and histology. The other half of the abomasum was digested with pepsin-HCl for six hours at 42°C. The digest was made up to 2L and ten 2ml aliquots were examined to estimate the number of larvae present (Herlich, 1956). At slaughter, the three uninfected control animals were free of parasites.

3.2.2.2 Female worm lengths and number of eggs in utero

Methods for assessing worm lengths and the number of *in utero* eggs are described in chapter two. Where possible 100 worms were measured but in 14 sheep less than 100 worms were found. In these sheep, all recovered worms were measured.

3.2.2.3 Faecal egg counts

The number of nematode eggs in faeces was determined with a modified McMaster technique as described in chapter two. Faecal egg counts were estimated three times each week during the challenge infection.

3.2.2.4 Preparation of parasite somatic extracts

Methods for the preparation of *T. circumcincta* L_3 and L_4 somatic extracts are given in chapter two.

3.2.3 Immune cell histology

Tissue samples were taken from the fundic and pyloric regions of the abomasum and fixed in 10% Neutral buffered formalin or Carnoy's fixative, processed in a standard cycle, then embedded in paraffin wax. Sections 5μm thick were cut and stained with haematoxylin and eosin for enumeration of globule leucocytes and eosinophils, or stained with 0.1% Astra Blue in 0.7N HCl and counterstained with 0.05% safranin for enumeration of mast cells.

The numbers of each cell type were estimated by systematic scanning of the complete tissue section from the basement membrane of the lamina propria to the mucosal surface. The area examined depended in part upon the tissue section and ranged form 2 to 16 mm^{-2}. At least 100 fields of view were counted for all cells in each animal.

3.2.4 Serological methods

3.2.4.1 Blood Sampling

Plasma and buffy coats were collected from blood samples as described in chapter two. Samples were taken before both infections, two or three times each week during infection and immediately before slaughter.

3.2.4.2 Peripheral blood eosinophil concentration

Peripheral blood eosinophil numbers were enumerated from each blood sample collected according to the method described in chapter two.

3.2.4.3 Plasma pepsinogen concentration

Plasma pepsinogen concentrations were determined once each week during the infection by the method of (Edwards *et al.*, 1960). The method is based on the transformation of pepsinogen into pepsin in an acid environment, and the digestion of protein to release tyrosine. Quantification of tyrosine was determined after protein precipitation and filtration by spectrophotometry using Folin Ciocalteau's method.

3.2.4.4 Cell culture of rat anti-sheep IgA monoclonal

The rat anti-sheep IgA monoclonal antibody used in the IgA ELISA was prepared as described in chapter two.

3.2.5 ELISA assays

Parasite specific host plasma IgA and IgG responses to infection were measured by ELISA as described in chapter two.

3.2.5.1 Summary of ELISA assays performed

To examine the kinetics of the antibody responses a preliminary assay was performed with seven plasma samples from each of only four animals. Plasma samples collected approximately every ten days were tested for IgA and IgG activity against both L_3 and L_4 *T. circumcincta* somatic extracts.

Based on the results of these preliminary assays the following assays were performed on plasma samples from all animals:

i. Nineteen plasma samples spanning the duration of the challenge infection were tested for IgA activity against L_3 somatic extracts.

ii. Plasma IgA and IgG activity were measured in samples taken at ten days post challenge against L_3 somatic extract. This was the time of the peak IgA response from the preliminary kinetic analyses.

iii. Plasma IgA and IgG activity were also measured in blood samples collected immediately before slaughter against both L_3 and L_4 somatic extracts.

3.2.6 Statistical analyses

In order for datasets to be used in parametric analyses they should be normally distributed (Sokal & Rohlf, 1995), therefore the normality of each dataset was checked

and transformed if necessary using the following procedure in Minitab v13.3 (Minitab Inc., State College, PA, USA).

The Anderson-Darling test for normality was initially used to assess the distribution of raw data. This test was preferred to the Kolmogorov-Smirnov test as it is more sensitive to deviations in the tails of the distribution (D'Agostino, 1986). Data were also plotted to graphically assess the normal probability plot. If the *P*-value of the Anderson-Darling normality test was greater than 0.05 then the data were considered not significantly different from the normal distribution and were not transformed. If the *P*-value was less than 0.05 then the data were considered to be significantly different from the normal distribution and required transformation.

The Box-Cox Transformation (Box & Cox, 1964) option in Minitab was then used to estimate the optimum transformation for normalising each dataset (Equation 3.1).

$$y' = \begin{cases} y^{\lambda} & (\lambda \neq 0) \\ \log y & (\lambda = 0) \end{cases}$$

Equation 3.1: Equation for Box-Cox transformation where y´ is the transform of the data y and λ is the transformation parameter lambda.

The Box-Cox transformation calculates the optimum lambda value for normalising the dataset and transforms the data accordingly. For simplicity, the λ value nearest to the values in Table 3.1 was used.

Logarithmic transformations were to the base ten of the count or the count plus ten if there were zero values. Following transformation, data were then subjected to the Anderson-Darling test to check that the data had indeed been normalised. All datasets used in subsequent analyses, either with or without the requirement for transformation were normally distributed.

Lambda (λ) value	Transformation
$\lambda = 2$	$y' = y^2$
$\lambda = 1$	$y' = y$
$\lambda = 0.5$	$y' = \sqrt{y}$
$\lambda = 0$	$y' = \log y$
$\lambda = -0.5$	$y' = 1 / (\sqrt{y})$
$\lambda = -1$	$y' = 1 / y$

Table 3.1: Summary of transformations for Box-Cox analysis using different values of λ.

Pearson product moment correlation coefficients were calculated in Minitab while the general linear model (GLM) program in the SAS package (SAS Institute, Cary, NC, USA) was used for regression analysis using adjusted (type III) sums of squares.

For all analyses results were considered significant if $P \leq 0.05$.

3.3 Results

3.3.1 Normalising data by Box-Cox transformation

Box-Cox analyses were used to provide suitable transformations to normalise data for further analysis. An example of the transformation process follows and utilises data from the total number of *T. circumcincta* counted at necropsy (adults + L_4 larvae + L_5 larvae). Raw data were first plotted to graphically assess the degree of normality, while the Anderson-Darling test gave a numerical estimate of normality (Figure 3.1). The *P*-value in this example was lower than the designated critical value of 0.05, therefore the distribution was considered significantly different from the normal distribution. Raw data were then subjected to the Box-Cox analyses which estimated the optimum lambda (λ) value for transforming the data to a normal distribution as 0.112 (Figure 3.2). The Box-Cox process also estimated the 95% confidence limits for the optimum λ value. As λ was close to zero, a log10 transformation was used.

Log transformed data were then plotted and the Anderson-Darling test used to determine whether the transformation had been successful in normalising the data (Figure 3.3). The log transformed data were much less skewed, and the *P*-value of 0.349 from the Anderson-Darling test demonstrated that the distribution was not significantly different from the normal distribution.

This process was applied to each variable in turn and data were transformed as necessary. Table 3.2 gives descriptive statistics about the different variables that were measured at post mortem, while Table 3.3 gives details about the relevant transformations that were used and how successful the transformations were in normalising the data. Data for worm lengths (WL), the number of mucosal mast cells (MMC) and the number of eggs per female worm (EPF) were not transformed as the

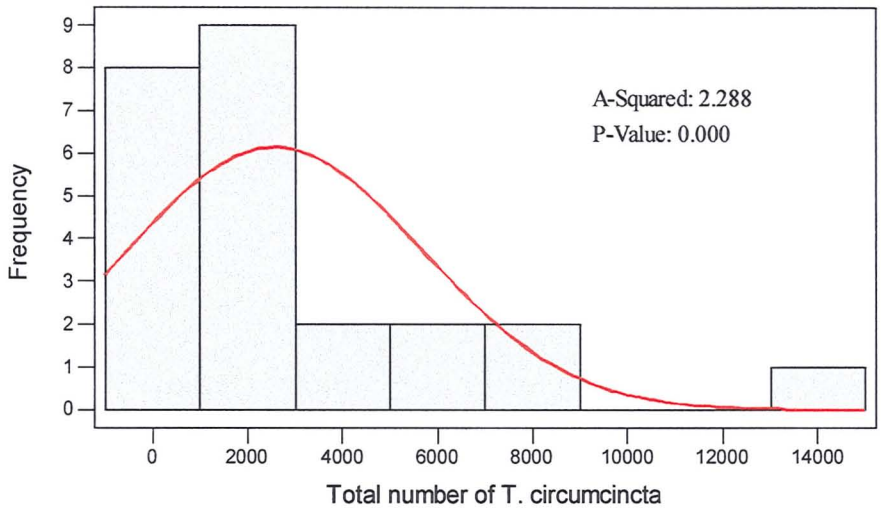

Figure 3.1: Histogram, overlaid with a normal curve with the same mean and variance (--), of total *T. circumcincta* numbers (adults + L_4 larvae + L_5 larvae) in Scottish Blackface lambs following challenge infection. A-squared is the Anderson-Darling statistic and *P* is the probability that the results were due to chance.

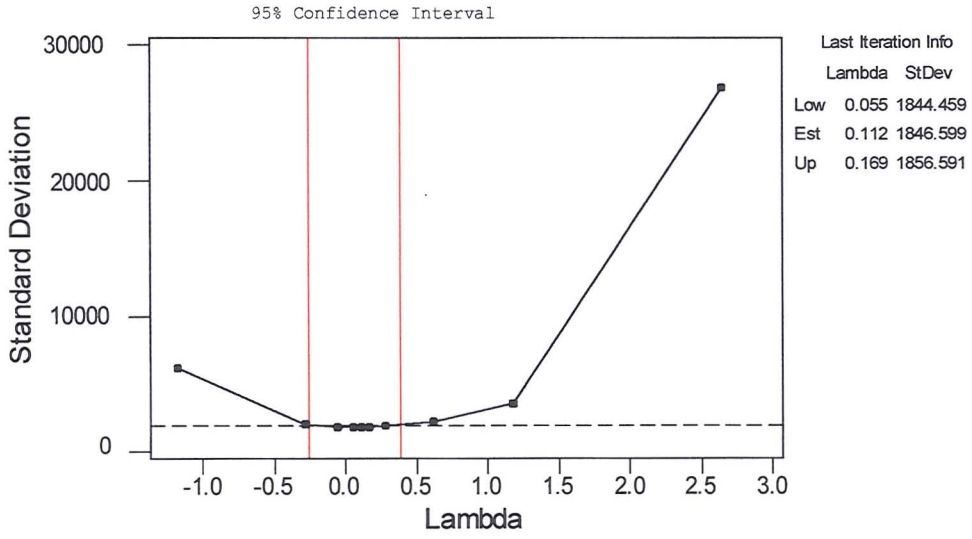

Figure 3.2: Box-Cox plot of total *T. circumcincta* numbers (adults + L_4 larvae + L_5 larvae) in Scottish Blackface lambs following challenge infection to ascertain the optimum transformation. Ninety-five percent confidence limits for the test are shown (--).

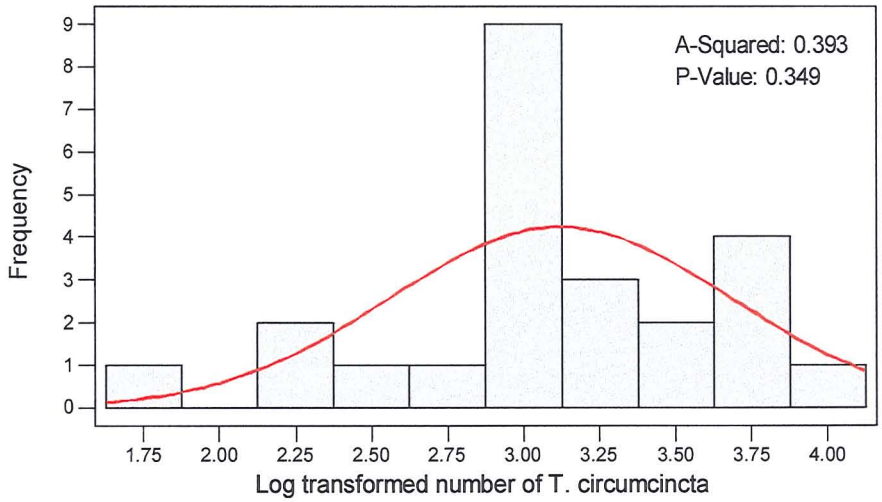

Figure 3.3: Histogram, overlaid with a normal curve with the same mean and variance (—), of the log transformed number of *T. circumcincta* (adults + L_4 larvae + L_5 larvae) in Scottish Blackface lambs following challenge infection. A-squared is the Anderson-Darling statistic and *P* is the probability that the results were due to chance.

Variable	Mean	Variance	Standard Error	Skewness	A^2	P	Transform Data
No. of L_4 larvae	95.833	48242	44.83	3.415	4.477	0.000	Yes
No. of adult larvae	2379	8614112	599	2.343	2.129	0.000	Yes
Total *T. circumcincta* burden	2589	9713691	636	2.098	2.288	0.000	Yes
Worm Length (cm)	0.951	0.028	0.034	1.404	0.543	0.146	No
Adult worm mass (No. of adults x WL)	2338	6862889	535	1.862	2.077	0.000	Yes
Eggs per female worm	31.22	259.33	3.29	0.254	0.346	0.453	No
Mucosal mast cells	12921	7.50 E+07	1768	0.536	0.339	0.471	No
Mucosal eosinophils	14339	6.47 E+08	5193	3.495	3.942	0.000	Yes
Globule leucocytes	9593	4.9 E+08	4516	3.854	5.419	0.000	Yes
IgA positive plasma cells	6136	1.45 E+08	2458	2.424	4.757	0.000	Yes

Table 3.2: Descriptive statistics of parasitological and histological variables measured at slaughter in Scottish Blackface lambs eight weeks after receiving a challenge infection with *T. circumcincta* L_3 larvae. A^2 is the Anderson-Darling statistic for normality and *P* the probability that the results were due to chance. *P* values under 0.05 were considered significantly different from the normal distribution and required transformation. *P* values greater than 0.05 indicated that the observed distributions were not significantly different from the normal distribution and so did not require transformation. All cell concentrations are cells mm^{-2}.

Variable	Optimum λ	λ used	Transformation	Skewness of transformed data	A^2 of transformed data	P
No. of L_4 larvae	–0.450	0	Log10(y)	0.874	4.053	0.000
No. of adult larvae	0.112	0	Log10(y)	–0.53	0.33	0.494
Total *T. circumcincta* Burden	0.112	0	Log10(y)	–0.51	0.393	0.349
Adult worm mass	0.113	0	Log10(y)	–0.78	0.447	0.257
Mucosal eosinophils	–0.112	0	Log10(y)	0.334	0.233	0.774
Globule leucocytes	–0.337	0	Log10(y)	1.002	0.594	0.110
IgA positive plasma cells	0.113	0	Log10(y)	0.242	0.244	0.738

Table 3.3: Summary of transformations to normality by Box-Cox analyses in parasitological and histological variables measured at slaughter in Scottish Blackface lambs eight weeks after receiving a challenge infection with *T. circumcincta* L_3 larvae. Transformations were carried out according to Equation 3.1 where λ is the transformation parameter. A^2 is the Anderson-Darling statistic for normality and *P* the probability that the results were due to chance. All but the number of L_4 larvae data were considered normally distributed after transformation as all *P* values were greater than 0.05.

raw data fitted the normal distribution (WL: $P = 0.146$; MMC: $P = 0.471$; EPF: $P = 0.453$).

Adult worm mass data were calculated by multiplying the number of adult worms (before transformation) by the average length of worms for each animal. The Box-Cox analyses were then run on this data set to see if it required transformation. Adult mass data were transformed using $\lambda = 0$, a log10 transformation. Data for the number of adult worms was similar to that of the total worm burden and used a log10 transformation. Data for the number of L_4 were highly skewed as several animals had no larvae. Subsequently the transformation (log10 +1) did not normalise the data. The untransformed data were used for analyses but must be taken with some caution. In any case there were few instances where there were apparent associations between the number of fourth stage larvae and any other variable.

Where variables were measured over time (faecal egg counts, peripheral eosinophil counts, plasma IgA activity and plasma pepsinogen concentrations) data within each time point were analysed separately to determine whether they were normally distributed.

Faecal egg count data were transformed using a λ value of 0.5 which gave a transformation of $\sqrt{}$ (FEC+10) or using a λ value of 0 which gave a transformation of log10(FEC+10). Ten was added to the count to overcome the problem with counts of zero.

Peripheral eosinophil counts varied in their distributions. Data from some time points did not require transformation (λ=1), while others required log transformations (λ=0) and square root transformations (λ=0.5).

Plasma IgA data were all transformed using λ=0, using the equation log10(IgA+1). One was added to each value before the logarithmic transformation to overcome the the fact that some IgA OD index values were negative or zero.

Data for plasma pepsinogen concentrations were log transformed (λ=0). There was no requirement to add one to the counts before transformation as all values were above zero.

3.3.2 Kinetics

3.3.2.1 Preliminary assays

Preliminary IgA and IgG ELISA assays using seven plasma samples from four animals were conducted in order to estimate their kinetics and to provide information for planning of subsequent assays.

Figures 3.4 and 3.5 show the plasma IgA and IgG responses of the four animals to L_3 and L_4 somatic extracts respectively. The pattern of the antibody responses was very similar against both extracts with the response against the L_4 extract being marginally stronger. IgA responses started quite low, peaked at 10 days and then declined while IgG responses changed little as the infection proceeded.

Further assays tested plasma from all infected animals. As the L_3 and L_4 responses were very similar, only the IgA responses against L_3 somatic extract were tested in all animals at all time points. However, the plasma IgG response to L_3 extract at 10 days post infection (the peak) and the plasma IgA and IgG responses to both L_3 and L_4 extracts at slaughter were also measured.

3.3.2.2 Peak and kill day IgA and IgG responses

These analyses correspond to data from all infected animals. Pearson product moment correlations between parasite specific IgA and IgG responses at ten days post infection (the time of the peak IgA response) and at the time of slaughter are shown in Table 3.4. All but one of the fifteen combinations of antibody response data gave significant correlations. Animals with strong IgA responses to L_3 somatic extract at 10 days post infection had strong IgA responses to both L_3 and L_4 extracts at the time of slaughter (L_3: $r = 0.703$, $P < 0.001$; L_4: $r = 0.536$, $P = 0.015$). Similarly, animals with high IgG responses to L_3 at 10 days post infection had high IgG responses at the end of the challenge infection (L_3: $r = 0.831$, $P < 0.0001$; L_4 $r = 0.924$, $P < 0.001$). There were very strong significant negative correlations between IgA responses and IgG responses at 10 days post infection and at slaughter. At 10 days post infection the correlation between IgA and IgG responses was –0.693 ($P < 0.001$).

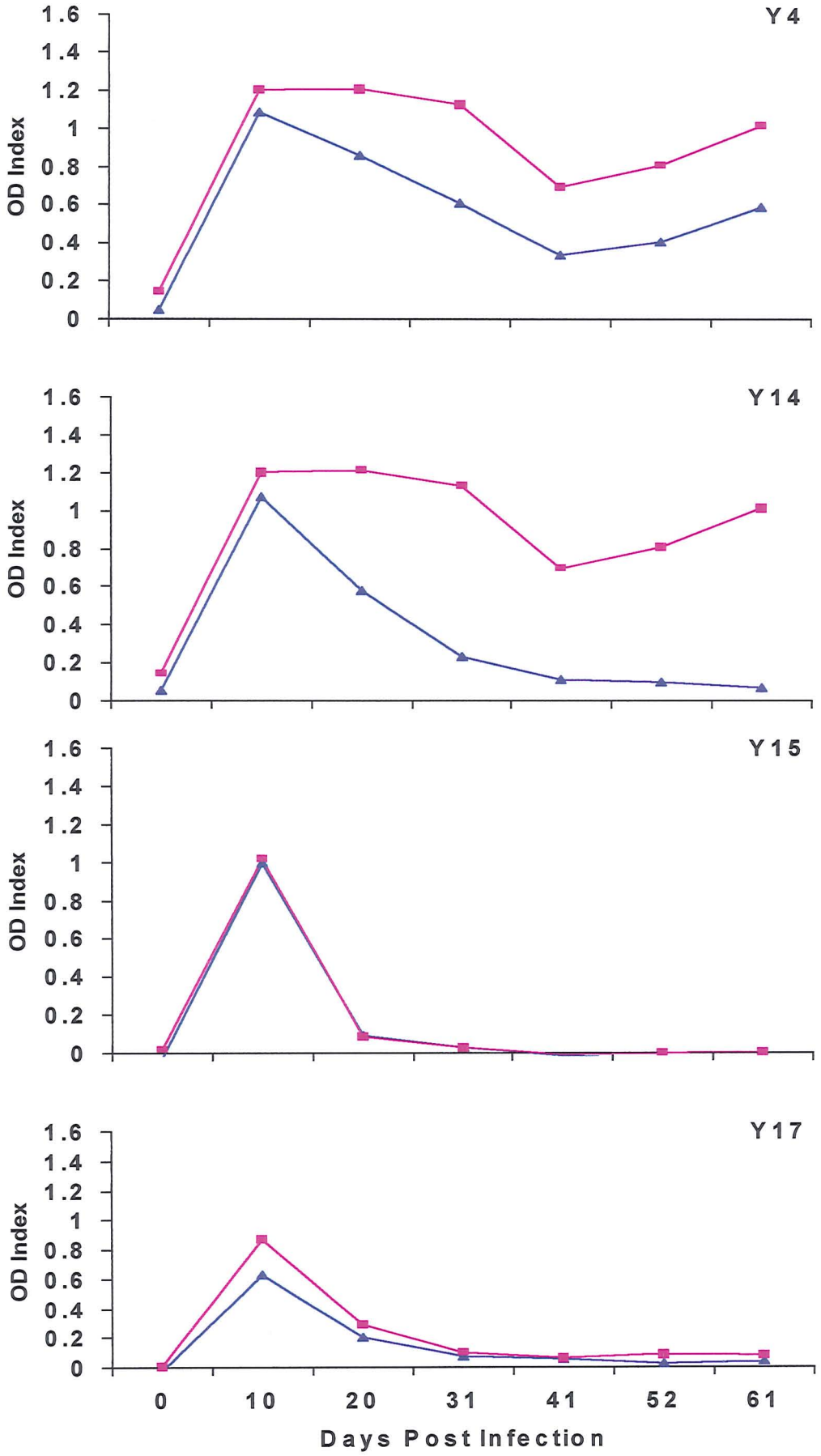

Figure 3.4: Plasma IgA activity against L_3 (▲) and L_4 (■) somatic extracts of *T. circumcincta* as measured by ELISA in four different animals following a challenge infection with *T. circumcincta* L_3 infective larvae.

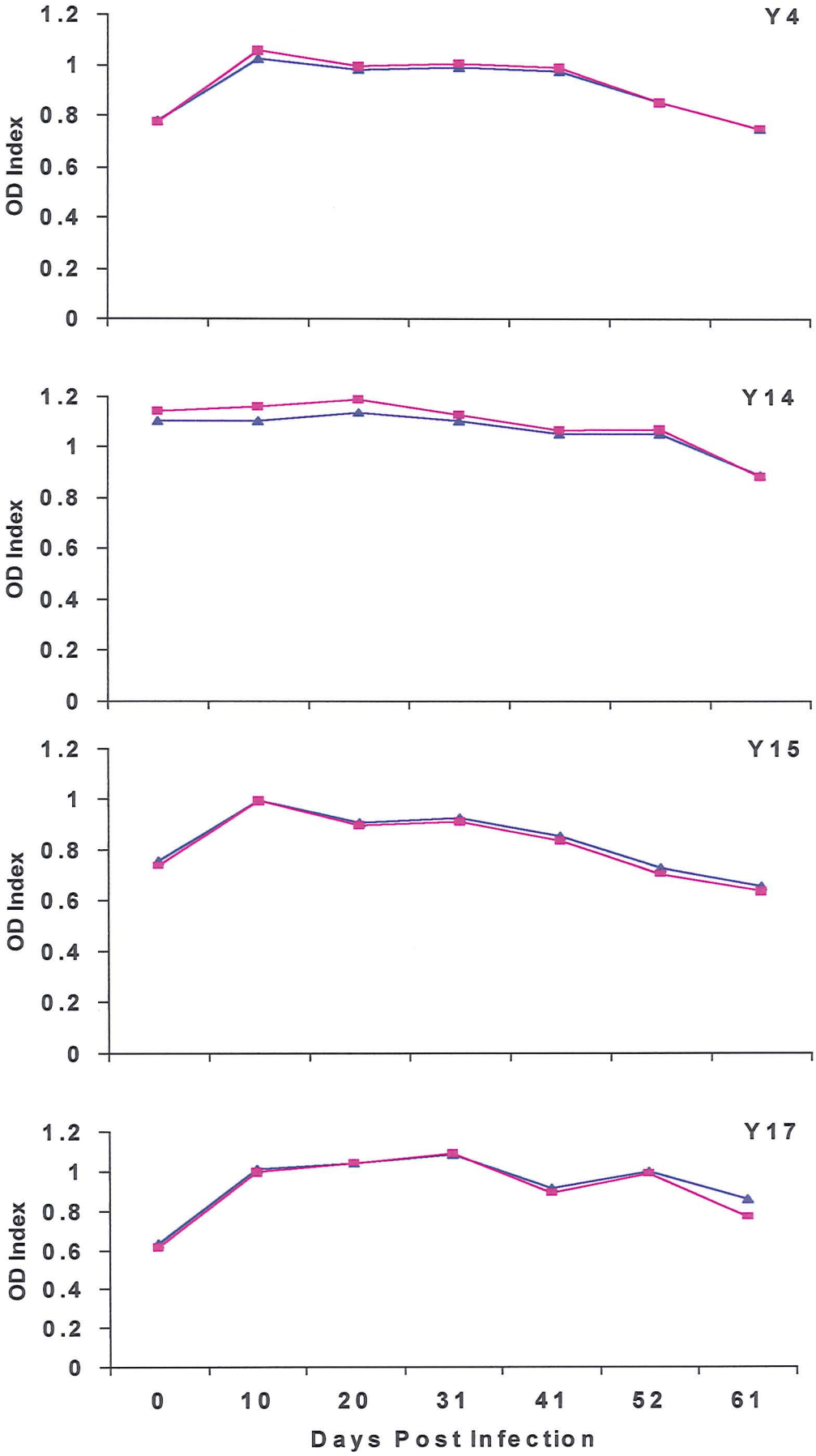

Figure 3.5: Plasma IgG activity against L_3 (▲) and L_4 (■) somatic extracts of *T. circumcincta* as measured by ELISA in four different animals following a challenge infection with *T. circumcincta* L_3 infective larvae.

	IgA $L_3$10	IgG $L_3$10	$IgAL_3$	$IgGL_3$	$IgAL_4$
IgG $L_3$10	–0.693***				
$IgAL_3$	0.703***	–0.670***			
$IgGL_3$	–0.588**	0.831***	–0.627**		
$IgAL_4$	0.536*	–0.458*	0.869***	–0.458*	
$IgGL_4$	–0.646**	0.924***	–0.517**	0.850***	–0.312

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

Table 3.4: Pearson correlation coefficients among parasite specific IgA and IgG responses to L_3 and L_4 somatic extracts of *T. circumcincta* in experimentally challenged lambs at 10 (IgA $L_3$10; IgG $L_3$10) and 61 days post infection.

3.3.2.3 IgA kinetics

Figure 3.6 shows the kinetics of the mean IgA responses of all animals after challenge infection. The kinetic pattern was similar in all animals and the standard errors are quite small. Initial IgA activity was low, but rose quickly following challenge, and peaked at 10 days post infection. Responses then declined to 20-22 days post challenge. IgA activity then remained stable for the rest of the experiment. Of the more than 150 combinations of IgA responses, only about 10 were not significantly correlated (data not shown). Animals which had the highest levels of L_3 specific IgA before this secondary challenge infection tended to have the highest levels of IgA throughout the infection and at slaughter. The correlation between IgA responses before and at the end of the challenge infection was 0.587 ($P = 0.004$).

3.3.2.4 Eosinophil kinetics

Peripheral eosinophil counts were carried out on blood collected from animals every 2-3 days during the challenge infection. Mean eosinophil counts following challenge infection are shown in Figure 3.7. Variance between animals in peripheral eosinophil count was relatively low therefore standard errors are quite small. Pre-challenge peripheral eosinophil counts were low and rose slightly in the first few days after infection. Between 6 and 8 days post challenge, peripheral eosinophil numbers increased rapidly reaching a maximum at 8 days. The number of eosinophils in the peripheral blood remained approximately the same for about another 5 days before gradually falling to pre-infection levels by day 45 and remaining at this level until slaughter. There were significant positive correlations between counts at each time point up to 22 days after challenge. Pre-challenge counts were significantly positively associated with counts up to 35 days post infection, but not subsequently. Therefore, although animals with high peripheral eosinophil counts before the infection started had more eosinophils during the early stages of the infection at about 8-14 days, they did not necessarily have high eosinophil numbers by the end of the infection. The correlation between peripheral eosinophil counts at the start and at the end of the infection was 0.057 ($P = 0.79$). From about 24 days post infection all counts were positively correlated and most significantly with each subsequent eosinophil count.

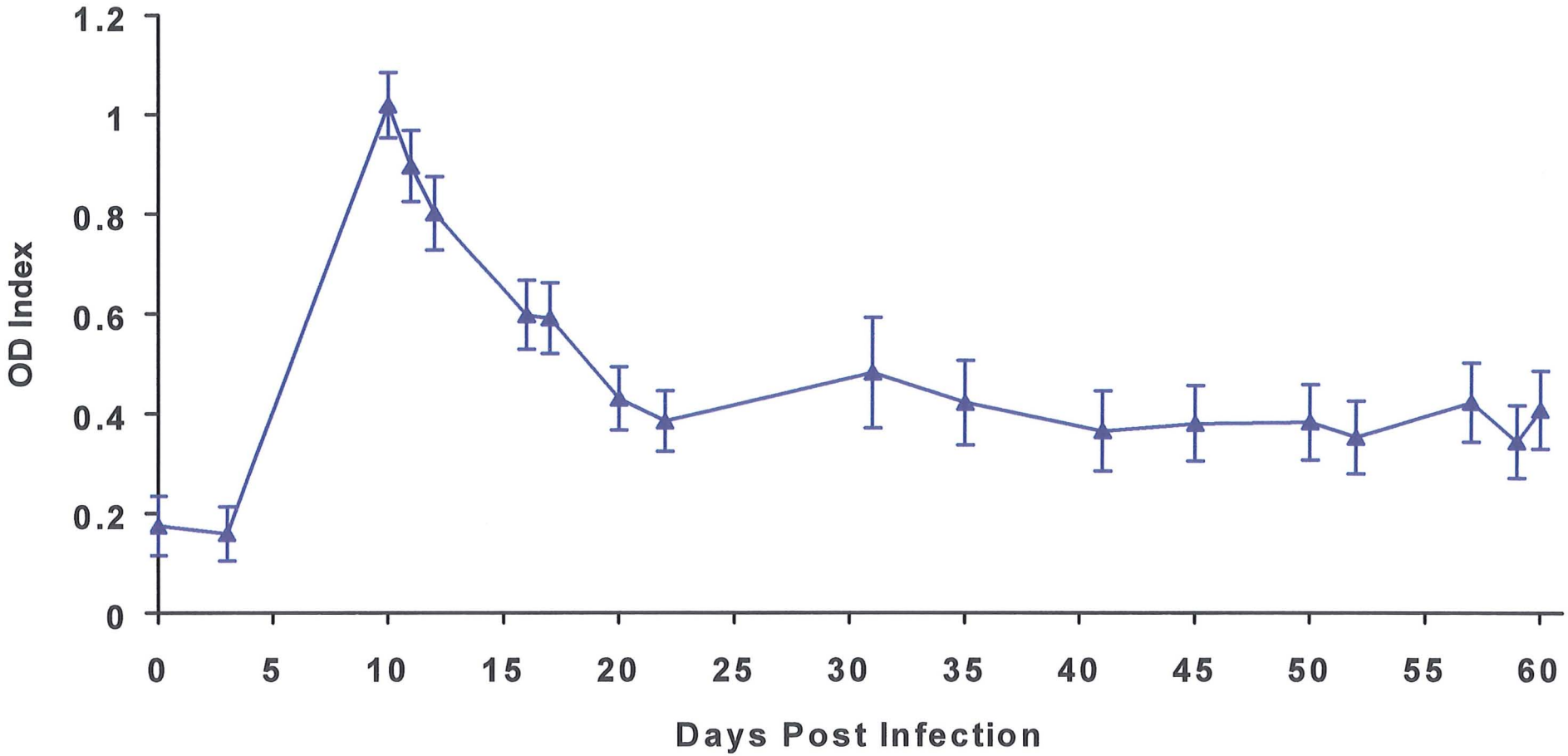

Figure 3.6: Kinetics of the mean (±S.E.) plasma IgA response against an L_3 somatic extract of *T. circumcincta* as measured by ELISA in a group of Scottish Blackface lambs following a challenge infection with *T. circumcincta* L_3 infective larvae.

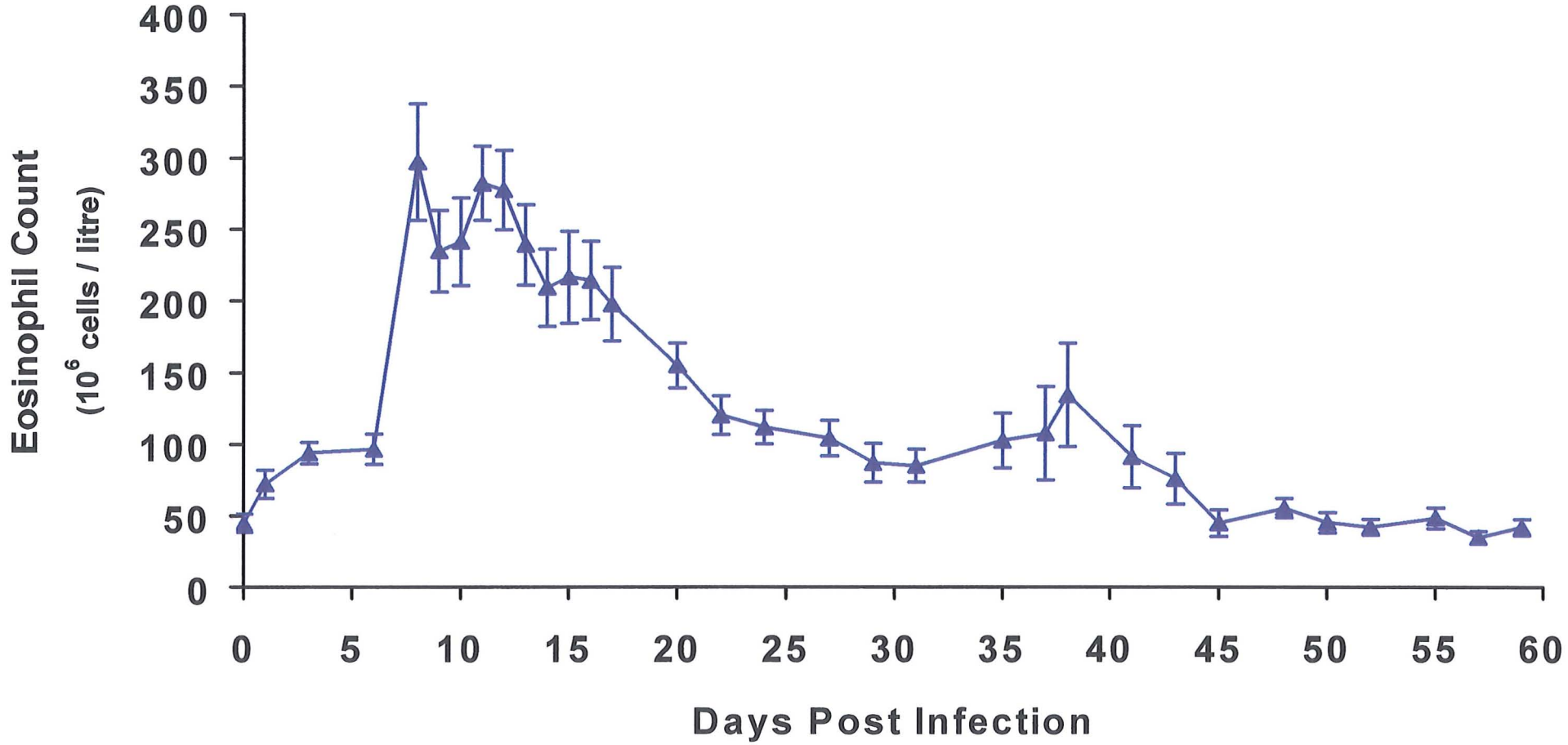

Figure 3.7: Kinetics of the mean (±S.E.) peripheral blood eosinophil count in a group of Scottish Blackface lambs following a challenge infection with *T. circumcincta* L_3 infective larvae.

3.3.2.5 Faecal egg count kinetics

All 24 experimental animals became infected and exhibited *T. circumcincta* eggs in faeces. The mean prepatent period for eggs to appear in faeces following the challenge infection was 29 days (± 2 days) and varied between 18 and 57 days. Mean FEC kinetics are shown in Figure 3.8. There was considerable variation among animals in FEC resulting in large standard errors on the counts. At 3 weeks after challenge, egg counts began to increase, peaking at approximately 7 weeks before declining towards the end of the experiment. At slaughter, 20% of the animals had FEC that had fallen to zero. There were very strong positive correlations between subsequent counts after day 22 and correlations tended to decrease as the time interval between counts increased (Figure 3.9). However, there were significant correlations between egg counts at 22, 25, 27 and 29 days after challenge and egg counts at slaughter (r = 0.426 to 0.562). All subsequent egg counts from day 41 onwards were significantly correlated with each other.

3.3.2.6 Plasma pepsinogen kinetics

Plasma pepsinogen concentrations were measured once each week during the challenge infection. The kinetics of the host plasma pepsinogen response during secondary challenge with *T. circumcincta* L_3 larvae are illustrated in Figure 3.10 and follow a similar pattern to that of the plasma IgA and peripheral eosinophil responses. Variation in pepsinogen concentrations among animals tended to increase as the infection proceeded so that standard errors were small at the start but increased considerably as the infection proceeded. Before challenge, plasma pepsinogen levels were approximately 900 mU, and rose by about 50% 7-14 days after infection. The trend was then a gradual decrease each week leading to a final plasma pepsinogen concentration at necropsy of just over 800mU, a value only slightly lower than the pre-challenge concentration. The mean pre-challenge concentration of plasma pepsinogen was significantly positively associated with the mean concentrations at one, two and eight weeks after infection (Figure 3.11). The correlation between plasma pepsinogen concentrations before and at the end of the challenge was 0.461 (P = 0.024). Plasma pepsinogen concentrations from the second and subsequent weeks of the challenge infection were strongly significantly positively correlated with each other.

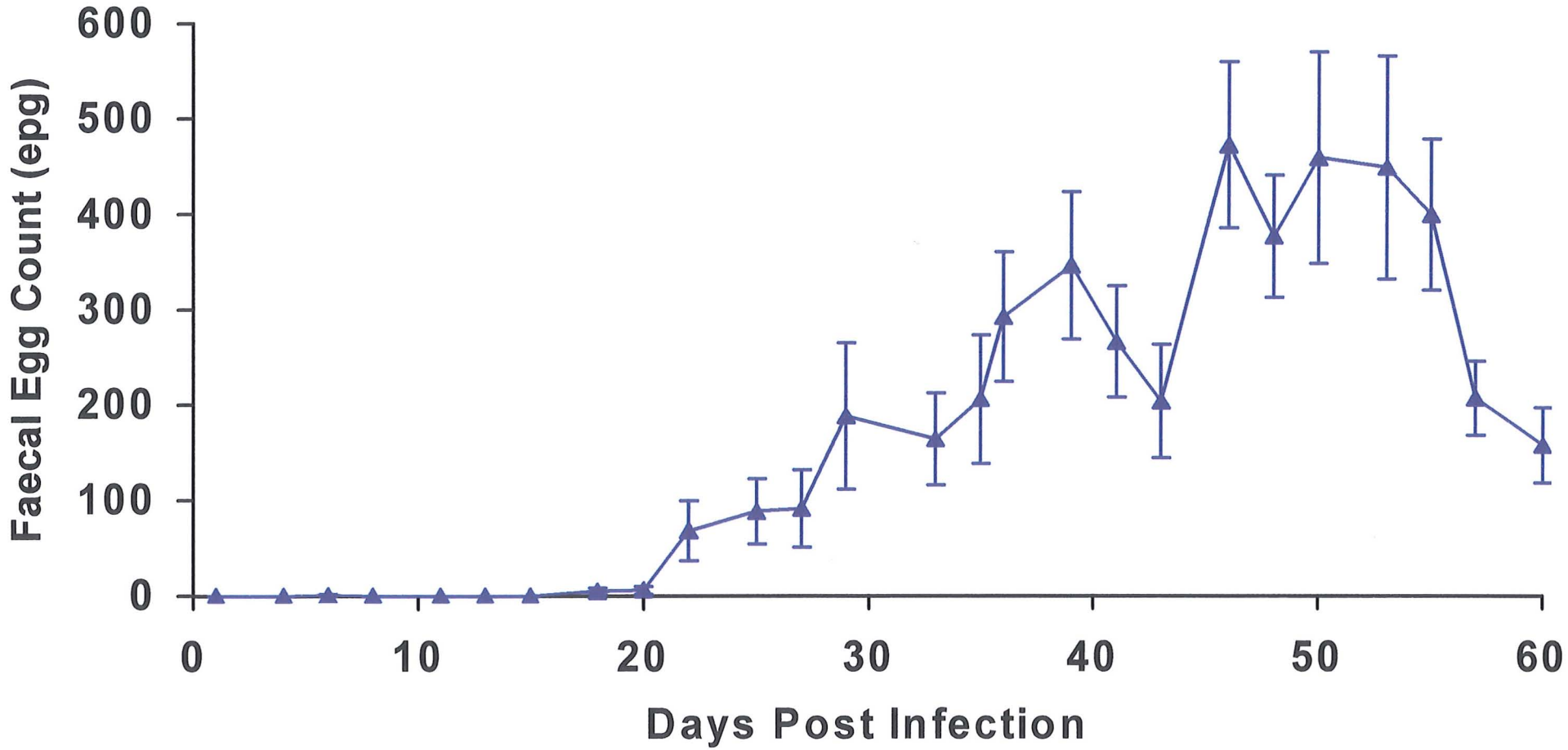

Figure 3.8: Kinetics of the mean (±S.E.) faecal egg counts (eggs per gram) in a group of Scottish Blackface lambs following a challenge infection with *T. circumcincta* L_3 infective larvae.

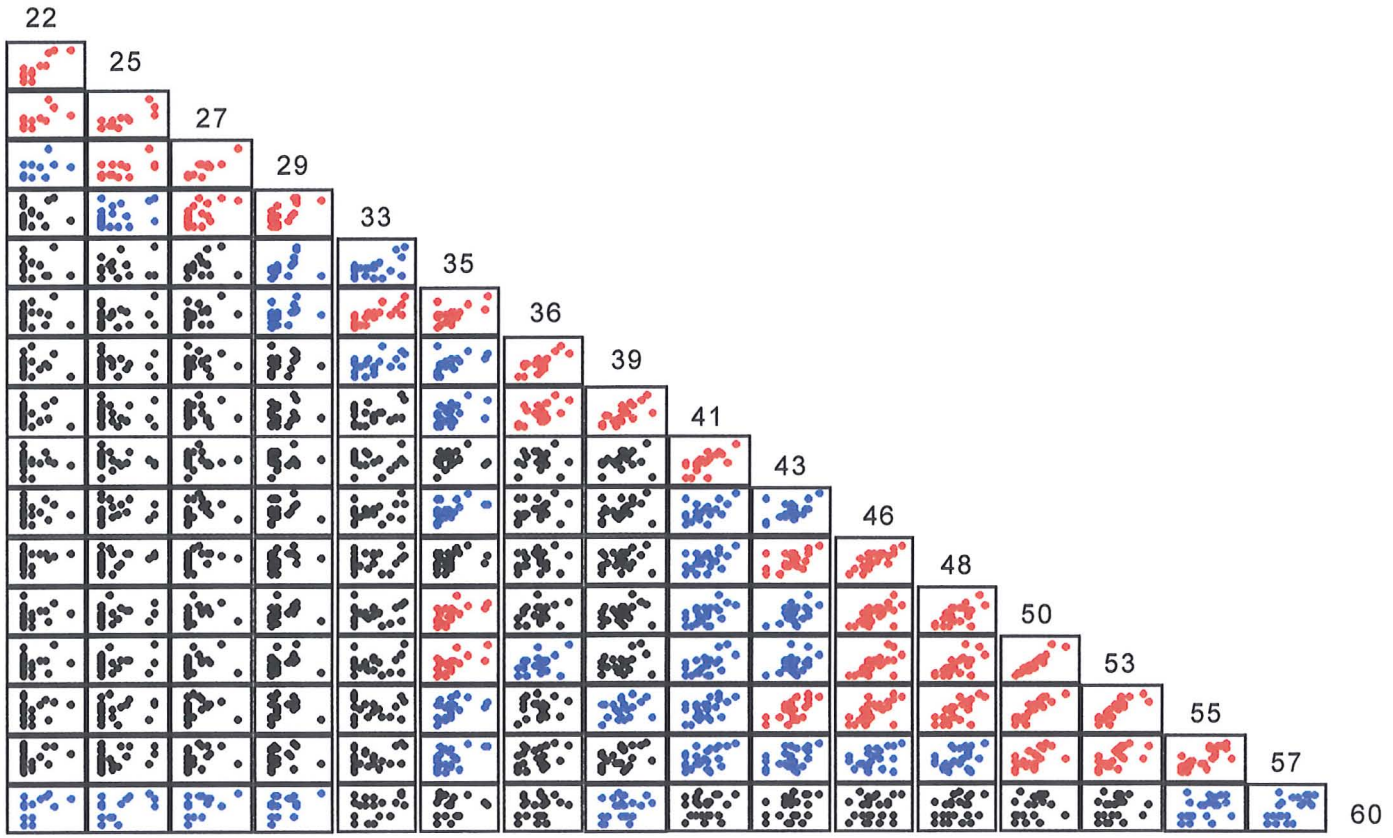

Figure 3.9: Table of matrix plots of correlations between mean faecal egg counts following secondary challenge with *T. circumcincta* in Scottish Blackface lambs. Numerical values are days after infection. In each matrix, each dot represents one animal. (•) $P > 0.05$, (•) $P < 0.05$, (•) $P < 0.001$.

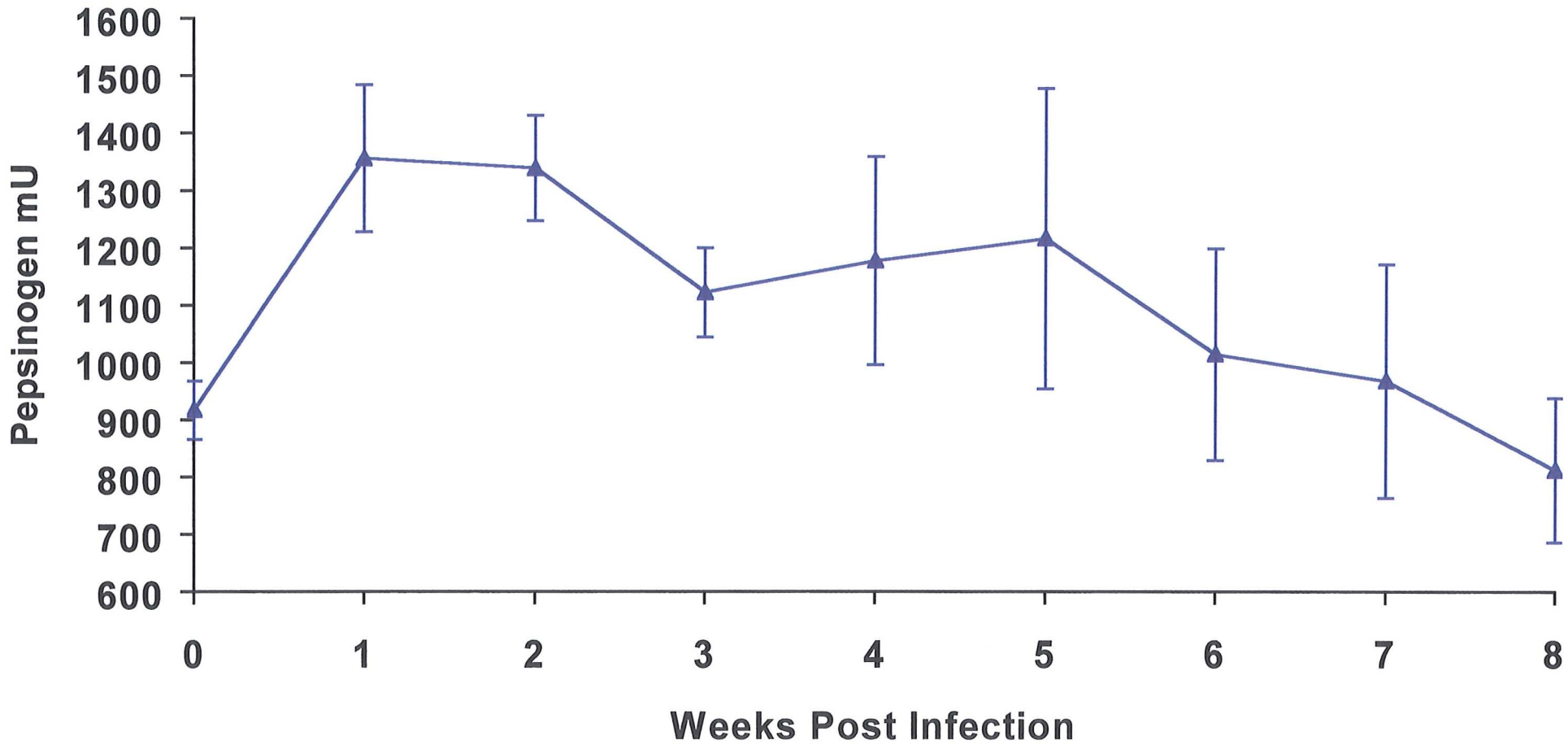

Figure 3.10: Kinetics of the mean (±S.E.) plasma pepsinogen concentrations in a group of Scottish Blackface lambs following a challenge infection with *T. circumcincta* L_3 infective larvae.

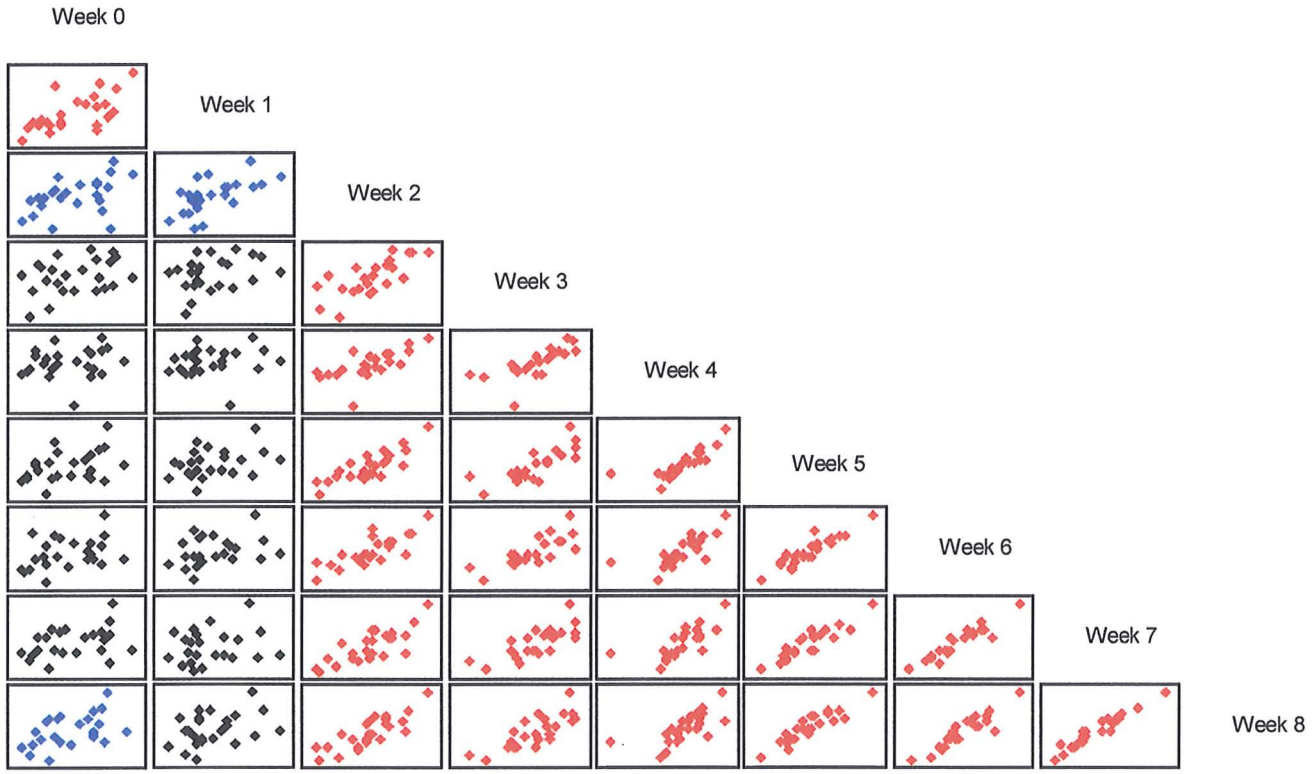

Figure 3.11: Table of matrix plots of correlations between weekly mean plasma pepsinogen concentrations following secondary challenge with *T. circumcincta* in Scottish Blackface lambs. Each dot in a single matrix represents one animal (•) $P > 0.05$, (•) $P < 0.05$, (•) $P < 0.001$.

3.3.2.7 Interaction of plasma IgA, peripheral eosinophilia, FEC and plasma pepsinogen kinetics

The kinetics of the host IgA response to *T. circumcincta* L_3 extract and the peripheral eosinophil counts following challenge appear similar and when they are shown together on the same graph the similarities are more clearly evident (Figure 3.12). Table 3.5 gives the mean plasma IgA responses and peripheral eosinophil counts at related points after infection. The Pearson product moment correlations between the responses are also shown. Although the responses follow the same pattern, there were only three occasions at which correlations were significantly correlated, these were at 41, 45 and 50 days post infection. By this time plasma IgA activity and peripheral eosinophil numbers had fallen to near pre-infection levels. There were significant negative correlations between the eosinophil counts at 10-12 days post infection, but not at the time of the peak eosinophil response at 8 days, and each subsequent IgA response.

The kinetics of mean plasma pepsinogen concentration was also very similar to the kinetics of the plasma IgA response and the peripheral eosinophil count. Plasma IgA responses were significantly positively correlated with plasma pepsinogen concentrations over the final few weeks of the infection.

There were no correlations between plasma pepsinogen concentrations and egg counts at related time points although animals that had high plasma pepsinogen concentrations after the first week of challenge tended to have higher egg counts as the infection proceeded.

In the final few weeks of the infection plasma IgA activity and peripheral eosinophil counts were both significantly negatively associated with faecal egg counts. Plasma IgA responses at 10, 11, 22, 20 and 31 days post infection were significantly negatively correlated with faecal egg counts at the end of the experiment (61 days) indicating that animals which mounted strong immune responses early after challenge were better at controlling egg output.

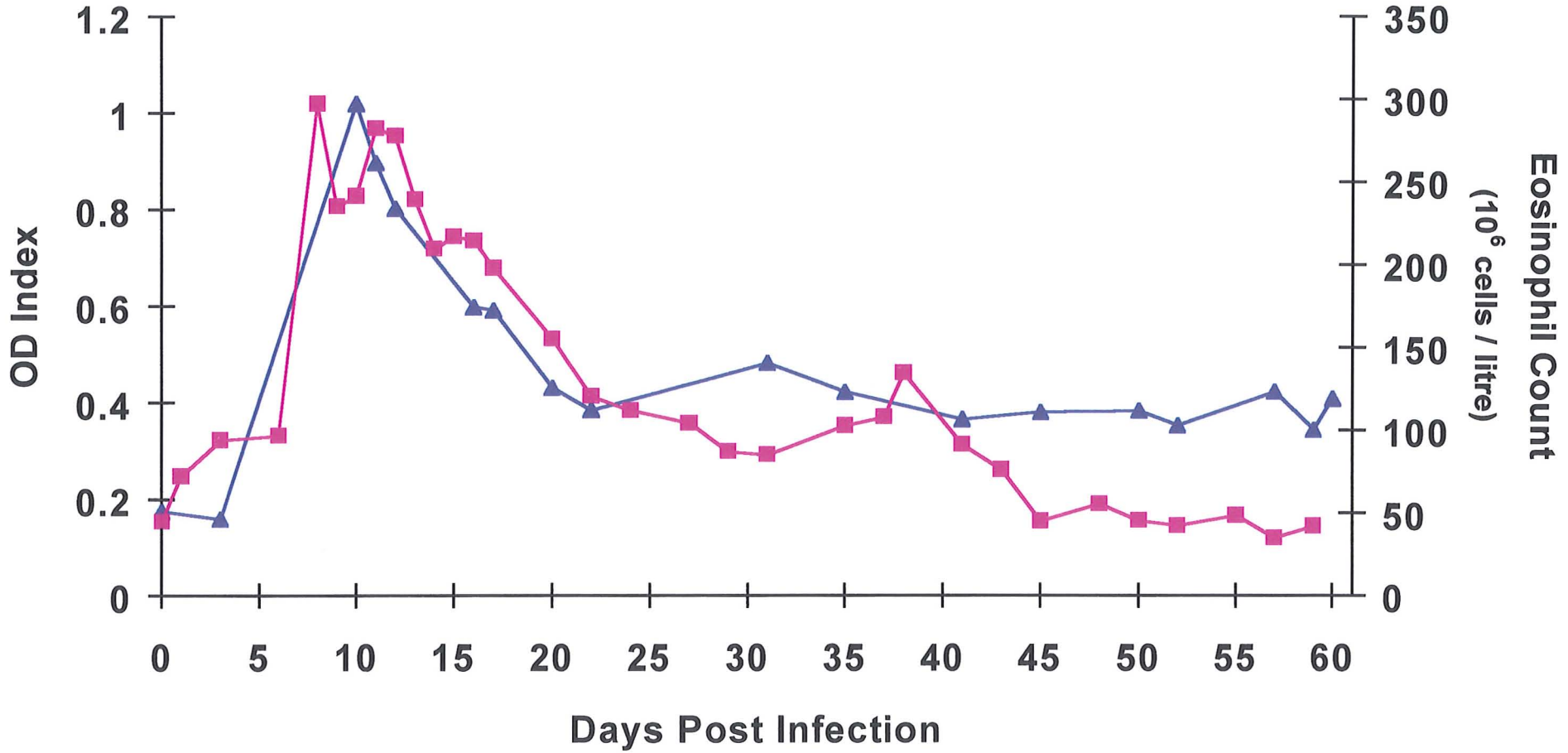

Figure 3.12: Kinetics of the mean plasma IgA response against an L_3 somatic extract of *T. circumcincta* as measured by ELISA (▲) and the mean peripheral blood eosinophil count (■) in a group of Scottish Blackface lambs following a challenge infection with *T. circumcincta* L_3 infective larvae.

Days post Infection	Mean IgA OD index	Mean eosinophil count	Correlation	P
0	0.176	45.15	0.104	0.637
3	0.160	94.27	0.165	0.464
10	1.020	241.73	–0.285	0.223
11	0.898	282.57	–0.188	0.402
12	0.803	277.9	–0.328	0.158
16	0.599	214.67	–0.341	0.153
17	0.592	198.10	–0.246	0.325
20	0.431	155.40	–0.098	0.689
22	0.386	120.63	–0.042	0.864
31	0.483	85.4	0.460	0.073
35	0.423	102.90	0.278	0.223
41	0.366	91.58	0.443	0.039
45	0.381	45.27	0.507	0.014
50	0.384	45.73	0.497	0.016
52	0.353	42.58	0.099	0.661
57	0.423	32.23	–0.094	0.662
59	0.345	42.35	0.284	0.2

Table 3.5: Mean anti-L_3 IgA OD indices and peripheral eosinophil counts in Scottish Blackface sheep following challenge infection with *T. circumcincta*. Pearson product moment correlations between the two measurements on each day are also shown.

3.3.3 Kinetics and post mortem data together

3.3.3.1 Associations between peak and kill day IgA and IgG responses and parasitological and histological data

Pearson correlation coefficients among the different parasitological and histological parameters measured during post mortem examination of experimental animals are shown in Table 3.6. Some of these results have been published previously (Stear *et al.*, 1995c) and are provided for reference since the present study has used additional data. Further analyses with the amended dataset gave results consistent with those previously published, hence only new analyses are shown.

Results from the three plasma IgA assays were negatively associated with worm burdens, worm lengths, worm fecundities and post mortem faecal egg counts while each of the plasma IgG assays were positively associated with worm length. However, only the IgA and IgG responses at 10 days were significantly correlated with worm burdens. Similarly, only the IgA and IgG responses at slaughter were significantly correlated with worm lengths and worm fecundities. The IgA and IgG responses at 10 days post infection were also significantly correlated with the adult worm mass (IgA: $r = -0.503$, $P = 0.012$; IgG: $r = 0.525$, $P = 0.009$). The IgA but not IgG response to L_3 extract at slaughter was also significantly correlated with the worm mass ($r = -0.426$, $P = 0.038$). There was no correlation between the IgA responses to L_4 extract at slaughter and the worm mass.

Previous analyses had demonstrated that decreased worms lengths were associated with increased anti L_4 IgA responses and increased numbers of mucosal eosinophils (Stear et al., 1995c).

Simple one way regression showed that the total parasite burden was negatively correlated with the plasma IgA response and positively with the plasma IgG response to L_3 extract at 10 days post challenge (but not to L_3 or L_4 extracts at post mortem), and negatively to the number of mucosal globule leucocytes. If all three variables are used in a regression equation (Table 3.7), then the model accounts for 53% of the variation in the total worm burden but only the globule leucocyte component remains statistically significant.

	No. of L_4	No. of adults	Worm burden	Worm mass	Worm length	Eggs per female	Mucosal mast cells	Mucosal eosinophils	Globule leucocytes
No. of adults	0.323								
Total no. of worms	0.420*	0.989***							
Worm mass	0.411*	0.98***	0.992***						
Worm length	0	–0.096	–0.072	0.054					
Eggs per female	–0.202	–0.131	–0.139	–0.019	0.912***				
Mucosal mast cells	–0.015	–0.238	–0.224	–0.242	–0.096	–0.261			
Mucosal eosinophils	–0.111	–0.088	–0.106	–0.196	–0.699***	–0.648***	0.192		
Globule leucocytes	–0.093	–0.562**	–0.556**	–0.623***	–0.501*	–0.476*	0.405*	0.638***	
IgA plasma cells	–0.262	–0.237	–0.238	–0.227	0.083	0.030	0.183	0.098	0.018

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

Table 3.6: Pearson correlation coefficients between parasitological and histological variables measured at slaughter in Scottish Blackface lambs following an 8 week challenge infection with *T. circumcincta* L_3 larvae.

Effect	Estimate	Standard error	Probability
Intercept	4.633	0.870	0.0001
Plasma IgA response to L_3 at 10 days p.i.	–0.930	1.026	0.376
Plasma IgG response to L_3 at 10 days p.i.	1.340	1.412	0.357
Globule leucocyte concentration	–0.455	0.167	0.015

Table 3.7: Multiple regression coefficients of plasma IgA and IgG responses to L_3 extract at 10 days p.i. and globule leucocyte concentration on the log transformed number of total *T. circumcincta* (adults + L_4 larvae + L_5 larvae) (R^2 = 0.532).

One way regression showed that parasite egg output at post mortem, measured as eggs per gram of faeces, was positively associated with the total worm burden and negatively with the number of globule leucocytes in the mucus, and the activity of IgA against L_3 at 10 days post infection. Combining the IgA and globule leucocyte components into a multiple regression equation accounts for 54% of the variation in the post mortem faecal egg count (IgA: P = 0.007; GL: $P = 0.004$), which is much higher than for either of the two variables when analysed independently. Introducing the total worm burden into the regression equation increases the proportion of the accounted variation to 57%, a very small increase, but only the IgA and globule leucocyte components remain significant (Table 3.8). When the mass of adult worms, which takes into account the worm length, was used in place of the worm burden in this equation the model still accounted for 57% of the variation in egg counts at this time, but none of the variables remained statistically significant. This emphasises the idea that both worm number and worm length control egg output in these data.

Using data from the peak and kill day assays with the parasitology and histology information demonstrates that the IgA response directed against L_3 larvae and the globule leucocyte response are higher in sheep with low worm numbers while the IgA response against L_4 larvae and the mucosal eosinophil response are higher in sheep with shorter worms.

3.3.3.2 Interactions between kinetic, parasitological and histological data

There was no significant correlation between the number of eosinophils in the abomasal mucosa and the number of eosinophils in the peripheral blood system at the time of post mortem examination ($r = 0.31$, $P = 0.14$). There were however significant negative correlations between the number of mucosal eosinophils counted during post mortem and nine of the eleven peripheral eosinophil counts made between days 6-17 post challenge (maximum on day 14, $r = -0.663$, $P = 0.0004$). This period of the infection at 6-17 days was at the time of the peak peripheral eosinophil response. Up to 24 days post infection there were positive correlations between the number of peripheral eosinophils and worm lengths. These associations were statistically significant at 6, 9 and 13-16 days after infection. This meant that animals which had high peripheral eosinophil counts during this phase of the infection actually had longer and more fecund worms at

Effect	Estimate	Standard error	Probability
Intercept	3.013	1.258	0.027
Plasma IgA response to L_3 at 10 days p.i.	–1.722	0.790	0.042
Total worm burden	0.259	0.214	0.241
Globule leucocyte concentration	–0.404	0.190	0.047

Table 3.8: Multiple regression coefficients of total worm burden, plasma IgA response to L_3 extract at 10 days p.i. and globule leucocyte concentration on post mortem faecal egg counts (R^2 = 0.572).

post mortem. At 24 days after challenge the association between peripheral eosinophilia and worm lengths became negative but only attained statistical significance on day 45 and 48.

The peripheral eosinophil count at slaughter was negatively but not significantly correlated with the number of L_4 larvae, the number of adult worms, the total worm burden and the adult worm mass. Throughout the challenge infection, peripheral eosinophil counts were negatively associated with the total worm burden and worm mass, but these only attained statistical significance at days 24, 27, 31, 48 and 52 after infection.

There was also a significant negative correlation between the time taken for eggs to appear in faeces and the subsequent cumulative total egg output ($r = -0.556$, $P = 0.005$). In other words animals which exhibited eggs in faeces the soonest after infection produced more eggs overall than animals which did not show eggs in faeces until later after challenge. These early egg-producing worms must either have remained in the host producing eggs and/or were replaced by developing fourth stage larvae which had arrested. The former explanation is more likely since there was no correlation between the prepatent period and the number of fourth stage larvae in the mucosa at post mortem. If fourth stage larvae were coming out of inhibition in these animals one might expect that in turn they would harbour fewer fourth stage larvae at post mortem but there was no evidence of this ($r = 0.074$, $P = 0.733$). If these worms had been expelled and were not replaced one would expect the total egg output to be similar in most infected animals. These results suggest that animals that are able to mount immune responses early in the challenge infection are able to control subsequent worm development and survival, and egg output.

Plasma pepsinogen concentrations in the 4^{th}-6^{th} but not final weeks of infection were significantly positively associated with total worm burdens at post mortem while plasma pepsinogen concentrations from the second week of infection were significantly negatively correlated with adult female worm lengths and subsequently worm fecundity. Worm mass at slaughter was only significantly correlated with the plasma pepsinogen concentration in the 5^{th} week of the infection, and then only weakly ($r = 0.422$, $P = 0.04$).

Each plasma IgA response from 31 days post infection was significantly negatively correlated with adult female worm lengths and worm fecundities until the end of the infection. Plasma IgA responses throughout the infection were negatively associated with the faecal egg count at slaughter, but only attained statistical significance at days 10, 11 and 25-30 post infection and at slaughter. Over the same period plasma IgA responses were significantly correlated with globule leucocyte and mucosal eosinophil counts.

3.4 Discussion

This chapter has investigated the kinetics of the immune responses to a challenge infection of 50,000 *T. circumcincta* L_3 larvae in Scottish Blackface lambs and whether readings taken during the infection rather than at the end may facilitate the identification of resistant animals.

While the kinetics of host antibody responses to parasitic infection as a whole have been widely studied, few studies have concentrated on gastrointestinal parasite infections in sheep and in *T. circumcincta* infections in particular. Plasma immunoglobulin responses against L_3 and L_4 somatic extracts of *T. circumcincta* were very similar in the present study. This suggests that there are many identical antigens in the two types of extract. Similar findings were reported by Charley-Poulain *et al.* (1984) in 7 month old Préalpes du Sud breed lambs immunised then challenged with *H. contortus*.

The results from the present study have demonstrated a distinct pattern in host plasma IgA in response to a challenge infection with *T. circumcincta* L_3 larvae (Figure 3.4). The sharp rise in antibody titre following challenge that peaked at 10 days and then declined, agrees well with the small number of other studies that have investigated the kinetics of sheep antibody responses to infections of this kind. Smith *et al.* (1983) studied total and anti-worm IgA activity in the gastric lymph of Greyface cross Suffolk lambs receiving a challenge infection with *T. circumcincta*. They found that total IgA concentrations in the gastric lymph started to increase on the third and fourth days following challenge then peaked at eight days before declining rapidly. Anti-worm antibody followed the same pattern but peaked at day seven. Animals in the study by Smith *et al.* (1983) were killed 10 days after challenge, but still had total and anti-worm IgA concentrations significantly higher than the IgA concentrations at day zero. A

similar study by the same group (Smith *et al.*, 1984) reported that the IgA concentration in gastric lymph peaked six to seven days after challenge before subsequently declining to near pre-challenge concentrations by day nine. Following challenge, lambs previously immunised with *T. circumcincta* L_4 and adult surface antigen extracts elicited strong serum IgA responses with peaks of activity at seven and fourteen days after challenge (Wedrychowicz *et al.*, 1994). In White Alpine mountain breed lambs challenged with *O. leptospicularis* following trickle infections, serum IgA peaked at 7 days before declining to pre-challenge levels (Hertzberg *et al.*, 1999). Gill *et al.* (1993a) reported peaks of anti-*H. contortus* larval plasma IgA activity at ten days post challenge in Merino lambs previously naturally infected with *H. contortus*. In general, these studies agree with the present study that responses peak 7-14 days post infection.

In all of these examples cited, and in the present study, sheep were being reinfected after previous exposure to parasite antigen, whether it be by natural or artificial infection. IgA activity during the challenge infections developed more rapidly and was stronger than during the first infection; this observation is consistent with our current understanding of the immune response.

The pattern of the plasma IgG response following challenge infection differed from the plasma IgA response and did not show a clear peak (Figure 3.5). Plasma IgG titres rose only slightly after challenge and persisted at relatively constant levels for the period of the infection. Previous studies measuring the kinetics of the IgG response have reported conflicting results. Wedrychowicz *et al.* (1994) showed a decline in the serum IgG response to both L_4 and adult *T. circumcincta* surface antigens in previously exposed sheep. Sutherland *et al.* (1999) reported a decline followed by an increase at 28 days post challenge in serum anti-*T. circumcincta* L_3 IgG in previously infected year old lambs. Gill *et al.* (1993a) reported significant increases in serum IgG1 at 10 and 17 days post challenge with infections of *H. contortus* which later declined, paralleling the serum IgA response that these authors described. The latter authors also noted that there was no change in the IgG2 response of the host. This confuses matters further as there is still conflicting evidence about which IgG antibody isotypes predominate in these gastrointestinal parasitic infections in sheep. Balic *et al.* (2000a) cites a list of papers which state that IgG1 is the predominant serum antibody isotype over IgG2 with different parasite extracts and stages. However Yong *et al.* (1991) stated that resistant sheep had high levels of IgG2 antibodies against L_3 and adult *T. circumcincta* antigens

as well as IgA but had little or no IgG1 while IgG2 levels were significantly raised against the L_4 stage of *O. ostertagi* in cattle (Hilderson *et al.*, 1993).

The production of different antibody isotypes is regulated by subsets of antigen-specific helper T cells through the production of cytokines (Janeway & Travers, 1994). In mice, T_H1 subsets of T cells stimulate IgG2a and IgG2b production while the T_H2 subset stimulates IgA, IgG1 and IgE production (Mosmann *et al.*, 1986), however the situation in sheep is not analogous and is unclear. Although the present study measured total IgG and did not discriminate between the two subclasses found in sheep, it is likely that this is predominantly IgG1 (Griebel, 1998). Assuming this is true then, this study has suggested a negative association between IgA and IgG1, whereas Yong *et al.* (1991) suggested a positive association between IgA and IgG2. Possibly, as suggested by Stear *et al.* (1995c), a T_H2 subset regulates mucosal mast cell/globule leucocyte, eosinophil and IgA responses whereas a T_H1 subset regulates IgG1 production.

A negative association between parasite specific IgA responses and worm lengths has been reported in both deliberate and natural infections. Deliberate infection studies have tended to use IgA measurements taken at the time of slaughter (Sinski *et al.*, 1995). In the present study IgA responses became significantly negatively associated with worm lengths by the start of the 5th week of the challenge infection and remained significant until the experiment was ended after eight and a half weeks. These findings support the use of IgA as a prospective marker of resistance. Continual, perhaps weekly monitoring of parasite specific plasma IgA responses could make identification of resistant animals easier and assist farmers in determining which animals required and when to administer anthelmintic treatment or otherwise cull the most susceptible animals. Currently, the assessment of antibody responses must be done in the laboratory, but if monitoring could be conducted by the farmer on the farm using a kit it would make selection based on antibody responses easier and possibly cheaper.

The kinetics of the peripheral eosinophil response following a primary infection is well documented but the response following a challenge infection has not been widely studied. Stevenson *et al.* (1994) reported that following challenge of Greyface cross Suffolk lambs with *T. circumcincta* peripheral eosinophil counts doubled in the ten days following challenge. In the present study peripheral eosinophil counts doubled in the first 3 days after challenge and by 8 days had increased 6-fold. Peripheral eosinophil

counts in the first 2 weeks of the present study were positively associated with worm lengths. It was not until the infection had proceeded for about 7 weeks that negative associations between peripheral eosinophil counts and worm lengths were observed. Eosinophil counts were significantly negatively correlated with egg output from the fourth week of infection until slaughter but not with worm burdens. This suggests that eosinophils might act in concert with IgA to reduce worm growth and fecundity. While a correlation between peripheral eosinophilia and resistance is not always observed (Woolaston *et al.*, 1996), Dawkins *et al.* (1989) and Buddle *et al.* (1992) both concluded that peripheral eosinophilia was a measure of a host's resistance to gastrointestinal nematodes, rather than an indication of worm burden, as did Stear *et al.* (2002) but only in lambs that were at least 3 months old.

Animals that developed challenge infections quickly (within 4 weeks of infection), as measured by faecal egg counts, tended to have high egg counts when the experiment was terminated at 8 weeks. The pattern of egg counts and the timing of the prepatent period of three weeks for experimentally challenged animals agrees with the published data. Fourteen-month old Romney lambs trickle infected and then challenged with *T. circumcincta* elicited positive faecal egg counts at three weeks after challenge (Sutherland *et al.*, 1999). The prepatent period in *H. contortus* previously infected then challenged Manchego and Castellana lambs was approximately 18 and 20-24 days (Cuquerella *et al.*, 1991 and Gómez-Muñoz *et al.*, 1999 respectively). In the present study, maximum faecal egg counts occurred at 46-53 post challenge before starting to decline as the infection was terminated. Sutherland *et al.* (1999) reported that egg counts were at a maximum at five weeks, and Cuquerella *et al.* (1991) reported maxima at six weeks. Unfortunately however, in both these studies maximum egg counts were achieved as the experiments were terminated. Therefore, there is no certainty that these were actual maximums, egg counts may have continued to rise for several more weeks or may have declined. As for eosinophilia, regular monitoring of egg counts would assist farmers in identifying the most susceptible animals so that they could be treated, culled or at least moved to separate pasture so as to reduce the possibility of these animals infecting and/or reinfecting the rest of the flock. An on-farm FEC monitoring system (FECPAK) was developed in 1994 by FECPAK International Ltd. in New Zealand and is now used quite widely. It has recently been extended for use in cattle and was claimed that farmers could save $7 per head by drenching less often after on-farm monitoring and could save the New Zealand cattle industry about $3 million per year

(Foundation for Research, 2000). It is however unclear how rigorously the FECPAK system has been tested as there is nothing in the scientific literature. The FECPAK system although encouraging should be evaluated under laboratory and field conditions in the UK before its use should be considered (Vipond, 1998).

The kinetics of the plasma pepsinogen response to challenge in this study was very similar to that of the plasma IgA response. Challenge infections of *O. leptospicularis* in sheep exhibited similar results while also showing that serum gastrin followed the same pattern (Hertzberg *et al.*, 1999). In the study of Anderson *et al.* (1985) adult *Ostertagia* spp. (predominantly *O. circumcincta*) were transferred via cannulae to the abomasum of previously worm free sheep. In these animals plasma pepsinogen concentrations rose in the first 24 hours after transfer and peaked at 8-12 days, thereafter declining towards pre-infection levels. *In vitro* studies showed that pepsinogen release from intact abomasal mucosal sheets (and smooth muscle contraction) could be stimulated by *T. circumcincta* ES antigens (Scott & McKellar, 1998). Additionally, adult *O. circumcincta* prevented from physical contact with the gastric mucosa by restraint in porous bags are able to raise the abomasal pH (Simpson *et al.*, 1999).

Pepsinogen release into the bloodstream is caused by damage to the abomasal epithelium (Stear *et al.*, 1999a), therefore animals with more and/or bigger worms would tend to have higher pepsinogen concentrations. Plasma pepsinogen concentrations in the present study were significantly negatively associated with adult female worm lengths as soon as 14 days after the infection was initiated. Worm lengths accounted for 46% and 35% of the variation in plasma pepsinogen concentrations at seven and eight week post infection respectively. A similar association was reported by Stear *et al.* (1999a) who demonstrated worm lengths accounted for 26% of the variation in plasma pepsinogen concentrations in six month old Scottish Blackface lambs naturally infected with predominantly *T. circumcincta*.

In the present study worm burdens were also significantly positively associated with plasma pepsinogen concentrations at 4-6 weeks after infection but not in the final two weeks. A parallel association between worm lengths and worm burdens did not cause this association as both traits had statistically significant probabilities when used together in a multiple regression equation. Worm burdens also had significant positive associations with plasma pepsinogen concentrations in the study of Stear *et al.* (1999a).

Additionally, animals which exhibited high plasma pepsinogen concentrations one week after challenge in the present study also tended to have fewer peripheral eosinophils and higher faecal egg counts in the final few weeks of infection suggesting that these animals were less resistant. Currently plasma pepsinogen must be determined in the laboratory and is time consuming and expensive. Development of an on-site kit would assist farmers greatly in identifying the most resistant/susceptible animals.

The kinetics and specificity of the host immunoglobulin response to primary and challenge infections clearly requires further study. Clarification of the timing of host responses as well as confirmation about which antibody isotypes predominate both locally and peripherally in the different gastrointestinal parasitic infections will significantly enhance our understanding of the host/parasite relationship and aid in future project design.

Faecal egg counts, plasma pepsinogen concentrations, peripheral eosinophil counts and parasite specific plasma IgA responses all vary between animals and have different degrees of association with resistance, particularly over time. The work in this chapter has investigated the kinetics of these responses following challenge infection and has demonstrated that continuous and simultaneous measurement of these markers of resistance could improve the identification of resistant and susceptible animals. However, testing would have to be economically viable and technically robust so that on-farm testing could be carried out. A need for these kinds of tests is particularly great at present given the increase in the incidence of anthelmintic resistant parasite strains. Selections indices could also be created based on the changing responses of the infected sheep to infection, so that animals could be bred for a reduction in parasite associated pathology, increased production, a reduction in the pasture larval load, or optimally, a combination of these factors.

Chapter 4

Recognition of *Teladorsagia circumcincta* larval antigens by plasma IgA from Scottish Blackface sheep following a challenge infection

4.1 Introduction

There has been a concerted effort to isolate and identify the antigens that are recognised by infected animals from the major gastrointestinal parasites of domesticated animals and these have been discussed in detail in chapter one. There is particular interest in characterising antigens which are preferentially recognised by resistant animals. Identification of these antigens and an increased understanding of how the host interacts with the parasite with respect to antigen recognition may aid vaccine design and could provide additional markers for the breeding of more resistant lines of animals.

McGillivery *et al.* (1989) extracted and isolated a 31 kDa glycoprotein antigen from the infective L_3 stage of *O. circumcincta* which was recognised by total antibody in sera of infected sheep as early as three weeks after experimental infection. This glycoprotein was subsequently found to be specifically located in the 'secretory organelles' within the cells of the oesophageal glands of the larvae and was also one of the major components of the ES complex (McGillivery *et al.*, 1990). The same group then reported that the purified 31 kDa antigen had been used to successfully immunise lambs against challenge infections with *O. circumcincta* (McGillivery *et al.*, 1992), but this could not be confirmed in three subsequent trials using the same 31 kDa antigen as an immunising agent (Morton *et al.*, 1995). Newton *et al.* (1997) later reported that this molecule was likely to be a beta- galactoside-binding lectin-like protein (galectin). While these authors did not attempt to test the galectin proteins as vaccine candidates, a galectin (Hco-gal-2) characterised from the gut of *H. contortus* larvae did not confer any protection against infection with *H. contortus* as judged by FEC and worm counts (Newlands *et al.*, 1999).

Coyne & Brake (2001) summarised the investigations involving characterisation and evaluation of various immunoprotective antigenic fractions from *H. contortus* larvae and stated that protection levels ranged from 17% up to 95% in studies using various isolated larval fractions.

Surface antigens *of O. circumcincta* L_3 larvae which were recognised by bile antibody were used to vaccinate Finn-Dorset lambs and subsequently the worm burdens of immunised and challenged lambs were significantly lower than in challenged control animals (Wedrychowicz *et al.*, 1995). The mucosal and bile IgM antibodies recognising

the L_3 surface antigens were more prevalent in the vaccinated lambs while but there was no difference between the vaccinated and control groups in the levels of mucosal and bile IgA which recognised the same surface antigens. Unfortunately, there was no attempt in the study of Wedrychowicz *et al.* (1995) to identify or characterise the individual surface antigens which constituted the larval extract or if any of the antigens were preferentially recognised by more resistant animals.

Huntley *et al.* (2001) also investigating *T. circumcincta* infections in sheep reported significant IgE responses to L_3 larvae but not to adult worms. Western blotting identified a strongly reacting band at 140-150 kDa (HMWTc) in *T. circumcincta* L_3 larvae which was recognised by host IgE. Reduction of HMWTc showed IgE reactive bands of 120, 50, 45 and 30 kDa, while N-linked deglycosylation abrogated the immunoreactivity of the 120 and 30 kDa bands. An analysis of a small number of naturally infected lambs in their first grazing season has shown that lambs which had low cumulative faecal egg counts had significantly higher IgE responses to HMWTc than lambs with high egg counts (Huntley *et al.*, 2001). However, this association could not be repeated in a different population of sheep (M.J. Stear, personal communication).

Immunoglobulin G1 was the predominant bovine antibody in calves experimentally infected with *O. ostertagi* recognising a range of molecules in L_3, L_4 and adult parasite extracts (Hilderson *et al.*, 1993). Although IgG2 and IgM responses were also observed, specific IgA antibody responses were hardly detectable. A 17 kDa globin-like molecule was further characterised from adult *O. ostertagi* but there were no differences in the responses to the antigen between calves infected with *O. ostertagi* and control animals (De Graaf *et al.*, 1996).

Three of fifteen L_3 larval molecules recognised by total plasma IgG in *T. circumcincta* challenged sheep were associated with differences in worm burdens (McCririe *et al.*, 1997). The same study reported that two molecules recognised in adult worm somatic extracts were associated with differences in worm lengths. Strain & Stear (1999) reported that recognition of *T. circumcincta* L_4 larval antigens with approximate molecular weights of 87 and 129 kDa by plasma IgA in infected sheep was associated with reductions in mean adult female worm lengths.

While the previous chapter and other studies have reported the importance of the host IgA response to infection with *T. circumcincta*, only the study of Strain & Stear (1999)

has investigated the role of IgA in the recognition of parasite antigenic molecules. McCririe *et al.* (1997) investigated the same set of animals as the study of Strain & Stear (1999), but utilised the plasma IgG responses of the sheep to identify potential immunogenic targets. Strain & Stear (1999) only investigated the recognition of 4th stage larval extracts while McCririe *et al.* (1997) investigated the recognition of 3rd stage and adult extracts. Additionally, antigen extracts were treated with periodate in the study of McCririe *et al.* (1997) to remove carbohydrate.

The work in the present chapter aims extend the studies by Strain & Stear (1999) and by McCririe *et al.* (1997) by investigating the plasma IgA responses to somatic extracts of 3rd stage larvae of *T. circumcincta* in experimentally infected Scottish Blackface sheep. These animals have been matched for breed, sex, age, date of birth, farm of origin and previous exposure to infection which makes this within-group analysis powerful at detecting real differences. Further, the method was modified to account for the differences in plasma antibody concentration among animals which may confound the interpretation of antigen recognition.

4.2 Materials and methods

4.2.1 Animals and Experimental Design

The experimental design is described in chapter 3.

4.2.2 Parasitological methods

4.2.2.1 Necropsy and total worm counts

Animals were necropsied and worms counted as described in chapter 3.

4.2.2.2 Female worm lengths and number of eggs in utero

Methods for assessing worm lengths and the number of *in utero* eggs were described in chapter two.

4.2.2.3 Faecal egg counts

The number of nematode eggs in faeces was determined with a modified McMaster technique as described in chapter two. Faecal egg counts were estimated three times each week during the challenge infection.

4.2.2.4 Preparation of parasite somatic extracts

Methods for preparation of *T. circumcincta* somatic extracts were given in chapter two.

4.2.3 Immune cell histology

Methods for assessing the numbers of mast cells, globule leucocytes, eosinophils and IgA positive plasma cells in the gastrointestinal mucosa were described in chapter 3.

4.2.4 Serological methods

4.2.4.1 Blood Sampling

Plasma and buffy coats were collected from blood samples as described in chapter two. Samples were taken before both infections, two or three times each week during infection and immediately before slaughter.

4.2.4.2 Peripheral blood eosinophil concentration

Peripheral blood eosinophil numbers were enumerated according to the method described in chapter two. Counts were performed on samples taken three times each week during the period of the challenge infection.

4.2.4.3 Plasma pepsinogen concentration

Plasma pepsinogen concentrations were determined as described in chapter 3.

4.2.4.4 Cell culture of rat anti-sheep IgA monoclonal

The rat anti-sheep IgA monoclonal antibody which was used as the primary antibody in the ELISA and Western assays was prepared as described in chapter two.

4.2.5 ELISA assays

Parasite specific host IgA responses to infection were measured by ELISA as described in chapter two.

4.2.6 Recognition of antigens from *T. circumcincta* by SDS-PAGE and Western blotting

The method used for the separation of parasite proteins on polyacrylamide gels (SDS–PAGE) followed by identification of proteins recognised by plasma from infected sheep (Western blotting) was described in chapter two. Where different techniques were employed to the method in chapter two, information is given here as necessary.

4.2.6.1 Kinetics of parasite antigen recognition following secondary challenge with T. circumcincta

To determine whether the profile of antigens recognised by infected sheep changed following challenge infection, seven plasma samples from different time points during the challenge infection were tested.

Ten microlitres of L_4 or 15µl of L_3 (approximately 12µg total protein in sample loading buffer) of somatic extracts of *T. circumcincta* were separated on 7.5% vertical polyacrylamide gels, transferred to nitrocellulose and processed as described in chapter two. Seven plasma samples (days 0, 10, 20, 31, 41, 52 and 61 p.i.) from four animals were tested for IgA specific recognition of parasite antigens. These were the same plasma samples that had been used determine the preliminary plasma IgA kinetics in chapter three (Figure3.4).

Blots were processed in Photoshop (Adobe) and TotalLab (Phoretix) as described previously. A band which each animal recognised was selected and the intensity of the

signal to that band was used for a comparison between the signal strength on Western blots and the concentration of IgA in the plasma samples as measured in the ELISA assay.

4.2.6.2 Adjustment of plasma antibody concentrations for Western blotting

The preliminary kinetic analysis revealed that the strength of signal on the Western blots was directly proportional to the activity of IgA in the plasma samples. Therefore, to reduce the possibility of wrongly attributing antibody concentration differences as recognition differences all subsequent Western assays employed plasma samples which had been adjusted to contain approximately the same amount of antibody.

Purified ovine IgA is not commercially available therefore samples could not be adjusted by comparing them to an IgA standard of known concentration. Sufficient signal strength and band recognition could be achieved from plasma samples which had exhibited OD index values of approximately 0.2 during earlier IgA specific ELISA assays. Samples could therefore be tested by Western analysis if plasma samples were adjusted to give an OD index of 0.2.

A standard ELISA was performed on all samples as described in chapter two with plasma samples diluted 1:10 in blocking buffer. The positive control (a sample that had previously given high optical densities) used in the ELISA assay was titrated and tested at doubling dilutions from 1:2.5 to 1:5120. The results from each dilution were transformed into OD indices based on the equation given previously (Equation 2.1), with the sample diluted at 1:10 being used as the positive control. This gave an OD index of 1.0 for the sample diluted at 1:10. Optical density indices were plotted graphically in Microsoft Excel 97 against the logarithm of the dilution factor. The equation of the resultant straight line was also calculated. In order for adjustments to be determined, it was assumed that all the test samples would follow the same decay pattern when diluted.

The gradient (m) from the straight line equation of the titration of the positive standard was used to calculate the intercept (c) of the hypothetical straight line of each test sample given the OD index and dilution factor according to Equation 4.1.

$$c = y - (m(\log_{10} x))$$

Equation 4.1: Equation for calculation of unknown intercept (c) of test samples for adjustment of plasma IgA concentrations. y is the OD index of the test sample before adjustment, m is the gradient of the straight line from the titration of the positive standard and x is the dilution factor of the test sample (i.e. for dilution of 1:10 x = 10; for dilution of 1:40, x = 40).

Once the intercept of the straight line of the test sample had been calculated, the dilution factor for adjusting the test sample to the required OD index could be determined by applying Equation 4.2.

$$X = 10^{\left(\frac{Y-c}{m}\right)}$$

Equation 4.2: Equation for calculation of dilution factor of test samples for adjustment of plasma IgA concentrations for Western blotting. X is the unknown dilution factor to use for the adjustment (i.e. 10 = 1:10, 40 = 1:40), Y is the OD index to adjust the samples to (usually 0.2), c is the intercept calculated in Equation 4.1 and m is the gradient of the straight line from the titration of the positive standard.

This adjustment process was applied to each plasma sample to be analysed by Western blotting. Before use in the Western assay, adjusted plasma samples were re-tested in the IgA ELISA assay to check that the adjustment did indeed work and that OD indices had been adjusted to approximately 0.2.

4.2.6.3 Recognition of third stage antigens of T. circumcincta *by Western blotting using plasma samples adjusted for differences in IgA concentration*

SDS-PAGE was performed as described in chapter two. Twenty-four microlitres of L_3 or parasite somatic extract diluted 1:1 in gel loading buffer (20µg protein) were run in each lane of a 5-20% resolving gel with a 4% stacking gel (Appendix A).

Following transfer, membranes were treated as described. Plasma samples were diluted according to the adjustments made above. Image processing and determination of band sizes were also performed as described. Two separate matrices for band recognition

were produced. One simply used positive or negative recognition of each band while the other took into account the strength of recognition by utilising the strength of the signal on the Western blot. Both sets of results are considered and details are given where necessary.

4.2.7 Statistical analyses

The method, with an example, used to normalise the parasitological and histological data is given in chapter three.

Student's two sample t-tests and Pearson product moment correlation coefficients were conducted using Minitab v13.3 (Minitab Inc.). Where variables had been logarithmically transformed before the application of t-tests, the means between groups were back transformed geometric means and were calculated in Microsoft Excel. The equality of variances between animals that recognised a particular band and those that did not for each parasitological parameter was checked using the TTEST procedure in SAS (SAS Institute, Cary, NC, USA). The general linear model (GLM) program in SAS was used for regression analysis using adjusted (type III) sums of squares. The test for the homogeneity of correlation coefficients (Gomez & Gomez, 1984; Sokal & Rohlf, 1995) was performed as described in chapter two using the Costat v6.003 computer package (CoHort Software, Monterey, CA, USA).

4.3 Results

4.3.1 *T. circumcincta* third and fourth stage antigens

Figure 4.1 demonstrates that *T. circumcincta* somatic larval extracts are complex and when reduced are comprised of proteins which span the molecular weight range. Antigens of similar sizes appear in all extracts, but these are not necessarily the same proteins. The antigenic profiles of the larval extracts used in this study are similar to those used previously by McCririe *et al.* (1997) since dominant bands are evident at similar sizes in both profiles.

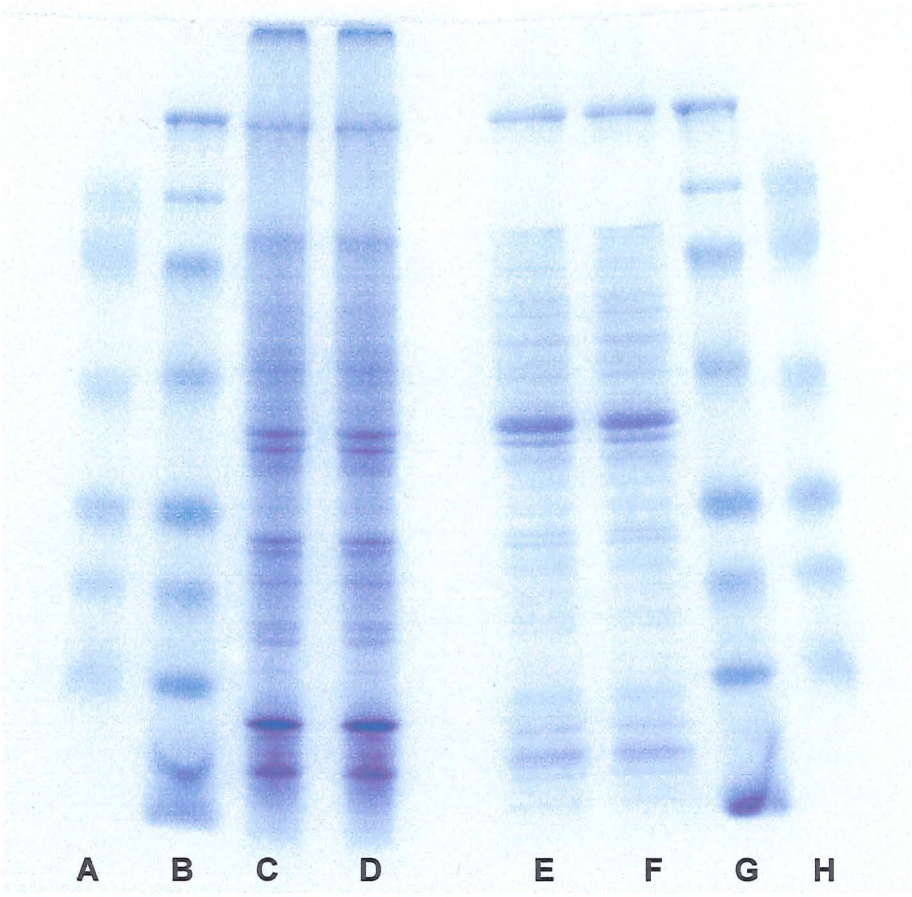

Figure 4.1: Coomassie stained 5-20% SDS-PAGE gel of *T. circumcincta* third and fourth stage larvae. Fourth stage larvae are in lanes C and D while third stage larvae are in lanes E and F (Duplicate lanes for each parasite extract). Lanes A, B G and H show molecular weight markers.

4.3.2 Kinetics of parasite antigen recognition following secondary challenge with *T. circumcincta*

A preliminary analysis was conducted to determine whether the pattern of bands recognised by plasma IgA in experimentally challenged animals changed as the challenge infection proceeded. Figure 4.2 shows the recognition of *T. circumcincta* fourth stage antigens in four challenged animals. The pattern of bands recognised by each animal did not change as the infection proceeded; rather it was the intensity of the signal on the blots which changed. The same set of bands was recognised at the end of the challenge infection as was recognised at the start of the challenge regime. A similar response was seen for the recognition of L_3 antigens of *T. circumcincta* (data not shown), with the differences in antigen profile over time due to differences in antibody concentration rather than antibody specificity.

A band that appeared in all blots was selected and the signal intensity recorded. The strength of the signal on each blot closely matched the strength of the antibody response when measured by ELISA (chapter three, Figure 3.4). The correlations between antibody responses as measured by ELISA and by Western blot analyses at each of the seven time points tested are shown in Table 4.1. The overall pooled correlation between the assays, calculated by the test for the homogeneity of correlation, was very high ($r = 0.905$). This confirmed what was assumed visually in that differences in some banding patterns on the Western blots could wrongly be attributed to differences in recognition when in fact the differences were due to the amount of antibody in the plasma samples.

Therefore, all subsequent Western assays employed plasma samples that had been adjusted to contain similar IgA activities.

4.3.3 Adjustment of plasma antibody concentrations

4.3.3.1 Titration of high standard for antibody adjustments

The high standard was titrated at varying dilutions in order to give a straight line equation from which the adjustment of test samples could be calculated (Table 4.2). The OD Index of each dilution was plotted against the logarithm of the dilution factor to

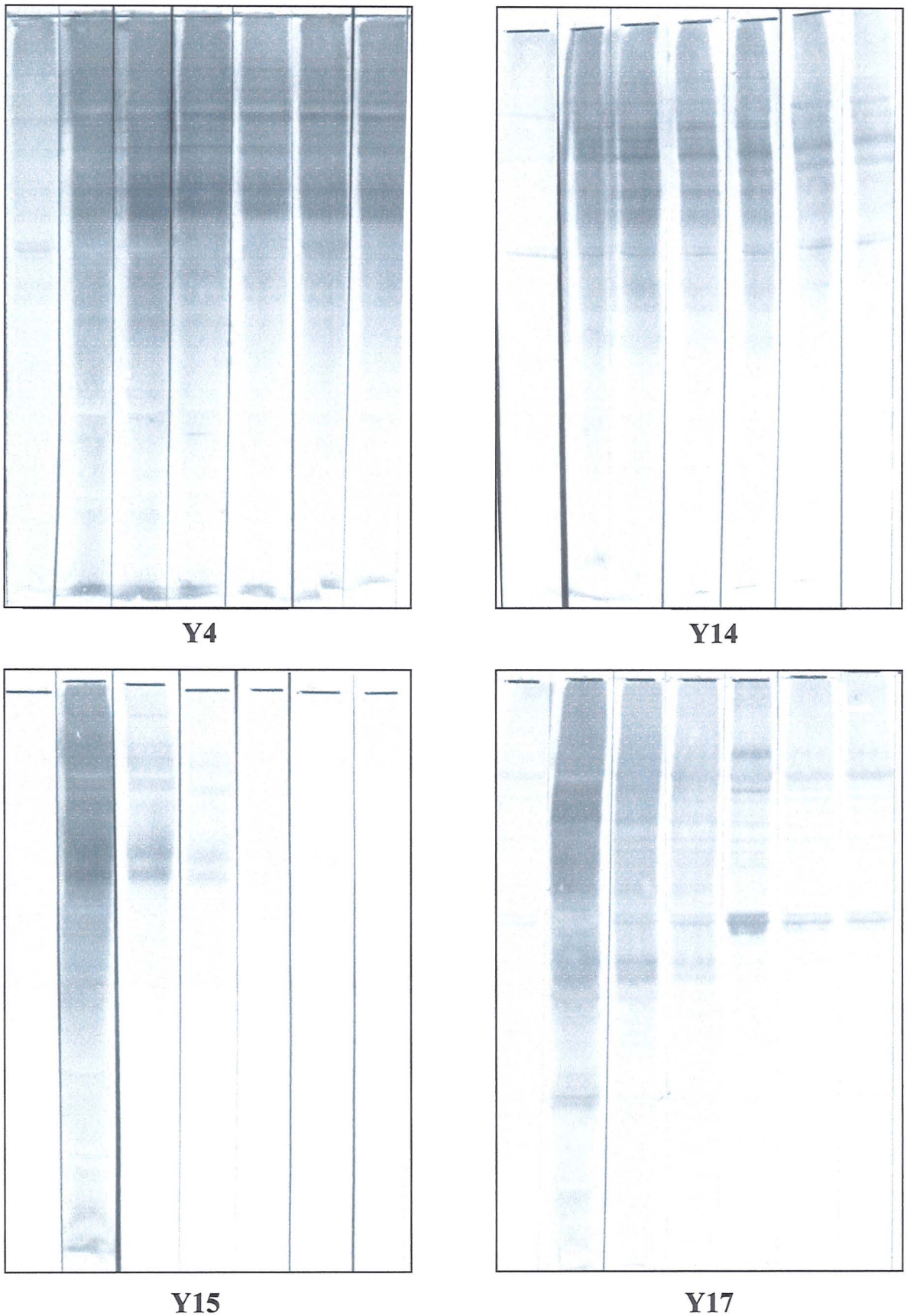

Figure 4.2: Western blot kinetics of L_4 specific plasma IgA responses in *T. circumcincta* challenged Scottish Blackface lambs. In each animal, from left to right, blots are from plasma samples from days 0, 10, 20, 31, 41, 52 and 61 after infection. 7.5% PAGE gels with plasma samples diluted 1:10 in PBS-TSM.

Animal	Days Post Infection 0	10	20	31	41	52	61
ELISA							
Y4	0.141	1.202	1.208	1.131	0.697	0.807	1.018
Y7	0.150	1.481	0.310	0.276	0.182	0.115	0.148
Y14	0.088	1.229	0.882	0.503	0.473	0.243	0.188
Y15	0.020	1.014	0.088	0.033	–0.005	0.000	0.009
Y17	0.010	0.876	0.293	0.098	0.064	0.089	0.079
Western							
Y4	53.51	130.60	167.10	161.24	141.00	142.56	139.39
Y7	51.27	140.46	75.79	89.18	85.52	78.64	65.90
Y14	17.01	89.04	115.39	76.80	79.03	57.96	42.82
Y15	2.16	148.42	81.83	44.69	12.77	1.25	5.59
Y17	10.30	91.48	74.12	45.56	19.90	4.45	5.02
Correlation (r)	0.941	0.345	0.942	0.961	0.915	0.890	0.933
P	0.017	0.571	0.017	0.009	0.029	0.043	0.020

Table 4.1: Correlations between ELISA and Western blot plasma IgA responses in Scottish Blackface lambs following a challenge infection with *T. circumcincta* third stage larvae. The test of homogeneity of the correlation coefficients estimated the common correlation as r = 0.905. The test gave a *P* value of 0.762 indicating that the coefficients should be considered homogenous, although the correlation at 10 days post infection (r = 0.345) appears quite deviant from the rest. This could be caused by saturation of the signal in the very strong samples in both the Western and ELISA assays.

Dilution	Sample average	1:10 high std average	Low std average	OD Index	Log dilution factor
1:10	1.840	1.840	0.035	1.000	1.000
1:20	1.629	1.840	0.035	0.883	1.301
1:40	1.447	1.840	0.035	0.782	1.602
1:80	1.251	1.840	0.035	0.674	1.903
1:160	0.980	1.840	0.035	0.523	2.204
1:320	0.724	1.840	0.035	0.381	2.505
1:640	0.492	1.840	0.035	0.253	2.806
1:1280	0.359	1.840	0.035	0.179	3.107
1:2560	0.251	1.840	0.035	0.120	3.408
1:5120	0.130	1.840	0.035	0.052	3.709

Table 4.2: Data from titration of the high standard used in anti-L_3 IgA ELISA assay. The high standard was titrated and the equation of the linear portion of the plotted line was used for the adjustment of plasma antibody concentrations for use in subsequent Western blotting assays.

produce the straight line (Figure 4.3). The gradient of the line (–0.368) was then used to adjust the plasma samples for use in the Western assay.

4.3.3.2 Pre- and post-adjustment anti-L_3 plasma IgA ELISAs

Table 4.3 shows the OD indices from the anti-L_3 plasma IgA ELISA assays before and after adjustment. Also shown in the table are the suggested changes to the dilution factor from the adjustment procedure to standardise the antibody quantity in each plasma sample. The arithmetic mean of the OD index decreased from 0.372 ± 0.062 to 0.200 ± 0.027 following the adjustment process.

The OD index of some samples (e.g. 24 and 28) did not reduce adequately after adjustment and remained high. These samples had pre-adjustment optical densities that were very high and at the upper tail of the titration graph. Small differences in OD indices at this end of the straight line equated to huge differences in suggested dilution adjustments because the adjustment has a logarithmic component on the x-axis of the graph. For example, the suggested dilutions for pre-adjustment OD indices of 0.3 and 0.4 are 1:18.7 and 1:34.95 respectively. When diluting the samples this equates to a difference of only a few microlitres of sample. On the other hand, the suggested dilutions for pre-adjustment OD indices of 1.1 and 1.2 are 1:2790 and 1:5216 respectively. Although the proportion between the dilutions is the same, the difficulty in accurately preparing solutions containing these different dilutions is greatly increased. The samples that gave spurious post-adjustment results were re-adjusted and re-tested to ascertain the appropriate dilution for the standardisation of the antibody concentration of each plasma sample.

4.3.4 Recognition of *T. circumcincta* third stage larval antigens with adjusted activity of plasma IgA

A digitised image of the Western blot of the third stage larval antigens of *T. circumcincta* recognised by immune sera from challenged Scottish Blackface lambs is shown in Figure 4.4. In all 63 bands were recognised, ranging in size from approximately 20 to over 200 kDa. No animals recognised all the bands but some bands were recognised by all the animals. The size and frequency of recognition of the 63

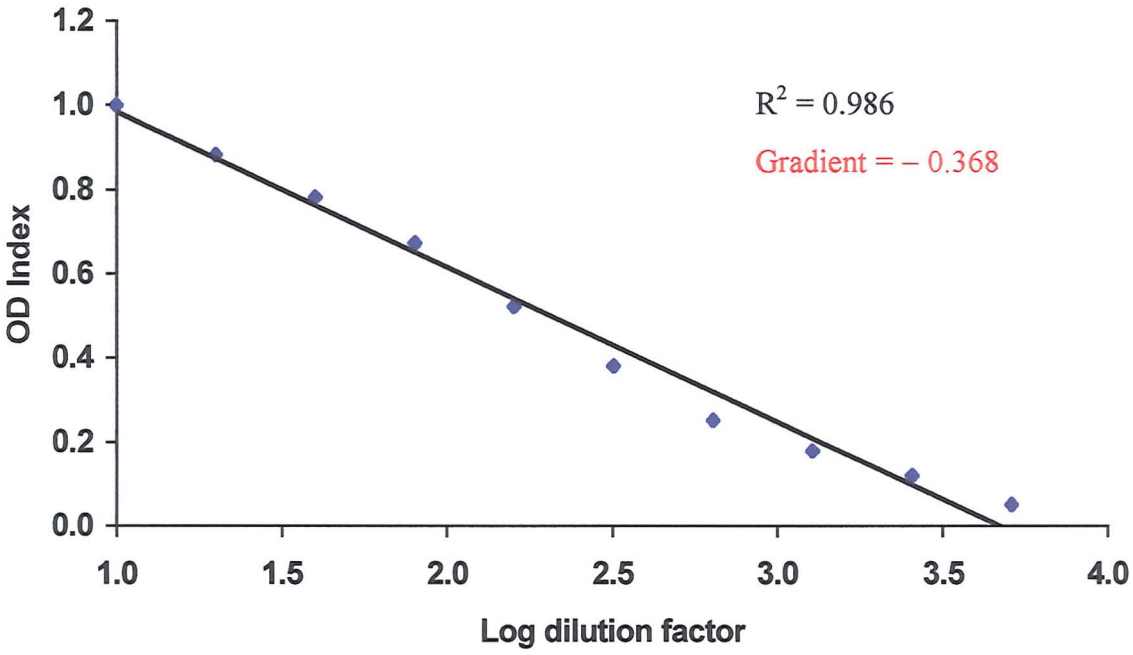

Figure 4.3: Plot of titration of the high standard used in anti-L_3 IgA ELISA assay for adjustment of plasma samples. The R^2 value and gradient of the line are also shown.

Animal	Before	Calculated dilution (1 : x)	Actual dilution (1 : x)	After
3	0.091	5.06	5.06	0.149
4	0.705	235.66	222	0.160
5	1.049	2027.99	1000	0.231
6	0.013	3.1	3.1	0.039
7	0.216	11.05	11.11	0.232
8	0.155	7.55	7.55	0.179
9	0.137	6.78	6.78	0.114
10	0.146	7.14	7.14	0.124
11	0.764	340.89	250	0.159
12	0.417	38.88	40	0.227
13	0.434	43.24	44.12	0.223
14	0.140	6.87	6.9	0.140
15	0.007	2.99	2.99	0.051
16	0.087	4.93	4.94	0.106
17	0.068	4.38	4.4	0.102
18	0.518	73.14	72.73	0.210
19	0.079	4.69	4.71	0.083
20	0.149	7.27	4.27	0.206
21	0.740	293.35	250	0.244
22	0.372	29.34	28.57	0.246
24	1.100	2790.31	1000	0.821
25	0.240	12.84	12.93	0.192
26	0.263	14.83	14.82	0.184
27	0.570	101.26	100	0.246
28	1.204	5348.86	1000	0.536
29	0.512	70.44	66.67	0.213
30	0.578	106.46	100	0.244
31	0.047	3.84	3.85	0.073
32	0.316	20.66	21.13	0.205
33	0.043	3.74	3.74	0.075
Average	0.372 (± 0.062)			0.200 (± 0.027)

Table 4.3: Mean (± S.E.) and individual optical density indices for pre- and post-adjustment anti-L_3 plasma IgA ELISAs. The OD index is shown for each animal before and after adjustment using the dilution given. Actual dilutions used were the calculated dilutions shown rounded to workable volumes. A maximum dilution of 1:1000 was used.

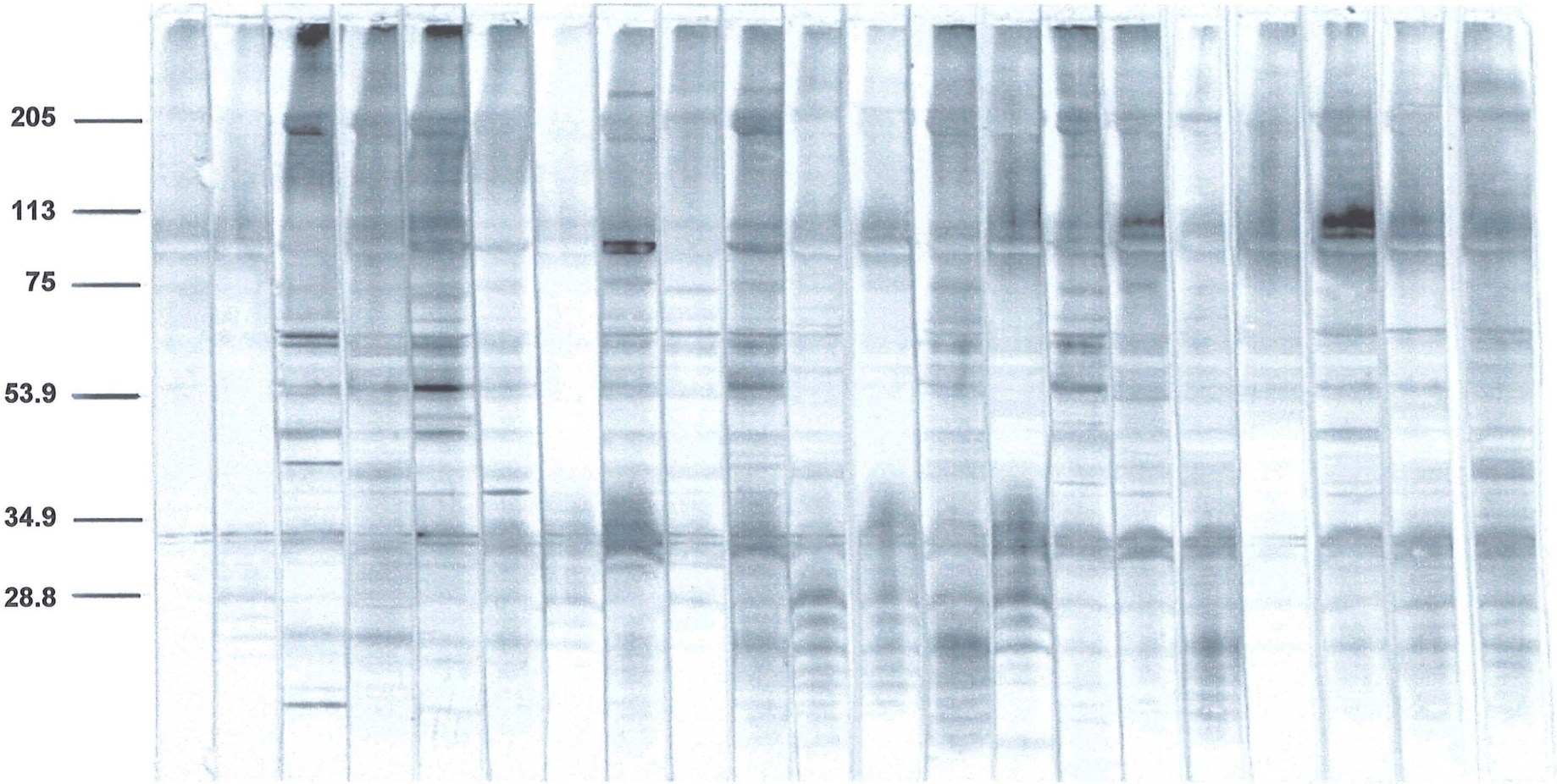

Figure 4.4: Digitised image of Western blot of plasma IgA recognition of *T. circumcincta* third stage larvae in experimentally challenged Scottish Blackface lambs. Plasma samples were taken at the time of slaughter, 8 weeks after secondary infection. Plasma IgA concentrations were standardised to contain the same amount of antibody. Each lane represents an individual animal. Molecular size markers (kDa) are shown on the left.

bands is shown in Table 4.4. The average number of bands recognised was 31.6 ± 1.79 (range 15-41).

There were no significant correlations between the total number of bands recognised and total worm burdens (r = 0.332, P = 0.178), worm lengths (r = 0.046, P = 0.855), adult worm mass (r = 0.301, P = 0.225), faecal egg counts at slaughter (r = 0.144, P = 0.568), the number of eggs per female worm (r = –0.003, P = 0.989), the number of mucosal mast cells (r = –0.051, P = 0.840), the number of globule leucocytes (r = –0.174, P = 0.490), the number of mucosal eosinophils (r = –0.98, P = 0.700), the number of mucosal IgA positive plasma cells (r = –0.207, P = 0.410) or the plasma pepsinogen concentration at slaughter (r = –0.057, P = 0.822).

Interestingly however, after having adjusted plasma samples to contain the same activity of IgA, there were negative correlations between the number of bands recognised and the IgA response to L_3 somatic extract at slaughter as measured by ELISA (r = –0.549, P = 0.023).

4.3.5 Associations between L_3 larval antigen recognition and parasitological and histological variables

Data for antigen recognition were collected as both positive/negative recognition of each particular band, and as the strength of the signal on the Western blot for each band. Results are presented here separately for each set of data in association with the various parasitological and histological parameters measured, as they provide different insights into the complexity of the host/parasite interaction.

4.3.5.1 Worm burden

4.3.5.1.1 Positive / negative recognition of bands

The mean log transformed total worm burdens were compared between animals that recognised each particular band and those that did not by Student's two-sample t-test. The equality of variances test was applied to the worm burden data for each band. The

Molecular Weight (kDa)	Frequency	Molecular Weight (kDa)	Frequency
367	56	56	94
335	6	54	22
280	67	51	83
251	56	50	72
212	94	48	6
191	94	47	17
180	11	46	22
168	50	45	33
155	50	43	78
144	67	42	22
133	17	41	72
113	100	39	67
106	83	38	56
101	22	37.5	50
96	94	36	67
93	39	35	44
91	11	34	72
87	11	32	94
82	28	31.5	100
80	56	30	67
78	11	29.5	17
76	11	29	50
74	50	28	100
73	22	27.5	6
71	17	27	78
69	44	26	100
68	17	25.5	67
67	11	25	39
64	67	24.5	50
62	83	24	44
60	83	23.5	11
57	17		

Table 4.4: Recognition of 63 third stage larval antigens of *T. circumcincta* by IgA. The sizes of bands recognised and the frequency of recognition are given.

test reported that worm burden data from only 5 of the 63 (8%) band associations had unequal variances. As this could be a chance effect of multiple comparisons, the t-test procedure was applied for all bands using equal variances.

The recognition of four different bands was significantly or nearly significantly associated with worm burdens (Table 4.5). The back transformed geometric mean worm burdens of animals which recognised individual bands of 68 and 47 kDa were 5.92 and 10.01 times lower respectively than animals which did not recognise any of these bands. Animals that recognised the two bands at 37.5 or 24 kDa had geometric mean worm burdens 4.67 and 4.53 higher respectively, than animals that did not recognise these bands. There was no correlation between recognition of each of these bands with any of the three others. This means that animals that recognised one of these bands were no more likely to recognise one of the other three bands than any other animal.

As shown previously, animals with higher numbers of GL tended to have lower total worm burdens ($r = -0.556$, $P = 0.005$). The plasma IgA response was also negatively associated with worm burdens. To determine the influence of these parameters on worm burdens, a multiple regression model which fitted the number of GL, plasma IgA activity and recognition of the four bands above was used. The model was highly significant ($P = 0.0009$) and accounted for 86% of the variation in the transformed total worm burden. This model accounted for considerably more of the variation in worm burdens than when the model was applied without the influence of the four bands ($R^2 = 0.32$, $P = 0.022$). This analysis suggests that the number of GL, antibody activity and antigen recognition all play a role in controlling worm numbers.

4.3.5.1.2 Strength of band recognition

If the strength of signal on the western blots are used rather than simply using the positive / negative recognition of bands then the analyses produce slightly different results. This data cannot be used in a t-test as signal strength is a continuous variable rather than a classification (positive or negative) variable. Subsequently the influence of signal strength of band recognition on worm burdens was analysed by one way regression. Analysis revealed that strong recognition of three bands was significantly associated with log transformed total worm burdens (Table 4.6). Of the three bands, two were the same bands that the previous analysis had identified as being statistically

Band (kDa)	Mean WB pos	Mean WB neg	Influence on WB	Probability
68	326	1933	–5.92	0.054
47	216	2164	–10.01	0.008
37.5	3127	669	4.67	0.018
24	3344	737	4.53	0.023

Table 4.5: Recognition of third-stage larval antigens of *T. circumcincta* and associations with total worm burden (WB) (adults + L_4 + L_5). The back transformed geometric mean total worm burdens were compared between animals that recognised the band (pos) and animals that did not recognise the band (neg). A –5.92 influence on worm burdens indicates that worm burdens were 5.92 times lower in animals that recognised this band then in animals that did not and vice versa.

Band	Correlation	Probability
47 kDa	–0.624	0.006
41 kDa	0.485	0.042
24 kDa	0.493	0.038

Table 4.6 Correlations between signal strength on Western blots of plasma IgA against different bands of *T. circumcincta* third stage larvae and log transformed total worm burden. Only significant associations are shown.

significant (47 and 24 kDa). The strength of recognition to the two other bands that were previously identified as important, were not significant in this analyses (68 kDa: r = –0.223, P = 0.373; 37.5 kDa: r = 0.341, P = 0.166) when tested for an association with worm burdens.

One additional band at 41 kDa was statistically significant in this analysis. Animals that recognised strongly the band at 47 kDa tended to have lower worm burdens, while animals that recognised strongly the bands at 41 and 24 kDa tended to have higher worm burdens. There was no correlation between recognition of each band and recognition of each of the other two bands. Neither was there any correlation between the strength of the reaction to each band and the strength of the reaction to each of the other two bands. In other words, an animal that recognised strongly one of the three bands was no more, or less likely to recognise either of the remaining two bands or produce strong responses against them.

A multiple regression model which fitted the strength of recognition of these three bands, the number of GL and the plasma IgA activity accounted for 83% of the variation in worm burdens. Again, this was considerably more than the variation in worm burdens accounted for by the model without the influence of the three bands.

4.3.5.2 Worm length

4.3.5.2.1 Positive / negative recognition of bands

The mean adult female worm lengths were compared between animals that recognised each particular band and those that did not by Student's two-sample t-test. The equality of variances test was applied to the worm length data for each band. The test reported that only 4 of the 63 (6.4%) band associations had unequal variances. As this could be a chance effect of multiple comparisons, the t-test procedure was applied for all bands using equal variances.

The analyses revealed 8 bands that were associated with differences in worm lengths at the 10% level. Of these 8 bands, 1 was recognised by only one animal while another was recognised by all but one animal. Both bands were not studied further. Of the remaining 6 bands only 3 gave significant associations at the 5% level (Table 4.7).

Band (kDa)	Mean WL pos	Mean WL neg	Difference	Probability
367	0.954	0.834	+0.120	0.026
73	0.794	0.932	–0.138	0.035
36	0.946	0.811	+0.135	0.017

Table 4.7 Recognition of third-stage larval antigens of *T. circumcincta* and associations with adult female worm length (WL). The mean worm lengths (in cms) were compared between animals that recognised the band (pos) and animals that did not recognise the band (neg).

Animals which recognised bands with molecular weights of approximately 367 and 36 kDa had adult female worms which were on average 0.120 and 0.135 cm longer respectively than animals which did not recognise these bands. Animals that recognised a band with an approximate molecular weight of 73 kDa had adult female worms that were on average 0.138 cm shorter than in animals that did not recognise the band. There was no significant correlation between recognition of each of these bands and recognition of the other two bands.

Previous analyses demonstrated that the number of mucosal eosinophils was significantly associated with adult female worm lengths. Additionally, the plasma IgA response was also associated with reduced worm lengths. A regression model which fitted the number of mucosal eosinophils, the plasma IgA activity and recognition of the three bands above accounted for 78% ($P = 0.002$) of the variation in adult female worm lengths.

4.3.5.2.2 Strength of band recognition

When the strength of the IgA response rather than just the positive or negative response was used to determine the relationship between band recognition and worm length the analyses gave slightly different results. Only the recognition of two bands gave statistically significant associations with worm lengths in this analysis. Strong recognition of the 73 kDa band from the previous analysis was also significantly correlated with worm lengths in this analysis ($r = -0.470$, $P = 0.049$). In addition, strong recognition of a band at 25 kDa was also significantly associated with reduced adult female worm lengths ($r = -0.558$, $P = 0.016$). The 367 and 36 kDa bands that had given significant associations with worm length in the previous analysis did not attain statistical significance when the strength of the response to the band was used (367 kDa, $P = 0.0603$; 36 kDa, $P = 0.285$).

There was no correlation between recognition of the 73 kDa band and recognition of the band at 25 kDa ($P = 0.103$). However, of the animals which did recognise both bands, there was a relatively strong and significant positive correlation between the strength of the IgA response to those bands ($r = 0.585$, $P = 0.005$).

A regression model which used the strength of recognition to these two bands as well as the number of mucosal eosinophils and the plasma IgA activity as independent variables accounted for 70% ($P = 0.004$) of the variation in worm lengths.

4.4 Discussion

This chapter has investigated the recognition of third stage larval antigens of *T. circumcincta* by Scottish Blackface sheep following deliberate challenge infection and whether preferential recognition of particular bands confers resistance or susceptibility.

The pattern of bands which were recognised by the animals did not change markedly as the challenge infection proceeded. The main differences between recognition in the same animal on different days was due more to the amount of antibody in the samples rather than actual differences in recognition. Other studies which have investigated the kinetics of the immune recognition of parasite antigens are hard to find, and for those which do exist the experimental regimes are usually quite different.

The larval antigen recognition profile from bile antibodies of 5-month old Finn-Dorset lambs did not change after initial immunisation and subsequent challenge with surface antigens of *O. circumcincta* in the study of Wedrychowicz *et al.* (1995). The only other comparable study which could be found was that of Cury *et al.* (2002) which investigated *Angiostrongylus vasorum* (the French heartworm) infections in dogs. The IgG responses of infected dogs were assessed by ELISA and Western blot analysis following primary challenge with infective larvae. On the day of infection ELISA IgG responses were negligible and few if any bands were recognised by Western analysis. By fourteen days most animals recognised a range of antigens and ELISA IgG responses were increased. The infection proceeded for a further twenty weeks but the pattern of antigens which were recognised did not change significantly, as was the case in the present study.

Additionally, in support of the results reported here, the differences in the banding patterns which were observed in the study of Cury *et al.* (2002) could also have occurred as a consequence of the amount of IgG in the serum samples. Although this idea was not considered by the authors, examining the ELISA results suggests that the strength of band recognition was closely associated with the level of serum IgG which

may have accounted for the minor differences in band recognition seen between animals. The present study showed that the strength of signal on a Western blot was highly correlated with the strength of the antibody response as measured by ELISA. The overall common correlation coefficient between IgA ELISA responses and IgA Western signals was 0.905 in samples spanning the duration of the infection. A search of the literature has identified only one other study which has directly considered this point. However, the study by Gómez-Muñoz *et al.* (1999), which investigated *H. contortus* infections in 2-month old Castellana lambs, reported a lack of correlation between the serum antibody response as estimated by ELISA and the degree of reactivity in Western blots. In studies using Manchego and Texel breeds respectively, Cuquerella *et al.* (1994) and Schallig *et al.* (1995) found more intense and complex immunodetection patterns in the Western blots of partially protected animals following *H. contortus* infection. These results are consistent with the hypothesis that intense Western staining is correlated with increased antibody activity which in turn is associated with protection.

The present study is an extension of the study of McCririe *et al.* (1997). Both studies utilised the same samples from a group of experimentally challenged animals but the present study measured plasma IgA responses while McCririe *et al.* (1997) measured total antibody (predminantly IgG). Additionally, larval extracts were treated with periodate to remove carbohydrates in the study of McCririe *et al.* (1997). The present study also adjusted antibody concentrations before Western blotting. Only 20 molecules were recognised by plasma samples from these animals using the technique of McCririe *et al.* (1997), while 63 different bands were recognised using the method in the present study. It is possible that the different antibody isotypes recognise different parasite antigens. This is a distinct possibility particularly since there was a considerable negative correlation between IgA and IgG activity in these animals. This was not tested in this thesis but should be a consideration for future work.

McCririe *et al.* (1997) found that no animal recognised all the potentially immunogenic molecules and that no molecule was recognised by all sheep. In the present study no animal recognised all the bands but in contrast to McCririe *et al.* (1997), there were four molecules recognised by all the animals, these were at 113, 31.5, 28 and 26 kDa. Standardising the IgA activity of the samples used in the present study and perhaps the periodate treatment of samples in the previous study are likely to explain the differences.

The 31.5 kDa band was the lower band of a doublet, with the 32 kDa band which was recognised by all but one animal. This doublet is likely to be the 31-33 kDa doublet initially proposed as a vaccine candidate by McGillivery *et al.* (1989) and later identified as the beta- galactoside-binding proteins (galectins) (Newton *et al.*, 1997). The galectin doublet is unlikely to be useful as a vaccine candidate since Morton *et al.* (1995) could not reproduce the initial results of the vaccination trials published by McGillivery *et al.* (1989). Also, Newlands *et al.* (1999) used a galectin (Hco-gal-2) characterised from the gut of *H. contortus* larvae in a vaccine study but it did not confer any protection against infection with *H. contortus* as judged by FEC and worm counts. However, a molecule, or group of molecules, which protects even a portion of the animals immunised could still be beneficial as herd immunity would reduce parasite transmission (Gasbarre *et al.*, 2001).

The present study determined that preferential recognition of four third stage larval molecules was associated with significant differences in total worm burdens. Animals which preferentially recognised the bands at 68 and 47 kDa had total worm burdens significantly lower than animals that did not recognise these bands while animals that recognised the bands at 37.5 and 24 kDa had significantly increased worm burdens. The study of McCririe *et al.* (1997) concluded that recognition of the three bands at 22, 31 and 48 kDa was associated with significant increases in worm burdens, although there was a significant association between recognition of the band at 22 kDa and the band at 31 kDa. No bands were associated with reduced worm burdens in this latter study. It is possible that the important bands in the study of McCririe *et al.* (1997) are the deglycosylated versions of those which are important in the present study. Technical differences could account for the differences in band sizes. In addition, the present study utilised digital imaging and analysis software to determine band recognition while the previous study assessed band recognition visually. While the present system is not faultless, it may be more consistent.

Preferential recognition of three third stage antigens in this study was also associated with significant differences in adult female worm lengths. The bands had approximate molecular weights of 367, 73 and 36 kDa and were associated with significant differences of +0.120, –0.138 and +0.135 cm respectively in adult female worm lengths. McCririe *et al.* (1997) found no significant associations between third stage antigen recognition by IgA and worm lengths. McCririe *et al.* (1997) did identify two adult

molecules which were associated with significant differences in worm lengths while Strain & Stear (1999) found that preferential recognition of two fourth stage bands was associated with reduced adult female worm length.

Recognition of these sets of bands in conjunction with the influence of GL or eosinophil numbers, as well as the plasma IgA activity accounted for a considerable proportion of the variation among worm burdens and adult female worm lengths respectively.

In addition to the positive/negative recognition of parasite antigens, the present study also determined whether the strength of band recognition was associated with resistance. Strong recognition of three third stage antigens was associated with significant differences in worm burdens while strong recognition of two antigens was associated with significant differences in worm lengths. The studies of McCririe *et al.* (1997) and Strain & Stear (1999) did quantify the level of staining reaction on the Western blots. Only the 47 kDa and 73 kDa bands from the previous analyses also gave significant associations with worm burdens and worm lengths respectively when using the strength of the band response. These two bands may be the most important and show up in both analyses while the other bands which do not show significant associations in both analyses may be less important.

The present study and those cited previously (McCririe *et al.*, 1997 and Strain & Stear, 1999) showed that preferential recognition of some parasite antigens was associated with increased worm burdens and/or worm lengths. Preferential recognition of a parasite molecule leading to improved parasite survival must mean that antibody binding is not deleterious for the parasites survival. This could be an evolutionary adaptation by which the parasite misdirects the host's immune response to non-essential parasite targets thereby reducing the possibility of terminal attack.

Alternatively, these proteins could be signalling molecules which are used to determine parasite density. The proteins might be released by the parasite and as the parasite density increased the concentration of the protein in the local environment would increase. Developing parasites might use these signals to determine if there will be enough space and/or resources for survival. If the concentration of the signal reached a threshold, this may cause larvae to go into inhibition. A similar mechanism has been observed in African trypanosomes (Vassella *et al.*, 1997). The authors showed that trypanosomes release a soluble low relative molecular mass protein, termed stumpy

induction factor (SIF), which triggers cell cycle arrest and halts development under increasing density conditions.

ES products of larvae and/or adult worms may play a role in parasite survival. The ES products of *T. circumcincta* have been implicated in the pathology associated with infection. They cause smooth muscle contraction and can affect gastrin production leading to the increase in abomasal pH (Scott & McKellar, 1998; Simpson *et al.*, 1999). Protection has been induced in sheep challenged with *H. contortus* following vaccination with adult derived ES proteins (Schallig *et al.*, 1997; Vervelde *et al.*, 2001), but this has not been demonstrated in *T. circumcincta* or even *T. colubriformis* infections.

Gut derived proteins have also recently been tested as potential vaccine candidates with encouraging success for *H. contortus* (Smith *et al.*, 2001b) but with less success for *T. circumcincta* (Smith *et al.*, 2001a). Smith *et al.* (2001a) also reported that the experimental vaccine containing *H. contortus* gut derived proteases did not provide any useful cross-protection against either *T. circumcincta*, *T. axei* or *C. oncophora* but in a reciprocal experiment where sheep were immunised with the equivalent glycoproteins from *T. circumcincta* there was some cross-protection against *Haemonchus* as measured by a significant reduction in worm egg output. The important proteins of interest from the present study may be gut proteins but only once localisation studies are conducted will this be known.

In conclusion, this chapter has investigated the recognition of third stage larval antigens of *T. circumcincta* by host plasma IgA following challenge infection. A different set of antigens were preferentially recognised by IgA compared to those recognised by IgG in the study of McCririe *et al.* (1997). Preferential recognition of these antigens accounted for a considerable proportion of the variation in worm burdens and worm lengths when the numbers of GL and eosinophils as well as the plasma IgA activity were included in the analyses. Additionally, a method for adjusting antibody concentrations in plasma has also been described which makes subsequent Western blot analysis more appropriate and reduces the possibility of confounding differences among animals in antibody activity with differences in antibody specificity.

Chapter 5

Recognition of larval antigens by plasma IgA from Scottish Blackface sheep following natural predominantly *Teladorsagia circumcincta* infection

5.1 Introduction

The previous chapter, and other studies, have demonstrated that a wide range of *T. circumcincta* proteins of different life cycle stages are recognised by antibody, and in particular IgA, in infected sheep. It has also been shown that preferential recognition of some of these proteins is associated with resistance or susceptibility to infection with the parasite.

However, previous studies have investigated the recognition of parasite antigens under controlled deliberate experimental conditions. It is not clear whether, under natural infection conditions, the same conclusions would be hold. The present author is unaware of any study that has attempted to investigate and verify whether recognition of particular bands that appear to be resistant in deliberate infections also confer resistance in naturally occurring infections with this parasite.

In fact, there are very few studies that have investigated the recognition of parasite antigens in naturally occurring infections at all for any gastrointestinal nematode species, let alone to back-up the results of deliberate infections of sheep with *T. circumcincta*. Studies such as these are much more common for human gastrointestinal parasitic infections where deliberate infection of experimental subjects would be unethical.

Atkins *et al.* (1999) were the first group to utilise quantitative immunoblotting in the analysis of *Strongyloides stercoralis* L_3 antigen-specific antibody isotype responses. Autoinfection in human strongyloidiasis is a major determinant in the progression to clinical disease as larvae may develop to the tissue invasive filariform third larval stage (L_3) before leaving the gut. This group showed that IgA reactivity with six immunodominant antigenic components of somatic extract of third stage larvae was significantly elevated in individuals with undetectable larval output by stool culture. This report supported previous work from the same group (Atkins *et al.*, 1997) which hypothesised an acquired effector role for IgA in human strongyloidiasis. Atkins *et al.* (1999) stated that the precise mechanisms by which the host modulates larval output are unknown although parameters such as worm fecundity, egg viability and infection intensity are likely to be involved.

Interestingly, Mansfield & Schad (1992) observed no difference in *S. stercoralis* parasitological parameters amongst intact dogs infected with *S. stercoralis* compared to those congenitally deficient in serum and mucosal IgA. Atkins *et al.* (1999) suggested that it seems likely that whilst IgA may not be critical in the clearance of infection, i.e. worm number, it may be involved in the modulation of worm fecundity and egg viability in chronic *S. stercoralis* infections.

Immunoglobulin A has also been shown to be a major humoral factor in reducing worm fecundity and egg viability in human schistosome infections (Grzych *et al.*, 1993; Capron *et al.*, 1994). Grzych *et al.* (1993) reported that IgA antibodies to the recombinant *S. mansoni* 28 kDa antigen glutathione-S transferase (Sm28GST) displayed a potent neutralising effect on the enzymatic properties of the molecule, and also markedly impaired schistosome fecundity by limiting both the egg laying of mature worms and the hatching capacity of schistosome eggs into viable miracidia.

A monoclonal antibody blocking activity of Sm28GST significantly reduced female worm fecundity and egg viability in experimentally infected mice, but had no effect on worm burdens (Xu *et al.*, 1991; Xu *et al.*, 1993). The 28 kDa GST of *Schistosoma haematobium* (Sh28GST) has recently been shown to have a high capacity for being bound by the sex steroid testosterone (Remoué *et al.*, 2002). In these infections, males are more resistant than females, so this research suggests a role for testosterone in binding GST and inhibiting its enzymatic ability and subsequently inhibiting egg production and egg viability.

The possibility for a role of GST in gastrointestinal nematode infections of sheep is however unclear. Sharp *et al.* (1991) raised antisera to GST from *H. contortus* and reported no effects on worm survival. The authors do not report whether there was any effect of the GST antisera on worm fecundity. This remains an area requiring further investigation.

Hernandez *et al.* (1999) reported that a 97 kDa band of adult soluble worm antigen was preferentially recognised by IgA in *Schistosoma japonicum* infected humans. Subsequent double immunoscreening revealed that the 97 kDa antigen was paramyosin, a myofibrillar protein found exclusively in invertebrates. Subsequent vaccine trials in mice and water buffalo using a bacterially expressed and purified form of *S. japonicum*

paramyosin (rec-Sj-97) demonstrated consistent decreases in the number of liver eggs following deliberate infection (McManus *et al.*, 2001b).

Helmy *et al.* (2000) noted enhanced recognition of antigens at 66, 40 and 14 kDa in immunopositive sera from Egyptians infected with the filarial nematode *W. bancrofti*. Only IgG responses were considered in this study with IgG4 being the most prominent IgG isotype. Previous studies however had shown no or limited band specificities in different geographical locations in the Far East (Day *et al.*, 1991; Zhang *et al.*, 1999). Characterisation of these potentially immunoprotective antigens has, as yet, been unpublished.

Only three bands from adult preparations (somatic and ES products) of *F. hepatica* were recognised by total IgG of cattle of different ages (Ortiz *et al.*, 2000). While the authors report that one of the bands is likely to be a cathepsin proteinase (28 kDa) and that the bands they reported confirm previous studies, no attempt was made to ascertain whether any of these molecules were preferentially recognised by resistant animals.

These studies have shown a role of IgA to control worm fecundity, and this is remarkably similar to the host/parasite system in the present study particularly given the major differences between nematodes and schistosomes. Additionally, and in support of the apparently similar mechanisms controlling these infections, the *S. japonicum*/human system is one of the few other host/parasite systems apart from that of the *T. circumcincta*/sheep system that has identified specific host MHC allelic associations between disease resistance and disease susceptibility (McManus *et al.*, 2001a). Additionally, Angyalosi *et al.* (2001) reported that there was a significant association between HLA polymorphism and pathology in *S. mansoni* infected mice which in turn correlated to differences in interferon gamma production. This also has considerable similarity to the *T. circumcincta*/sheep system (Coltman *et al.*, 2001).

The few studies reported here demonstrate a gap in our understanding of the recognition of specific antigens that are associated with disease resistance/susceptibility in a variety of host/parasite systems. Identifying parasite antigens that are preferentially recognised by resistant or susceptible animals under natural as opposed to experimental infection conditions is a necessity in all parasitic infections. There may be differences between the recognition of antigens in the two infection regimes caused by for example environmental factors. Breeding and vaccine/drug design programmes based

exclusively on deliberate experimental studies may select for or target parasite proteins that actually confer susceptibility on a host and/or promote parasite survival under natural conditions.

Therefore this chapter aims to extend on the results of the previous chapter and the other studies by investigating the recognition of different life cycle stage antigens of *T. circumcincta* by sheep experiencing natural mixed nematode infections which comprise predominantly *T. circumcincta.*

5.2 Materials and methods

5.2.1 Animals and experimental design

Approximately 200 straightbred Scottish Blackface lambs from a commercial, upland farm in Southwest Strathclyde were studied. All husbandry procedures followed standard, commercial practice. Lambs were born in a 16-day period in late April to early May. Within a week of lambing the ewes and lambs were moved onto one of two fields. Gastrointestinal nematode infection had previously been diagnosed in sheep on all fields for ten consecutive years. At four months of age the lambs were separated from their mothers and moved to the larger of the two fields.

Every four weeks from the age of four to twenty weeks lambs were weighed, faecal sampled, blood sampled and were given a broad spectrum anthelmintic (albendazole sulphoxide (Rycoben, Young's Animal Health, Leyland, UK) at a dose rate of 5mg kg^{-1} according to the manufacturers' recommendations. In order to determine anthelmintic efficacy faecal egg count reduction tests were performed on a small number of lambs which consistently gave high faecal egg counts. Ten days after anthelmintic treatment these animals were resampled to check for eggs in faeces. No evidence of parasite drug resistance was observed on this farm.

Six or seven weeks after the last anthelmintic treatment approximately half of the lambs were slaughtered in the local abattoir and the gastrointestinal tract was removed for analysis.

5.2.2 Parasitological methods

5.2.2.1 Necropsy and total worm counts

Standard parasitological procedures were used to identify and count all nematodes in the small intestine and abomasum (Armour *et al.*, 1966). The abomasum and small intestine were washed separately and the number of adult worms present in a 2% sample of the washing fluid was multiplied by 50 to estimate the worm burden of each particular parasite species. The number of fourth and fifth stage larvae were counted as well the number of adults. The large intestine was not investigated for the presence of parasites since the pre-patent period for all common large intestinal dwelling species exceeded the four-weekly anthelmintic treatment regime (Stear *et al.*, 1998). In addition, larval cultures of faecal samples from animals on the same farm did not reveal any large intestinal species in previous years.

5.2.2.2 Female worm lengths and number of eggs in utero

Methods for assessing worm lengths and the number of *in utero* eggs are described in chapter two. The length of 25 randomly selected female *T. circumcincta* worms were measured.

5.2.2.3 Faecal egg counts

The concentration of nematode eggs in faeces was estimated with a modified McMaster technique (Gordon & Whitlock, 1939). Eggs were counted from a 3g rectal sample and each egg counted represented 50 eggs per gram of faeces. Faecal egg counts were estimated once each month during the duration of the study at the time of anthelmintic treatment.

5.2.2.4 Preparation of parasite somatic extracts

Methods for preparation of *T. circumcincta* third and fourth stage somatic extracts that were used in the ELISA and Western assays are given in chapter two.

5.2.3 Serological methods

5.2.3.1 Blood Sampling

Plasma and buffy coats were collected from blood samples as described in chapter two. Blood samples were taken once each month during the duration of the study at the time of anthelmintic treatment. All assays described in this chapter utilised the plasma samples collected in September 1993, six weeks before the animals were slaughtered.

5.2.3.2 Peripheral eosinophil count

Peripheral eosinophil counts were performed as described in chapter two on each blood sample collected.

5.2.3.3 Preparation of anti-sheep IgA monoclonal

The preparation and cell culture of the rat anti-sheep IgA monoclonal antibody used in the ELISA and Western assays has been described in chapter two.

5.2.4 Parasite specific IgA responses

The plasma IgA responses to third and fourth stage larvae were assessed by ELISA as described in chapter two.

5.2.5 Parasite antigen recognition

5.2.5.1 Adjustment of plasma IgA activity

Previous assays (chapter four) had shown that the activity of IgA in a plasma sample could considerably affect the recognition of parasite antigens. Therefore, Western assays in this chapter used plasma samples that were diluted to contain approximately the same activity of IgA. The method used for the adjustment of plasma IgA concentrations is described in chapter four. Titration of high standards was performed separately for both third and fourth stage larvae.

5.2.5.2 Parasite protein separation and Western blotting

Third and fourth stage somatic larval extracts of *T. circumcincta* were separated under reducing conditions by SDS-PAGE (5-20%) according to the method described in chapter two. Twenty micrograms of protein (L_3 24µl or L_4 17.5µl) were run per lane.

Western blots were processed as described using plasma samples that had been adjusted for IgA activity. Developed blots were scanned and images analysed as described previously.

5.2.6 Statistical analyses

All datasets were tested to check whether they were normally distributed and then transformed as necessary using the Box-Cox power transformation in Minitab (v13.3) according to the method described in chapter three. Details of transformations are given in the results section.

Student's two sample t-tests and Pearson product moment correlation coefficients were conducted using Minitab. Where variables had been logarithmically transformed before the application of t-tests, the means between groups were back transformed geometric means. The equality of variances between animals that recognised a particular band and those that did not for each parasitological parameter was checked using the TTEST procedure in SAS. The mixed linear model (MIXED) program in SAS was used for regression analysis for identifying preferential recognition of parasite proteins and fitted sire and dam as random variables where appropriate.

5.3 Results

5.3.1 Data normalisation

Data for *T. circumcincta* adult female worm lengths did not require transformation as the untransformed data fitted the normal distribution ($A^2 = 0.372$, $P = 0.413$).

Data for adult worm burdens (male and female worms) were not normally distributed ($A^2 = 3.656$, $P < 0.000$). Box- Cox analyses suggested a transformation with lambda =

0, giving the transformation as log10 (adult worm burden + 1). The transformed dataset did not differ significantly from the normal distribution ($A^2 = 0.413$, $P = 0.332$).

Data for the adult worm mass (worm burden x worm length) also required transformation ($A^2 = 3.271$, $P = 0.005$). Using the transformation log10 (adult worm mass) the dataset did not differ significantly from the normal distribution ($A^2 = 0.314$, $P = 0.250$).

Data for the number of eggs per adult female worm did not follow the normal distribution ($A^2 = 3.982$, $P < 0.000$). Data were transformed according to the equation log10 (eggs per female + 1). Analyses showed that the transformed dataset was normally distributed ($A^2 = 0.332$, $P = 0.507$).

Data for peripheral eosinophil counts and faecal egg counts were transformed by taking the logarithm of the count plus one.

Data for the IgA activity against somatic extracts of third and fourth stage larvae of *T. circumcincta* were not normally distributed (L_3: $A^2 = 4.672$, $P < 0.001$; L_4: $A^2 = 3.663$, $P < 0.001$). These data sets were transformed using log10 (IgA OD index + 0.2). The transformed L_4 dataset was not significantly different from the normal distribution ($A^2 = 0.589$, $P = 0.123$). The transformation did not quite normalise the L_3 dataset ($A^2 = 0.883$, $P = 0.023$), but it was closer to a normal distribution than the untransformed dataset.

5.3.2 Relationships between parasitological and immune responses

There was a very strong significant positive correlation between the plasma IgA responses to third and fourth stage somatic extracts of *T. circumcincta* ($r = 0.905$, $P < 0.0001$), so that animals that reacted strongly against one antigen preparation tended to react strongly against the other antigen preparation (Figure 5.1).

Correlations between the different parameters measured are given in Table 5.1. As expected from the results of the deliberate infection described in chapters three and four, there was a significant positive correlation between worm lengths and the number

of eggs *in utero* (r = 0.866, $P < 0.0001$). There were significant negative correlations between the two IgA responses and worm lengths (L_3: r = –0.237, $P = 0.025$; L_4: r = –0.235, $P = 0.033$) but not with the number of eggs *in utero* (L_3: r = –-0.188, $P = 0.077$; L_4: r = –0.116, $P = 0.300$).

There were significant positive correlations between worm lengths and faecal egg counts (r = 0.404, $P < 0.0001$) and between faecal egg counts and the number of eggs *in utero* (r = 0.317, $P = 0.002$). There was a positive but non-significant correlation between the *T. circumcincta* adult worm burdens and faecal egg counts (r = 0.65, $P = 0.111$). There were also significant but weak negative correlations between the IgA responses to third and fourth stage larval extracts and egg counts (L_3: r = –0.155, $P = 0.042$; L_4: r = –0.176, $P = 0.028$).

There was a significant negative correlation between worm lengths and adult worm burdens (r = –0.283, $P = 0.005$). This is in contrast to the results of the deliberate infection (chapter three) which showed no significant correlation between worm lengths and worm numbers, although the association was also negative in the previous chapter. There was also a significant negative association between worm burdens and the number of eggs *in utero* under the natural infection conditions (r = –0.288, $P = 0.004$). Significant positive correlations also existed between the IgA responses to third and fourth stage larvae and worm burdens (L_3: r = 0.209, $P = 0.048$; L_4: r = 0.265, $P = 0.016$). These results are also in contrast to the results described for the deliberate challenge infection.

While worm lengths were positively correlated, and adult worm burdens negatively correlated with worm fecundity, the product of these two variables, the adult worm mass, was only weakly negatively and insignificantly associated with worm fecundity (r = –0.149, $P = 0.146$). The adult worm mass was significantly positively correlated with faecal egg count (r = 0.227, $P = 0.029$). These data suggest that it is the size (mass) of the worm population which is more important in determining egg output rather than the number of worms in the population. Although the plasma IgA responses were significantly correlated with both worm lengths and worm burdens there was only a weak and insignificant correlation between the IgA responses and the adult worm mass.

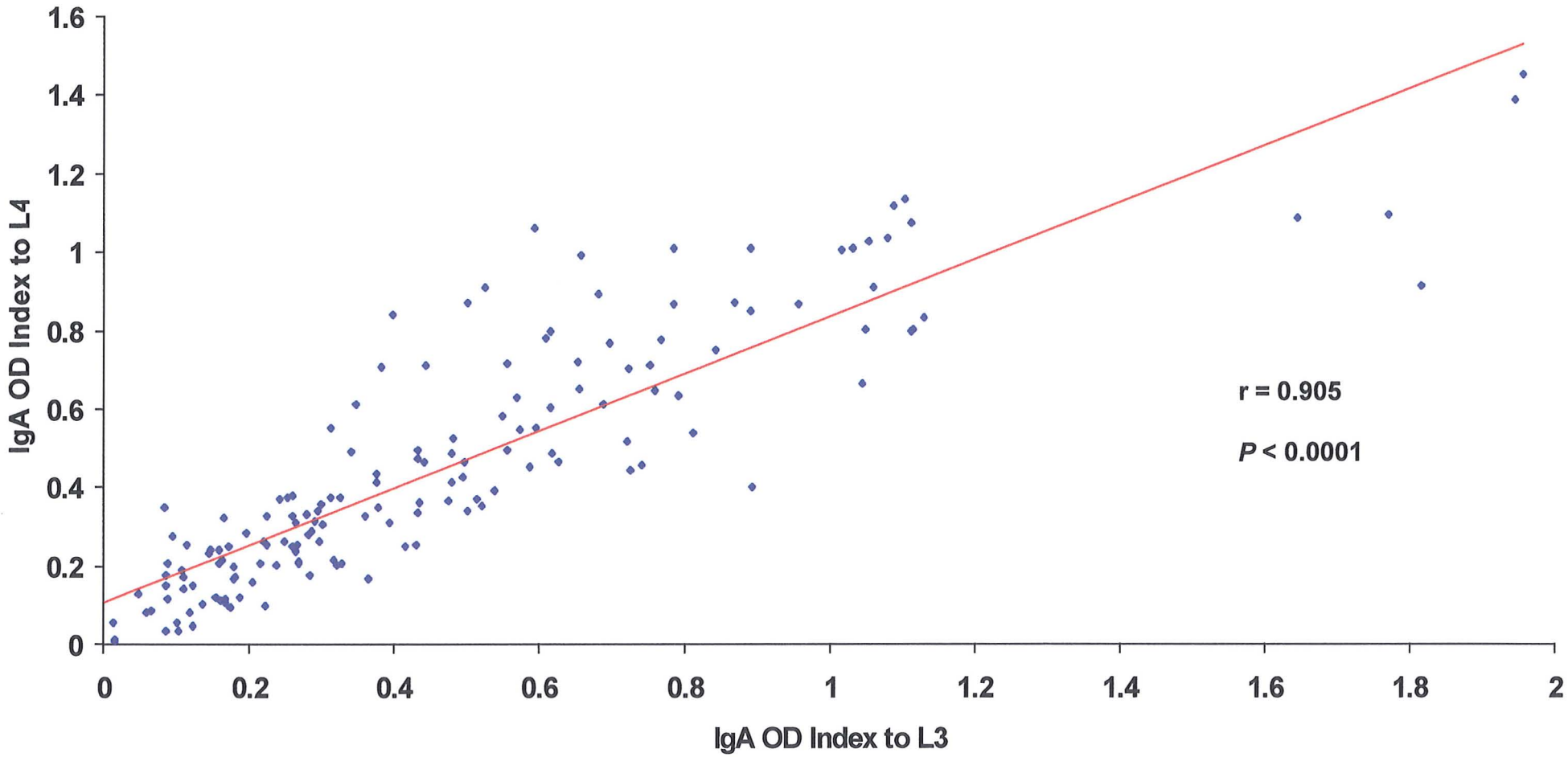

Figure 5.1: Scatter plot of ovine plasma IgA responses (OD Index) to third and fourth stage larvae of *T. circumcincta* following natural predominantly *T. circumcincta* infection. The linear regression line is shown in red (R^2 = 0.819). The points corresponding to the highest L_3 responses tend to be below the calculated trendline, this is because the L_3 assay was reaching saturation at these levels.

	WL	Worm burden	Worm mass	Eggs/female	FEC	L_4 burden	IgA L_3
Worm burden	–0.283**						
Worm mass	–0.122	0.986***					
Eggs/female	0.866***	–0.288**	–0.149				
FEC	0.404***	0.165	0.227*	0.317**			
L_4 burden	–0.277***	0.208*	0.188	–0.246*	0.028		
IgA L_3	–0.236*	0.209*	0.175	–0.187	–0.155*	0.101	
IgA L_4	–0.235*	0.265*	0.185	–0.116	–0.176*	0.179	0.905***

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

Table 5.1: Pearson product moment correlations among parasitological and immunological parameters from Scottish Blackface sheep following natural predominantly *T. circumcincta* infection. IgA assays were conducted on plasma samples collected at the end of September while the parasitological measurements were carried out approximately 6 weeks later.

Animals which harboured higher numbers of adult larvae at slaughter also tended to have higher numbers of fourth stage larvae in the mucosa of the abomasum ($r = 0.208$, $P = 0.040$). Because this was a natural and presumably continuous infection it is unclear whether these fourth stage larvae were inhibited larvae picked up at the beginning of the infection in early October or were newly acquired parasites which had not yet matured to the adult form but had not entered the inhibited stage. In either case the positive correlation between adult worm burdens, but interestingly not worm masses, suggests that the fourth stage larvae can monitor the size of the adult population and can become inhibited if the population of adults is too large and so would make survival and optimum breeding capacity difficult.

5.3.3 Adjustment of plasma IgA concentration

5.3.3.1 Titration of high standard against 3rd stage larvae

The high standard (a sample that had given a high OD Index in preliminary assays) was titrated at doubling dilutions against third stage larvae from 1:10 to 1:5120 (Table 5.2). The OD Index of each dilution was plotted against the logarithm of the dilution factor to produce a straight line from which the gradient (–0.409) was then used to adjust the plasma samples for use in the Western assay.

5.3.3.2 Titration of high standard against 4th stage larvae

The high standard was titrated at doubling dilutions against fourth stage larvae from 1:2.5 to 1:5120 (Table 5.3). The OD Index of each dilution was plotted against the logarithm of the dilution factor to produce a straight line from which the gradient (–0.362) was then used to adjust the plasma samples for use in the Western assay.

5.3.3.3 Pre- and post-adjustment anti-L_3 plasma IgA ELISAs

Plasma samples from September were tested by ELISA for IgA specific responses to third stage larvae. The OD Index was used with the gradient of the high standard titration (–0.409) to estimate a dilution to standardise the amount of the IgA between samples that would give an OD Index of 0.2. Samples were re-tested following the adjustment to check that the estimation was accurate. The OD Indices before and after

Dilution	Sample average	1:10 high std average	Low std average	OD Index	Log dilution factor
1:10	1.814	1.814	0.044	1.000	1.000
1:20	1.698	1.814	0.044	0.934	1.301
1:40	1.464	1.814	0.044	0.802	1.602
1:80	1.106	1.814	0.044	0.600	1.903
1:160	0.781	1.814	0.044	0.416	2.204
1:320	0.508	1.814	0.044	0.262	2.505
1:640	0.322	1.814	0.044	0.157	2.806
1:1280	0.177	1.814	0.044	0.075	3.107
1:2560	0.116	1.814	0.044	0.041	3.408
1:5120	0.053	1.814	0.044	0.005	3.709

Table 5.2: Titration of high standard for adjustment of plasma IgA against third stage larvae. The OD Index of each dilution was plotted against the logarithm of the dilution factor to produce a straight line from which the gradient was calculated (R^2 = 0.958).

Dilution	Sample average	1:10 high std average	Low std average	OD Index	Log dilution factor
1:2.5	1.872	1.768	0.127	1.063	0.398
1:5	1.810	1.768	0.127	1.026	0.699
1:10	1.768	1.768	0.127	1.000	1.000
1:20	1.630	1.768	0.127	0.916	1.301
1:40	1.371	1.768	0.127	0.758	1.602
1:80	1.174	1.768	0.127	0.638	1.903
1:160	0.893	1.768	0.127	0.467	2.204
1:320	0.680	1.768	0.127	0.337	2.505
1:640	0.494	1.768	0.127	0.223	2.806
1:1280	0.337	1.768	0.127	0.128	3.107
1:2560	0.223	1.768	0.127	0.058	3.408
1:5120	0.159	1.768	0.127	0.020	3.709

Table 5.3: Titration of high standard for adjustment of plasma IgA against fourth stage larvae. The OD Index of each dilution was plotted against the logarithm of the dilution factor to produce a straight line from which the gradient was calculated (R^2 = 0.979).

adjustment are shown in Appendix B. The average OD Index before adjustment was 0.536 ± 0.031. Following adjustment, the average OD Index was 0.205 ± 0.006.

5.3.3.4 Pre- and post-adjustment anti-L_4 plasma IgA ELISAs

Plasma samples were also tested by ELISA for IgA specific responses to fourth stage larvae. The OD Index was used with the gradient of the high standard titration (–0.362) to estimate a dilution to standardise the amount of the IgA between samples that would give an OD Index of 0.2. Samples were re-tested following the adjustment to check that the estimation was accurate. The OD Indices before and after adjustment are shown in Appendix C. The average OD Index before adjustment was 0.456 ± 0.025. Following adjustment, the average OD Index was 0.204 ± 0.007.

5.3.4 Plasma IgA recognition of *T. circumcincta* third stage larvae

A digitised image of a Western blot showing the recognition of third stage *T. circumcincta* antigens by plasma IgA from a sample of the sheep tested is shown in Figure 5.2.

In all 54 third stage larval bands were recognised. The size and frequency of recognition of the 54 bands is shown in Table 5.4. While no band was recognised by all the animals, as with the deliberate infection all but a couple of animals recognised both bands of the 30-31 kDa putative galectin doublet. The mean number of third stage bands recognised was 23.5 ± 1.9 (range 3-41).

There was no difference between the number of bands recognised by male and female lambs ($P = 0.666$). There was however a significant association between the number of bands recognised and the pre-weaning field that each lamb was on with its mother before all the lambs were put together on the same field. Animals that had been on field one recognised on average 21.6 ± 0.7 bands compared to animals that had been on field two which recognised on average 27.4 ± 0.9 bands ($P < 0.0001$). The influence of pre-weaning field on the number of bands recognised remained significant when a mixed model analysis which also fitted sex, sire and dam was used ($p < 0.0001$).

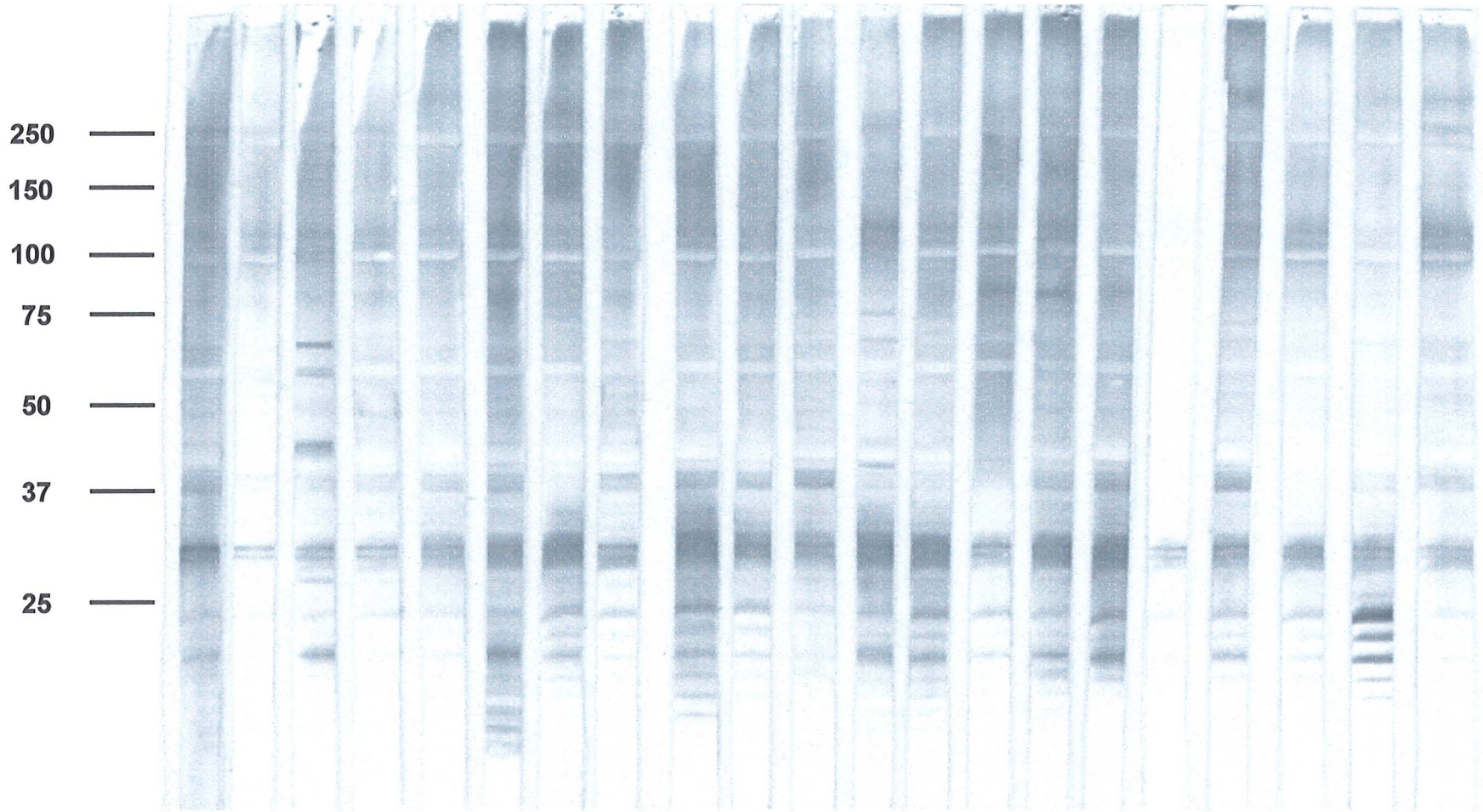

Figure 5.2: Digitised image of Western blot of plasma IgA recognition of *T. circumcincta* third stage larvae in naturally infected Scottish Blackface lambs. Plasma IgA concentrations were standardised to contain the same amount of antibody. Each lane represents an individual animal. Molecular size markers (kDa) are shown on the left.

Molecular weight	Frequency	Molecular weight	Frequency
427	18.1	50	91.9
284	24.4	47	78.1
257	20.0	44	91.9
225	48.8	42	24.4
210	3.1	40	52.5
198	51.9	39	15.6
183	1.9	37	87.5
180	1.3	36.5	3.1
170	2.5	36	83.1
153	5.0	35.5	67.5
149	24.4	35	83.1
142	21.3	33	36.9
122	11.9	32.5	48.8
109	51.9	32	30.6
100	52.5	31	99.4
90	52.5	30	99.4
85	13.8	29.5	57.5
76	1.3	29	60.0
74	44.4	28.5	42.5
67	56.3	28	97.5
62	50.6	27	88.8
59	20.6	26.5	96.9
58	53.1	26	78.1
56	50.6	25.5	63.8
54	52.5	25	29.4
53	2.5	24.5	6.3
52.5	5.0	24	2.5

Table 5.4: Frequency of recognition of *T. circumcincta* third stage larval antigens by plasma IgA from naturally infected Scottish Blackface lambs.

Although the lambs were born over a period of only 16 days there still remained a significant effect of age on the number of bands recognised by plasma IgA collected in September (r = 0.229, P = 0.004). The animals which were born first and so were older at the time of sampling recognised more bands than those animals born later. There was no significant difference between the ages of lambs that had been on each field (P = 0.089), so the differences seen between fields could not be attributed to the age of the animals.

5.3.5 Plasma IgA recognition of *T. circumcincta* fourth stage larvae

A digitised image of the a Western blot showing the recognition of fourth stage *T. circumcincta* antigens by plasma IgA from a sample of the naturally infected sheep tested is shown in Figure 5.3. Only 37 bands were recognised by these animals. The sizes of the bands recognised and the frequency of recognition are shown in Table 5.5. Again all animals recognised a 30-31 kDa doublet which is believed to be galectin.

The mean number of fourth stage bands recognised was 12.9 ± 0.4 (range 2-27). There was no difference in the number of bands recognised by male and female sheep (P = 0.924). As for the recognition of third stage antigens, animals which grazed on field two early in the experiment recognised more bands than animals which had grazed on field one (14.0 ± 0.7 compared to 12.3 ± 0.5 respectively), however the difference was not significant (P = 0.054). Contrary to the result for the recognition of third stage bands, there was no significant effect of age on the number of fourth bands recognised although the trend was in the same direction (r = 0.151, P = 0.084).

5.3.6 Number of bands recognised and association with resistance

There was a positive but weak correlation between the number of third stage bands recognised and the number of fourth stage bands recognised by plasma IgA from these naturally infected animals (r = 0.291, P = 0.001). Correlations between the numbers of bands recognised and the parasitological parameters measured are given in Table 5.6.

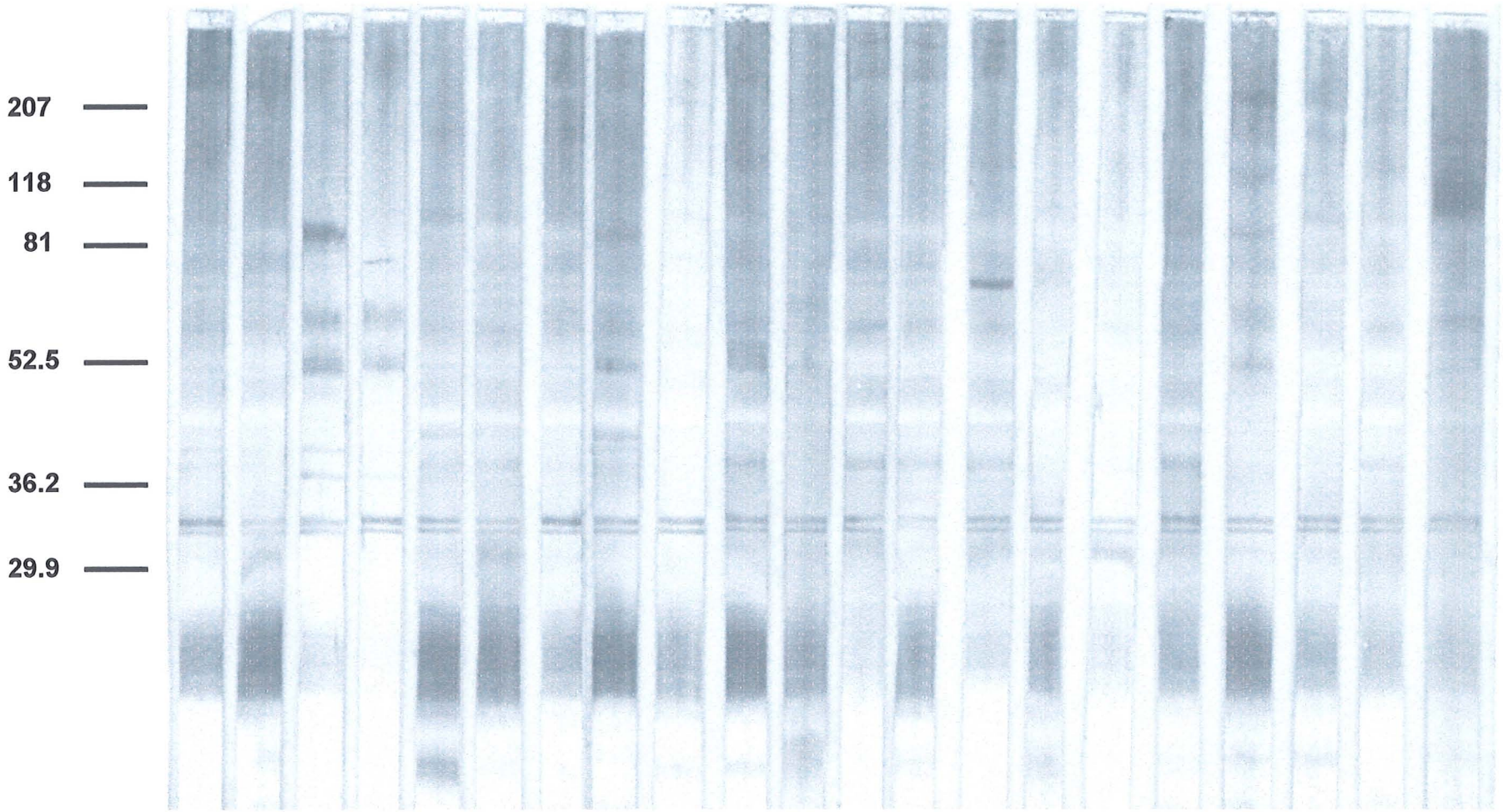

Figure 5.3: Digitised image of Western blot of plasma IgA recognition of *T. circumcincta* fourth stage larvae in naturally infected Scottish Blackface lambs. Plasma IgA concentrations were standardised to contain the same amount of antibody. Each lane represents an individual animal. Molecular size markers (kDa) are shown on the left.

Molecular weight	Frequency	Molecular weight	Frequency
384	59.0	57	44.0
286	4.5	55	9.0
264	11.2	54	30.6
232	10.4	50	17.2
211	23.1	48	78.4
197	20.1	47	14.2
180	54.5	45	16.4
161	59.0	43	11.2
147	17.9	38	53.0
131	29.1	37	32.1
123	14.2	36	31.3
114	20.9	35	26.1
100	47.8	34	14.2
90	30.6	33	6.7
85	18.7	31	100.0
78	39.6	30	100.0
72	50.0	29	44.8
67	70.9	24	28.4
61	53.0		

Table 5.5: Frequency of recognition of *T. circumcincta* fourth stage larval antigens by plasma IgA from naturally infected Scottish Blackface lambs.

	No. of L_3 bands	No. of L_4 bands
WL	–0.137	0.105
Worm burden	–0.221	–0.258*
Worm mass	–0.251*	–0.249*
EPF	–0.101	0.037
EPG	–0.179*	–0.011
IgA (L_3)	–0.267***	–0.396**
IgA (L_4)	–0.221***	–0.474***

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

Table 5.6: Pearson product moment correlations between the numbers of third and fourth stage antigens of *T. circumcincta* recognised by plasma IgA from Scottish Blackface sheep following natural predominantly *T. circumcincta* infection. The IgA (L_3) and IgA (L_4) variables were the plasma IgA responses to each extract as determined by ELISA.

Additionally and in support of the results from the previous chapter, there were significant negative correlations between the number of bands recognised by plasma IgA and the corresponding IgA responses as determined by ELISA. Again, this association occurred after plasma samples had been adjusted to contain the same activity of antibody.

5.3.7 Band recognition and worm length

Previous analysis on the animals used in this study (Stear *et al.*, 1996b) revealed that there was no significant effect of sex or age of a lamb on the length of adult female worms at slaughter ($P = 0.313$ and $P = 0.677$ respectively). The effect of dam was not significant ($P = 0.263$) while the effect of sire on worm lengths was significant ($P = 0.046$). The effect of pre-weaning field did not significantly affect worm lengths ($P = 0.954$). When the effects of sire and dam were fitted in a mixed model as random effects, the influences of sex, age and field remained non-significant at the 5% level.

As described previously, worm lengths were significantly influenced by the adult worm burden and by the plasma IgA responses to larval extract as measured by ELISA. The influence on worm lengths of these variables remained statistically significant when sire and dam were fitted in the mixed model (worm burden: $P = 0.015$; IgA against L_3: $P = 0.029$; IgA against L_4: $P = 0.028$).

Since the effects of sire, dam, worm burden and the plasma IgA responses were all significantly associated with worm lengths they were all fitted in the mixed model analyses which attempted to identify if preferential recognition of particular bands was associated with increases or reductions in adult female worm lengths.

5.3.7.1 Third stage T. circumcincta antigens

Preferential recognition of four third stage bands of *T. circumcincta* was associated with significant differences in adult female worm lengths (Table 5.7). Animals that recognised the bands at 50, 37, 32.5 or 29 kDa had on average worms that were respectively 12.6%, 9.7%, 7.6% and 7.1% shorter than there counterparts that did not recognise these bands. These bands were recognised by 92%, 88%, 49% and 60% of all animals respectively. There were no bands for which preferential recognition gave a

Band (kDa)	Worm length (cm) positive	Worm length (cm) negative	Difference (cm)	Probability
50	0.816	0.919	–0.103	0.024
37	0.814	0.893	–0.079	0.041
32.5	0.792	0.852	–0.060	0.027
29	0.801	0.858	–0.057	0.037

Table 5.7: Recognition of *T. circumcincta* third stage antigens by plasma IgA from naturally infected Scottish Blackface lambs and associations with adult female worm length. The average length of worms for animals that recognised each band (positive) and those that did not (negative) are shown. Band recognition was analysed as a fixed effect in a mixed model analysis on worm length. The model also fitted sire and dam as random effects and worm burdens and plasma IgA responses as fixed effects. Only significant associations between band recognition and worm lengths are shown.

significant increase in worm lengths. There were significant correlations between recognition of some of these bands. This meant that animals which recognised the band at 50 kDa were also likely to recognise the other three bands. There was also a small but significant positive correlation between recognition of the 37 kDa band and the 32.5 kDa band.

When the affinity or strength of the plasma IgA recognition of each band was used in the same model in place of the positive/negative recognition used above, there were no significant associations between band recognition and worm lengths. This included the four bands that had given significant associations in the previous analysis.

5.3.7.2 Fourth stage T. circumcincta *antigens*

Preferential recognition of three fourth stage larval *T. circumcincta* bands was associated with significant differences in worm lengths (Table 5.8). Animals that recognised fourth stage antigens at approximately 232 and 131 kDa had worms that were respectively 13.6% and 9.3% shorter than animals that did not recognise these antigens. Animals that recognised the band at approximately 34 kDa had worms that were 13.1% longer than animals that did not recognise the band. These three bands were recognised respectively by 10, 29 and 14% of animals. There was no correlation between recognition of these three bands.

When the strength of band recognition was considered rather than positive/negative recognition, the same three bands gave significant associations with worm lengths. In addition, three other bands also gave significant associations with worm lengths. Animals that recognised strongly the bands at approximately 232, 211, 131 and 123 kDa had significantly shorter worms than animals that reacted weakly to these bands while animals that recognised strongly the bands at 34 and 30 kDa had significantly longer worms than did those that reacted weakly to these bands. There were weak but significant positive correlations between recognition of the bands at 211 and 123 kDa and between the bands at 131 and 123 kDa while there was a weak negative correlation between recognition of the bands at approximately 211 and 34 kDa.

Additionally, correlations between the strength of the IgA responses to the six bands of interest are given in Table 5.9. There were several significant, although relatively weak

Band (kDa)	Worm length (cm) positive	Worm length (cm) negative	Difference (cm)	Probability
232	0.736	0.836	–0.100	0.035
131	0.777	0.849	–0.072	0.036
34	0.918	0.812	+0.106	0.033

Table 5.8: Recognition of *T. circumcincta* fourth stage larval antigens and associations with adult female worm length. The average length of worms for animals that recognised each band (positive) and those that did not (negative) are shown. Band recognition was analysed as a fixed effect in a mixed model analysis on worm length. The model also fitted sire and dam as random effects and worm burdens and plasma IgA responses as fixed effects. Only significant associations between band recognition and worm lengths are shown.

kDa	232	211	131	123	34
211	0.059				
131	0.661***	0.268**			
123	0.216*	0.354***	0.352***		
34	–0.062	–0.099	–0.047	0.115	
30	–0.086	–0.104	0.044	–0.044	0.186*

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

Table 5.9: Pearson product moment correlations between the strength of recognition of selected *T. circumcincta* fourth stage antigens by plasma IgA (bands associated with worm length).

positive correlations between the strengths of band recognition amongst these six bands, but there were no significant negative correlations.

5.3.8 Band recognition and worm burdens

In this group of animals *Teladorsagia circumcincta* adult worm burdens were not significantly influenced by sire (P = 0.473), dam (P = 0.423), sex (P = 0.847) or pre-weaning field (P = 0.097) (Stear *et al.*, 1996b), but were significantly positively influenced by the plasma IgA response to third stage (P = 0.048) and fourth stage (P = 0.016) larval preparations.

The effect of band recognition on worm burdens was assessed using a mixed model analysis which fitted sire and dam as random variables and band recognition and the IgA response as measured by ELISA as fixed variables. Sire and dam were fitted in the model even although they were not directly associated with differences in worm burdens. They were fitted to remove any associations which could be attributed to sire and dam and made the analysis of band recognition more sensitive.

5.3.8.1 Third stage T. circumcincta *antigens*

Recognition of four third stage antigens was associated with significant differences in the number of adult *T. circumcincta* at slaughter (Table 5.10). Animals which recognised the bands at approximately 183, 170, 50 and 26 kDa had worm burdens that were 74%, 85%, 55% and 41% lower respectively than animals that did not recognise the bands.

However, the bands at 183 and 170 kDa were recognised by only 3 (1.9%) and 4 (2.5%) of all the animals respectively, making them too infrequent for the associations to be statistically sound. The bands at 50 and 26 kDa were recognised by 147 (91.9%) and 125 (78.1%) of animals. There was a significant positive correlation between recognition the band at 50 kDa and the band at 26 kDa (r = 0.230, P = 0.003).

Recognition of five bands gave significant associations with worm burdens when the strength of the IgA response against the bands was tested in place of positive/negative recognition. Animals that recognised strongly the bands at 170, 122, 27 and 24.5 kDa

Band (kDa)	Worm burden positive	Worm burden negative	% difference	Probability
183	600	2265	–74%	0.031
170	342	2300	–85%	0.004
50	2039	4509	–55%	0.007
26	1898	3216	–41%	0.022

Table 5.10: Recognition of *T. circumcincta* third stage antigens and associations with adult *T. circumcincta* worm burdens. The back transformed geometric mean worm burdens of animals that recognised each band (positive) and of those that did not (negative) are shown. Band recognition was analysed as a fixed effect in a mixed model analysis on worm burdens which also fitted sire and dam as random effects and plasma IgA responses as fixed effects. Only significant associations between band recognition and worm lengths are shown.

had lower worm burdens than animals that did not recognise the bands, while animals that recognised strongly the band at 54 kDa had significantly more worms. There was a significant correlation between recognition of the band at 170 kDa and the band at 24.5 kDa ($r = 0.455$, $P < 0.0001$). This was the only significant correlation between recognition of these bands. Similarly the only significant correlation between the strength of recognition of these bands was between the bands at approximately 170 and 24.5 kDa ($r = 0.383$, $P < 0.0001$).

5.3.8.2 Fourth stage T. circumcincta *antigens*

Preferential recognition of only one fourth stage antigen gave a significant association with adult worm burdens. Animals that recognised the band at 85 kDa had worm burdens 61% lower than animals that did not recognise the bands. This band was recognised by 19%.

Additionally, animals that recognised strongly the fourth stage band at 72 kDa had significantly fewer adult *T. circumcincta* worms than animals that reacted weakly to this band ($P = 0.005$). This was the only band that gave a statistically significant association with worm burdens when the strength of the reaction to the band was used in the analysis in place of the positive/negative recognition of the band.

5.4 Discussion

This chapter has investigated the recognition of third and fourth stage larval antigens of *T. circumcincta* by plasma IgA from Scottish Blackface lambs following natural predominantly *T. circumcincta* infection and whether preferential recognition of specific antigens might confer resistance or susceptibility. The main aim was to investigate whether the same antigens which appeared to confer resistance/susceptibility under deliberate infection conditions did so during a natural infection.

In addition to the assessment of antigen recognition, it was observed that there was a positive correlation between the number of adults and the number of fourth stage larvae recovered at slaughter. As suggested previously it is unclear whether these fourth stage larvae were newly acquired larvae about to develop to adults or were previously acquired larvae which had become inhibited. In any case this suggests that the fourth

stage larvae might be able to monitor the size of the adult worm burden and enter inhibition if space is limited. How the worms are able to determine if conditions are favourable is unclear but the chemosensory pheromone signalling system seen in *C. elegans* dauer larva development has been suggested (Ren *et al.*, 1996) and has been implicated in the arrested development of *H. contortus* fourth stage larvae (Ashton *et al.*, 1999). A similar system has not yet been identified in *T. circumcincta* but it is probable that one exists.

The author believes that this study is one of the first to investigate the recognition of parasite antigens of *T. circumcincta* under natural infection conditions and has been possible by using the new adjusted antibody technique described in the previous chapter. The animals in this part of the study were younger and exposed to much lower parasite challenges than in the previous chapter and in turn produced weaker antibody responses. These antibody responses have been difficult to test by Western blotting using previous techniques (MJ Stear, personal communication).

There were 54 third stage and 37 fourth stage bands recognised by plasma IgA in these Scottish Blackface lambs which were sampled in September when the animals were approximately 5 months old. Using the same technique 63 third stage bands were recognised by the deliberately infected animals from the previous chapter which were over one year old. It might have been expected that the results would be the other way round since the present work utilised nearly ten times more animals. However, it is more likely that the differences are still due to differences in antibody concentration with the deliberately infected animals having higher plasma IgA concentrations even after adjustment.

McCririe *et al.* (1997) reported that 20 third stage bands and 31 adult bands were recognised by IgG from the deliberately infected animals using the unadjusted antibody technique, while Strain & Stear (1999) reported that 49 fourth stage bands were recognised by IgA in the same animals also without adjusting antibody concentrations. Strain & Stear (1999) also stated that no proteins were recognised which had molecular weights between 40 and 70 kDa. This is in contrast to the present study which identified bands throughout this size range. Technical differences and differences in the ages of the animals used between the two studies are the most likely candidates for the differing numbers of bands recognised. In addition, as stated in the previous chapter, the study of

McCririe *et al.* (1997) measured IgG responses rather than IgA responses and also treated larval extracts with periodate before blotting.

An interesting observation from the present and the previous chapter was that there was a negative correlation between the number of parasite larval antigens recognised after adjusting for antibody concentration and the activity of IgA in the unadjusted samples. This meant that animals which had the highest plasma IgA concentrations actually recognised the fewest bands. Since animals with increased plasma IgA concentrations are more resistant to infection (Stear *et al.*, 1995c) one would expect these same animals to recognise more parasite antigens than animals with weak antibody responses. However, when one considers what might actually be happening during these immune responses, in particular the process of affinity maturation, this phenomenon is not inconceivable.

The antibodies produced in a primary response to a T-dependent antigen generally have a low average affinity. However, during the course of the response, the average affinity of the antibodies increases or matures. As antigen becomes limiting, the clones with the higher affinity will have a selective advantage (Roitt *et al.*, 2001). The degree of affinity maturation is inversely related to the dose of antigen administered. It has been suggested that, in the presence of low antigen concentrations, only B cells with high affinity receptors bind sufficient antigen and are triggered to divide and differentiate. However, in the presence of high antigen concentrations, there is sufficient antigen to bind and trigger both high and low affinity B cells (Roitt *et al.*, 2001). This could explain the observations seen in this study. If only a proportion of parasite antigens was targeted by host antibody in the present system, affinity maturation would dramatically increase the levels of antibody to these antigens and account for the high levels of IgA seen in plasma. Animals which targeted many parasite antigens would tend to have low affinity antibodies which would not be selected for, so an increase in antibody production would not be observed, and in turn these animals would be less able to clear the infection. A similar hypothesis has been posed by Dunne *et al.* (1994) who investigated the antibody responses of mice following vaccination and infection with *S. japonicum*. This could also explain why whole parasite preparations have not been effective as vaccine candidates in promoting protection, particularly in young animals with undeveloped immune systems (Knox, 2000), while preparations consisting of a

single or a small number of defined antigens show much more promise (Knox & Smith, 2001).

The animals used in this study were receiving their fourth or fifth independent antigenic challenge. The process of affinity maturation during these secondary responses could also be promoted since the number of larvae establishing will be reduced in the more resistant animals. This could act in a kind of positive feedback loop in the resistant animals. Hypersensitivity immune responses to incoming larvae in the secondary responses of resistant animals would mean that fewer larvae established, leading to a reduced antigen challenge, in turn this would lead to greater affinity maturation and increased antibody levels and subsequently more worms would be lost and the worms that did establish would be shorter. In the more susceptible animals, the system could have the opposite effect, namely a negative feedback loop. More larvae would establish in secondary infections, giving a large antigen load which would not lead to affinity maturation and increased antibody levels. In turn these animals would have more and larger worms and would contribute significant numbers of infective larvae to the pasture. After each antigenic challenge the feedback system would intensify the immune response of resistant animals so that they would have high concentrations of high affinity antibodies to specific parasite targets while susceptible animals would consistently be unable to mount effective responses due to their having low concentrations of low affinity antibodies targeted to large numbers of unimportant parasite proteins.

Again, all but a couple of animals recognised both bands of the galectin doublet which appeared in both third and fourth stage larval extracts. Since virtually all animals recognise these antigens, it is impossible to determine whether there is any association between recognition and resistance. It may be that generations of selective breeding have unintentionally selected for recognition of these antigens. It is interesting to note that in the present study there was a significant association between the strength of the IgA response to the lower band of the doublet in fourth stage larvae and increased worm lengths. In other words, animals which reacted strongly to this band had significantly longer worms than animals which reacted weakly to this band.

The analyses revealed that preferential recognition of four third stage antigens with approximate molecular weights of 50, 37, 32.5 and 29 kDa were each associated with

reduced worm lengths. This is in contrast with the results of the previous chapter which identified that recognition of the bands at approximately 367 and 36 kDa were associated with increased worm lengths while recognition of the band at 73 kDa was associated with reduced worm lengths. Additionally, there were no associations between the strength of the IgA response to any bands in the current analyses compared to two significant associations in the previous analyses.

Preferential recognition of four third stage bands was also associated with significant reductions in adult worm burdens in the present study. Again, this is in contrast to the results of the previous chapter which identified two bands positively and two bands negatively associated with differences in worm burdens. It is possible however that the 50 kDa band in the present analysis is equivalent to the 47 kDa band from the previous analysis.

Three fourth stage bands were associated with significant differences in worm lengths. Animals which recognised the bands at 232 and 131 kDa had shorter worms and those that recognised the band at 34 kDa longer worms than those that did not recognise these bands. Recognition of the band at 85 kDa was also associated with reduced worm burdens in the present study. Strain & Stear (1999) found that bands at 87 and 129 kDa bands were associated with reduced worm lengths in the deliberate challenge infection study described previously. It is possible that the 85 and 131 kDa bands from this study are the same bands as the 87 and 129 kDa from the study of Strain & Stear (1999) with the small size differences being of a technical nature.

When the recognition of parasite antigens was compared in association to differences in adult worm mass the results were identical to those for the worm burden despite the fact that the worm mass was the product of the worm burden and the average worm length. This suggests that the worm burden is much more important in determining worm mass than worm length.

The results from this chapter, the previous chapter, and other published studies demonstrate why as yet there is no commercially available vaccine to combat gastrointestinal parasitism in ruminants. The inconsistency of the results proves that this is a very complex system about which we still need to learn a great deal. The differences between the results of these different studies could have been caused by the animals being exposed to slightly different parasite strains on pasture. Subsequently the

animals' immune responses could have targeted different antigens leading to the spurious Western blotting results. This is not inconceivable since the existence of different *T. circumcincta* morphotypes has been described, particularly in different geographical locations (Stevenson *et al.*, 1996). Also and perhaps more importantly the strain of parasite used as the primary antigen in the ELISA and Western assays may have differed from that with which the sheep were originally infected. Before efficient vaccines or selective breeding programmes can be designed, results must be confirmed in different farming situations, different geographical locations and different breeds of animal. There is little point in manufacturing a vaccine which is effective only in one breed of sheep in an isolated region of the country which just happens for example to be warmer or wetter than another and so the parasite population differs slightly. The same arguments are true for selective breeding programmes.

Molecular typing of parasite species and morphotype is being developed (Silvestre & Humbert, 2000; Zarlenga *et al.*, 2001). If these techniques become rapid and economically viable they could be used as part of vaccine development and selective breeding programmes to determine which strains of parasite are found in particular areas. When testing for antigen recognition in the future in may be more useful to use larval extracts from different locations. This could either be done individually with each animal being tested against a panel of different antigen extracts. Additionally, more suitable might be a single test which consisted of a mixture of different extracts. These tests would determine whether animals recognised different antigens from parasites which came from different locations and whether selection/vaccine design based on these results would be effective.

Teladorsagia circumcincta had been confirmed as the predominant gastrointestinal parasite for several years on the farm used in the present study. Interestingly there was a significant effect of pre-weaning field in the present study on the number of bands recognised. Unfortunately, data is not available as to the specific larvae present on each field at the time of weaning. It is very possible that even if small differences existed between the species of parasites which were present on the fields then this may confound the results presented here, particularly since there are likely to be many common antigens between the different parasite species. This later fact may benefit potential vaccines if an antigen or panel of antigens can be found which confers resistance against a range of parasite species.

In conclusion, this chapter has investigated the recognition of larval extracts of *T. circumcincta* by Scottish Blackface lambs following natural predominantly *T. circumcincta* infection. Preferential recognition of several larval antigens gave significant differences with adult worm burdens or worm lengths. These results differed from the results of the deliberate infection of the last chapter and from other published material (McCririe *et al.*, 1997; Strain & Stear, 1999). The results suggest we may have underestimated how complex this host/parasite system really is, particularly in the field, and that more research must be conducted before we can be certain that any potential vaccines or breeding programmes will be effective in different farming situations, locations and breeds.

Chapter 6

Associations between MHC polymorphism, antigen recognition and resistance to *Teladorsagia circumcincta*

6.1 Introduction

The MHC complex is among the most polymorphic gene complexes known in vertebrates (Bodmer *et al.*, 1990). Products of these genes present antigenic peptide to T cells, initiating an immune response and clearance of the foreign material. Evolutionary and population studies have led to the general idea that the great diversity and even distribution of allelic frequencies observed in the class I and class II genes of the MHC are maintained through selective forces (Carrington *et al.*, 1999). The hypothesis of overdominant selection (heterozygote advantage) at the MHC proposes that individuals heterozygous at MHC loci are able to present a greater variety of antigenic peptides than are homozygotes, resulting in more productive immune responses to a diverse array of pathogens (Carrington *et al.*, 1999).

The idea of heterozygote advantage was first postulated by Doherty & Zinkernagel (1975) who in 1975 suggested that in mice there was an evolutionary advantage of both gene duplication and heterozygosity at the H-2 gene complex, in the absence of positive selection for any particular H-2 haplotype. Although thirty years has now passed there have still been relatively few studies which have confirmed that MHC heterozygosity does confer resistance. Many of the studies have concentrated on viral infections. Homozygosity at the human DRB1*1301 MHC class II allele is associated with susceptibility to chronic active hepatitis C infection (Höhler *et al.*, 1997) while persistence of hepatitis B virus infection is associated with class II allelic homozygosity in African Americans (Thio *et al.*, 1999). MHC class II homozygosity has also been associated with disease progression in simian immunodeficiency virus (SIV) infected macaques (Sauermann *et al.*, 2000) and in human common variable immunodeficiency (CVID) (De La Concha *et al.*, 1999). Carrington *et al.* (1999) also reported that maximum HLA heterozygosity of class I loci (A, B, and C) delayed acquired immunodeficiency syndrome (AIDS) onset among patients infected with human immunodeficiency virus-type 1 (HIV-1), whereas individuals who were homozygous at one or more loci progressed rapidly to AIDS and death. The extended survival of 28 to 40 percent of HIV-1-infected Caucasian patients in the study of Carrington *et al.* (1999) who avoided AIDS for ten or more years could be attributed to their being fully heterozygous at HLA class I loci, to their lacking the AIDS-associated alleles B*35 and Cw*04, or to both.

Interestingly Rulicke *et al.* (1998) found that virus-infected mice produced more MHC-heterozygous embryos than sham-infected mice did. This suggests that parents are able to promote specific combinations of MHC-haplotypes during fertilisation according to the presence or absence of an infection. Offspring of infected parents would therefore be more capable of mounting effective immune responses against the antigens which infected their parents. However, in house mice Potts *et al.* (1994) were able to measure a fitness decline associated with inbreeding, but were unable to detect fitness declines associated with MHC homozygosity. These authors suggested that inbreeding avoidance may be the most important function of MHC-based mating preferences and is therefore the fundamental selective force diversifying MHC genes in species with such mating patterns.

There are also examples of non-MHC genes for which heterozygote advantage has been demonstrated. Two examples are cystic fibrosis (CF) and sickle cell anaemia (SSA). Cystic fibrosis is caused by a mutation in a single gene, the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Riordan *et al.*, 1989). Affected individuals which inherit only one mutated allele from a parent are considerably healthier then those who inherit two mutated alleles from their parents. Sickle cell anaemia is caused by a mutation in the haemoglobin protein. A mutant form of the beta chain (allele S) differs from the wildtype allele by only a single amino acid, out of a total of 146 amino acids in the polypeptide chain (Steinberg & Mitchell, 1999). The mutation changes the amino acid at position six in the polypeptide chain from glutamic acid to valine. Since valine is non-polar the folding of the polypeptide chain is affected and causes the mutant forms of the haemoglobin molecules to clump together and alter the shape of the red blood cell (sickle shape). Wildtype allele homozygotes (AA) show the normal phenotype and transport O_2 correctly. Wildtype/mutant (AS) heterozygotes show only slightly reduced ability to transport O_2 while mutant homozygotes (SS) show severely reduced O_2 transport efficiency.

Theory suggests that natural selection would gradually decrease the frequency of the susceptibility alleles for CF and SSA. However there is evidence to suggest that these alleles remain in the population as the susceptibility alleles for CF and SSA are also actually resistant alleles for the effects of diseases associated with diarrhoea and malaria respectively. The gene for cystic fibrosis codes for a protein that forms ion channels in cell membranes, especially the cells lining the intestines and airways. Gabriel *et al.*

(1994) suggested that the CF mutation, by halving the number of ion channels in heterozygotes, would in turn decrease by half the amount of fluid lost into the intestines. Gabriel speculated that might be just enough water to rid someone of an infection such as cholera without making him succumb to the dehydrating and fatal effects of diarrhoea. A similar theory exists for sickle cell anaemia. The mutant S allele has been maintained in the population as it confers resistance to malaria (Aidoo *et al.*, 2002). However, the heterozygote advantage, and hence the maintenance of the S allele in the population, occurs only in presence of malaria. These MHC allele trade-offs between resistance and susceptibility have also been demonstrated in mice. For example, mice with the *k* MHC haplotype are resistant to *Taenia* and *Giardia* infection but are susceptible to *Trichuris*, Theiler's virus and murine acquired immune deficiency syndrome (MAIDS) while mice with the *q* haplotype are resistant to *H. polygyrus* infection but are also susceptible to Theiler's virus and MAIDS (Penn & Potts, 1999).

These last points raise the concern that breeding for resistance based on selection at a single gene may confer susceptibility to other related or unrelated disease conditions and might also be associated with reduced production. Although not using a genetic approach and mentioned previously, there have been some reports of unfavourable associations between selection for reduced FEC in sheep with gastrointestinal nematode infections and increased diarrhoea, and reduced wool production.

The present author is aware of only one published study which has investigated MHC heterozygosity advantage in sheep gastrointestinal infections. Paterson *et al.* (1998) determined that the presence of individual MHC alleles rather than MHC heterozygosity was the critical factor determining mortality in lambs and yearlings in the population of feral Soay sheep on the Outer Hebridean island if St. Kilda.

MHC haplotype as well as heterozygosity is important in disease resistance/susceptibility. In the case of gastrointestinal parasitism of sheep there is evidence that polymorphisms at the *DRB1* locus of the MHC Class II are associated with resistance to *T. circumcincta* in Scottish Blackface sheep (Schwaiger *et al.*, 1995). In particular 6-month old animals with the G2 allele had FEC 58 times lower then animals with the most common (I) allele. No association between MHC polymorphism and FEC could be found in *H. contortus* infected Merino sheep using a different method of genetic typing (Blattman *et al.*, 1993).

Schwaiger *et al.* (1995) explained that the polymorphisms in the class II *DR* molecules are mainly restricted to the first domain of the β chain and according to the 3-dimensional structure of the class II molecules these polymorphic residues are either located in the antigen binding groove or they interact with the TCR for antigen. Polymorphisms in this antigen binding groove would differentiate the molecules which are able to bind specific peptides. Indeed a single amino acid deletion in the antigen binding site can significantly alter peptide binding (Sitte *et al.*, 2002).

This theory suggests that specific MHC alleles should be associated with recognition of specific parasite antigens. A clear correlation between MHC alleles and antigen recognition has been demonstrated in inbred rodents (Kennedy, 1990) but was not apparent in a small study of deliberately infected sheep (McCririe *et al.*, 1997).

In the studies of Schwaiger *et al.* (1995) and McCririe *et al.* (1997) there was no indication of whether animals that were homozygous at the MHC class II loci were more susceptible than their heterozygous counterparts. Consequently, this chapter aims to investigate whether there is any MHC *DRB1* class II heterozygote advantage/resistance association in parasitised sheep under deliberate and natural infection conditions. The MHC data generated from the studies of Schwaiger *et al.* (1995) and McCririe *et al.* (1997) have been used in conjunction with the Western blot data from the previous chapters.

Also, to confirm and extend the work of McCririe *et al.* (1997) the data were analysed to assess whether there were any significant associations between MHC polymorphisms and recognition of specific parasite antigens. Additionally, and for the first time this was also done in a group of naturally infected animals.

6.2 Materials and methods

6.2.1 Animals and Experimental Design

The experimental designs, parasitological and Western blot methods of the deliberate and natural infection experiments have been described in chapters three and five respectively.

6.2.2 MHC *DRB1* typing

Animals were typed for differences at the MHC class II *DRB1* locus as described in chapter two. The MHC typing data for the deliberate and natural infections used in this chapter were generated by Schwaiger *et al.* (1995).

6.2.3 Statistical analyses

Student's two sample t-tests and Pearson product moment correlation coefficients were conducted using Minitab v13.3 (Minitab Inc.). Where variables had been logarithmically transformed before the application of t-tests, the means between groups were back transformed geometric means. The equality of variances between animals that recognised a particular band and those that did not for each parasitological parameter was checked using the TTEST procedure in SAS (SAS Institute, Cary, NC, USA). Fisher's Exact test (Sokal & Rohlf, 1995) was conducted using SAS to determine associations among MHC *DRB1* polymorphisms and antigen recognition.

6.3 Results

6.3.1 MHC class II *DRB1* alleles expressed

6.3.1.1 Deliberately infected animals

Animals were typed for differences at the MHC class II *DRB1* locus and of 19 possible published alleles (Schwaiger *et al.*, 1995) only 10 were expressed in these animals (Table 6.1). The I and D alleles were respectively the most common and accounted for 56% of all the alleles expressed. Eighteen different genotypes were expressed in these animals and this represented 10.5% of the 171 possible combinations. There were six (25%) homozygotes which accounted for four different haplotypes (C, D, G1 and I).

6.3.1.2 Naturally infected animals

One hundred and seventy nine animals were typed for polymorphisms at the MHC class II *DRB1* locus. The frequency of the alleles that were expressed are given in Table 6.2.

All but one of the alleles (Q) was present in at least one animal. As with the deliberate infection the I and D alleles were the most common and accounted for 49% of the alleles expressed. Twenty eight (15.6%) of the animals were homozygotes and these constituted only three different haplotypes (D, F, and I). Of these 28 homozygotes 23 (82%) were homozygous for the I allele. Of the 171 possible combinations, 52 (30%) were represented in this group of animals. Homozygotes were split equally among male and female lambs.

6.3.2 Associations between MHC class II *DRB1* heterozygosity and resistance

6.3.2.1 Deliberately infected animals

Parasitological and histological parameters were compared between *DRB1* homozygotes and *DRB1* heterozygotes by Student's t-tests to assess whether there were any significant differences between the two groups (Table 6.3). There were no statistically significant differences between homozygotes and heterozygotes in any of the parameters measured. However, in each case homozygotes did show a tendency towards increased susceptibility. Homozygotes had higher worm burdens, higher FEC, lower numbers of GL and lower levels of IgA. If the effect of homozygosity was weak then the small number of animals may not be large enough to detect the differences. In other words, there may be a lack of statistical power.

6.3.2.2 Naturally infected animals

Student's t-tests were used to detect differences in parasitological and serological parameters between *DRB1* homozygotes and heterozygotes (Table 6.4). Heterozygote animals had higher adult and L_4 burdens, more eggs per female worm, and higher FEC but the differences were not statistically significant. Additionally however, heterozygote animals also had shorter mean worm lengths and increased eosinophil counts compared to homozygotes. Again, these tests were not statistically significant at the 5% level. The analysis did reveal however that heterozygotes had 46% higher anti-L_3 and 42% higher anti-L_4 plasma IgA responses than homozygotes ($P = 0.033$ and $P = 0.043$ respectively) when all animals were used in the analysis. Although heterozygote animals produced significantly more IgA than homozygotes, there was no evidence that this was done in

Allele	Frequency	Percent
A	3	6.25
C	2	4.17
D	13	27.08
F	3	6.25
G1	4	8.33
G2	4	8.33
H2	1	2.08
I	14	29.17
L	3	6.25
O	1	2.08
Total	48	100

Table 6.1: MHC *DRB1* allele frequencies of the 24 deliberately infected Scottish Blackface sheep used in this study. Only nine of the possible 19 alleles were expressed in this group of animals. There are a total of 48 alleles expressed as each animal expresses two alleles hence 2 x 24 = 48.

Allele	Frequency	Percent
A	40	11.17
B	4	1.12
C	6	1.68
D	49	13.69
E	3	0.84
F	35	9.78
G1	9	2.51
G2	21	5.87
H1	3	0.84
H2	6	1.68
I	125	34.92
K	3	0.84
L	25	6.98
M	7	1.96
N	10	2.79
O	8	2.24
P	1	0.28
Q	0	0
R	3	0.84
Total	358	100

Table 6.2: MHC *DRB1* allele frequencies of the 179 Scottish Blackface sheep naturally infected with predominantly *T. circumcincta*. All but one (Q) of the possible 19 alleles were expressed in this group of animals. A total of 358 alleles were counted because each animal expresses two alleles (2 x 179 = 358).

Parameter	Homozygote Mean	Heterozygote mean	Probability
Total worm burden	1703	1222	0.599
Worm length	0.947	0.953	0.943
Adult worm mass	1497	1015	0.550
Mast cells	15348	12113	0.441
Mucosal GL	1761	3850	0.215
Mucosal eosinophils	4397	6620	0.521
Plasma IgA OD index	0.293	0.387	0.586
FEC at slaughter	119	54	0.303
Time for FEC to become positive	28.5	28.8	0.925
IgA plasma cells	4250	831	0.066
Plasma pepsinogen concentration	553	700	0.451

Table 6.3: Comparison of mean values of parasitological and histological parameters between MHC *DRB1* homozygotes and heterozygotes in Scottish Blackface sheep following challenge with *T. circumcincta*. . All cell concentrations are cells mm^{-2}.

Parameter	Homozygote mean	Heterozygote mean	Probability
Adult worm burden	1900	2129	0.673
Worm length	0.845	0.825	0.578
Adult worm mass	1583	1813	0.579
L_4 burden	43	89	0.447
Eggs per female	13.9	14.3	0.913
FEC	128	151	0.483
Peripheral eosinophils	6.6	7.0	0.652
Plasma anti-L_3 IgA OD index	0.314	0.459	0.033
Plasma anti-L_4 IgA OD index	0.289	0.411	0.043

Table 6.4: Comparisons of mean values of parasitological and serological parameters between MHC *DRB1* homozygotes and heterozygotes in Scottish Blackface sheep following natural predominantly *T. circumcincta* infection.

detriment to growth since the mean weight gains between heterozygotes and homozygotes from the start to end of the experiment were not significantly different ($P = 0.999$).

Although the I allele is the most common it is also a relatively susceptible allele (Schwaiger *et al.*, 1995)). Since most homozygotes had the II haplotype, the significant differences seen between heterozygotes and homozygotes could have been due to the large number of relatively susceptible alleles in the homozygote population. This was not the case however as D and F homozygotes actually had lower, although not significantly, plasma IgA responses than their I homozygote counterparts. Additionally D and F homozygotes had higher worm burdens and longer worms.

Student's t-tests were also used to determine whether MHC *DRB1* heterozygosity was associated with resistance to the other individual species of gastrointestinal parasites found in these animals and also to overall resistance to the entire worm population (Table 6.5). The was no evidence that heterozygosity at the MHC *DRB1* locus was associated with resistance to any of the parasite species or to gastrointestinal parasitism as a whole in this population of naturally infected animals ($P > 0.05$ in each case)

6.3.3 Associations between MHC polymorphism and antigen recognition

6.3.3.1 Number of bands recognised

6.3.3.1.1 Deliberately infected animals

There was no significant difference between the total number of third stage parasite antigen bands recognised by heterozygotes and the number recognised by homozygotes. Heterozygotes did recognise more bands compared to homozygotes (34.6 compared to 30.2) but the difference was not statistically significant ($P = 0.295$).

There were also no significant associations between expression of the different alleles and the number of third stage bands recognised (Table 6.6). Again this may have been due to the number of animals used and hence the small numbers of each allele.

Parasite species	Homozygote mean	Heterozygote mean	Probability
T. circumcincta	1900	2129	0.673
Cooperia	23.18	63.40	0.269
Nematodirus	122.34	38.03	0.243
Trichostrongylus	2.83	3.53	0.836
Total worm population	2904	2820	0.904

Table 6.5: Comparisons of mean adult worm burdens of gastrointestinal parasite species between MHC *DRB1* homozygotes and heterozygotes in Scottish Blackface sheep following natural but predominantly *T. circumcincta* infection.

Allele	Correlation	P
A	0.003	0.992
C	–0.241	0.335
D	0.012	0.962
F	–0.027	0.917
G1	0.084	0.740
G2	–0.422	0.081
H2	0.147	0.559
I	0.197	0.435
O	0.245	0.328

Table 6.6: Correlations among MHC *DRB1* allele expression and the number of third stage larval antigens recognised by plasma IgA from Scottish Blackface sheep following challenge infection with *T. circumcincta*.

6.3.3.1.2 Naturally infected animals

In the naturally infected animals, homozygotes actually recognised slightly more larval antigens than heterozygotes (L_3: homozygotes = 24.14 and heterozygotes = 23.67; L_4: homozygotes = 14.78 and heterozygotes = 12.47) but the differences were very small and were not statistically significant at the 5% level (P = 0.786 and P = 0.06 respectively).

Additionally there were no significant associations among the number of third or fourth stage bands recognised and the presence of the different alleles ($P > 0.05$ in each case).

6.3.3.2 Individual band recognition

6.3.3.2.1 Deliberately infected animals

Fisher's exact test was used to determine if expression of particular alleles was associated with recognition of specific parasite antigens. Only alleles which occurred at a frequency greater than 5% were considered, of which there were seven. There were only four apparently significant associations ($P < 0.05$) between class II MHC polymorphisms and recognition of third stage parasite antigens. However, sixty three bands were recognised by these animals which expressed the seven alleles so that purely by chance alone one would expect 22 of the 441 (7 x 63) comparisons made to fall below the 5% level. Therefore, there is no evidence in these data for any specific associations between MHC polymorphism and antigen recognition although the number of animals is likely to be a limiting factor.

6.3.3.2.2 Naturally infected animals

There were six alleles which occurred at a suitable frequency (> 5%) for analysis with band recognition. Fisher's exact test determined that there were 17 significant associations ($P < 0.05$) between the six alleles and recognition of *T. circumcincta* third stage bands. Fifty four third stage bands were recognised in total by these animals so that by chance alone one would expect 16 of 324 (6 x 54) comparisons to fall below the 5% level of significance. By chance alone one would expect 3 of 324 comparisons to fall below the 1% level of significance. When this lower value of P is used, there are still six significant associations between allele expression and band recognition. Of

these six significant associations, five involved the G2 allele and the other the D allele (Table 6.7). Animals which possessed the G2 were more likely to recognise the five bands than those that did not possess the allele while animals with the D allele were less likely to recognise the associated band than animals without the D allele. This is particularly interesting as the G2 allele has been previously identified as the most resistant allele in this population of animals. More research in additional flocks is required to confirm these associations.

There were only four significant associations between allele expression and recognition of *T. circumcincta* fourth stage antigens ($P < 0.05$). By chance alone one would expect 11 of 222 (6 x 37) associations to fall below the 5% level. Two of the four associations remained significant at the 1% level. One might expect two associations to occur by chance at the 1% level. Although it is unclear whether these associations are real or may have occurred by chance, it is interesting to note that both associations involved recognition of the fourth stage band at 180 kDa.

6.4 Discussion

This chapter has investigated whether there was any association between MHC homozygosity and disease susceptibility. It has also investigated the associations between MHC class II polymorphism and antigen recognition of *T. circumcincta* larval antigens in both deliberate and natural infections of Scottish Blackface lambs.

This study has been unable to demonstrate heterozygote advantage in both experimentally challenged and naturally infected animals. Although there have been recent examples of MHC heterozygote advantage in viral infections there is surprisingly little evidence from population surveys and experimental infections to support the heterozygote advantage hypothesis for parasitic infections (Penn & Potts, 1999). For example, Hill *et al.* (1991) actually found that MHC heterozygotes had a disadvantage in a large survey study of human malaria. The present study supports that of Paterson *et al.* (1998) which concluded that the presence of individual MHC alleles rather than MHC heterozygosity was the critical factor determining mortality in the feral Soay sheep population of the island of St. Kilda. Interestingly, Paterson *et al.* (1998) also stated that the most common MHC allele (which was investigated), in the Soay population was also the most susceptible allele, however the associations were not

Allele	L_3 band (kDa)	% of animals which recognised band		P
		With allele	*Without allele*	
D	40	33	58	0.009
G2	198	89	49	0.002
G2	109	89	49	0.002
G2	100	89	50	0.002
G2	90	89	50	0.002
G2	28.5	72	37	0.009

Table 6.7: Associations between MHC *DRB1* allele expression and recognition of *T. circumcincta* third stage larval antigens by plasma IgA from Scottish Blackface sheep following natural predominantly *T. circumcincta* infection. Associations were determined using Fisher's Exact test. Only those associations where $P < 0.01$ are shown.

found to be consistent between lambs and yearlings. In the population of animals used in the present study the most common allele was also one of the most susceptible. Paterson *et al.* (1998) has suggested that different MHC alleles might exhibit different associations at different stages during the animals' life, possibly reflecting the complex interplay between helminth parasites and the vertebrate immune system.

Penn & Potts (1999) suggested that there are several reasons why the MHC heterozygote advantage hypothesis has not been adequately tested. Firstly, they suggest that the protective effect of MHC heterozygosity may only occur when individuals are infected with multiple parasites (strains or species) as occur in the wild. Recently, Potts (2002) has demonstrated that MHC heterozygotes have an advantage when coinfected with Salmonella and Theiler's virus while heterozygotes had no advantage when infected individually with either pathogen. The author claims that this is the first experimental confirmation that MHC heterozygote advantage may emerge because heterozygotes have greater overall resistance during coinfections. The same group recently confirmed this observation in *Salmonella* and *Listeria* infected mice (Penn *et al.*, 2002). This however was not true in the present study or the study of Paterson *et al.* (1998) which both involved infections with the major gastrointestinal parasites found in temperate areas although both studies found *T. circumcincta* to be the most prevalent. In the present study, heterozygotes had no significant advantage, as measured by worm number, against the individual species of *Teladorsagia*, *Cooperia*, *Nematodirus* or *Trichostrongylus*, or against the worm population as a whole.

Secondly, MHC heterozygotes may be protected against rapidly evolving parasites, such as HIV, that evade the immune system by diverging into multiple strains since heterozygotes can potentially recognise a given parasite in more ways than homozygotes, therefore successful evasion of immune recognition may be more difficult (Penn & Potts, 1999). This hypothesis may explain why heterozygote advantage has been demonstrated more easily for viral diseases such as HIV, SIV and hepatitis which may be evolving very quickly, but has not been demonstrated for complex multicellular macroparasites which will evolve much more slowly.

Penn & Potts (1999) also suggested that MHC-heterozygote advantage may be overlooked if the benefit of heterozygosity lies in reduced immunopathology rather than increased immune responsiveness. The results of the present study go some way to

negate this hypothesis on two fronts. While immunopathology is difficult to assess directly in the infections used in this study, indirect measures such as growth could be used since animals suffering considerable immunopathological effects are unlikely to grow as much as unaffected animals. The present study showed that MHC heterozygotes produced significantly more parasite specific plasma IgA than MHC homozygotes but this did not appear to divert resources away from growth since the growth rates of both heterozygote and homozygote animals was near identical over the span of the infections. This was true for both male and female lambs. This is in contrast to the recent work of Penn *et al.* (2002) who demonstrated that MHC-heterozygous male mice gained significantly more weight than homozygotes during experimental infections with multiple strains of *Salmonella*.

As just stated, and particularly interesting, was the fact that the present study demonstrated that MHC heterozygotes produced significantly more parasite specific plasma IgA than did homozygotes, but as explained previously this did not manifest in significant differences in parasite resistance. The previous chapters showed that there was a significant correlation between increased plasma IgA responses and resistance so the lack of an association between MHC heterozygosity and resistance is very perplexing.

It is very possible that the lack of a correlation between heterozygosity and resistance is yet again due to a lack of statistical power. Since only approximately half of the animals in this study underwent post mortem to determine parasite numbers, there were only 82 animals for which MHC and parasitological data were available. However, plasma IgA and MHC data were available for 163 animals. For example, the association between heterozygosity and the plasma IgA responses to L_3 larvae, although significant was quite weak ($r = -0.167$, $P = 0.033$). When the analysis of IgA/resistance data was restricted to the same animals for which post mortem data was available the association remained weakly negative but was no longer significant ($r = -0.186$, $P = 0.095$). It is therefore likely that the significant effect of heterozygosity would have been observed had more animals been tested. These analyses suggest that heterozygote advantage does exist, although the effect is weak. Careful selection of animals for breeding could increase the proportion of heterozygotes and would theoretically increase overall flock immunity which in turn would lead to a reduction in pasture contamination and parasite

transmission. How this might affect the balance between host and parasite evolution would require investigation, particularly in the long term.

MHC heterozygote animals recognised slightly, but not significantly more larval antigens than homozygotes in the challenge infection part of this chapter but the opposite was true for the natural infection where homozygotes recognised more bands, but again the differences were not significant. In the naturally infected animals, heterozygotes produced more IgA than homozygotes, and the previous chapter described that animals with high plasma IgA responses recognised fewer bands. Therefore, heterozygotes should recognise fewer bands. Although the results did show that heterozygotes recognised fewer bands, this observation is very difficult to explain since heterozygotes should recognise a wider range of antigens.

Affinity maturation was given as a possible explanation for the negative association between the number if parasite bands recognised and the plasma IgA response but it is unclear how the results of this chapter fit this hypothesis. As explained previously affinity maturation only occurs when the amount of presented antigen is limited. However, heterozygotes will recognise more antigen and so potentially the amount of presentable antigen is increased and affinity maturation is unlikely to occur. Penn & Potts (1999) suggested that heterozygote advantage might be overlooked if the optimal number of MHC molecules expressed in an individuals immune system is less than complete heterozygosity. Individuals with more heterozygous MHC loci present more antigens to the immune system; however, they probably have smaller T-cell repertoires (because of thymic selection), that is, there is a trade-off between maximising the number of different antigens presented by MHC and the number recognised by T-cells (Penn & Potts, 1999). It is possible then that the most MHC heterozygotic animals could present less antigen to B-cells from the limited number of T-cells and so in turn produce high amounts of antibody to only a limited cohort of the presented antigens. In this study, the naturally infected animals were receiving their fourth independent infection by the end of the experiment which gave sufficient time for affinity maturation to occur. By this fourth infection heterozygote animals might produce large amounts antibody to a restricted cohort of important parasite antigens and undetectable amounts of antibody to those unimportant parasite antigens which were not selected. Homozygote animals on the other hand might produce small but detectable amounts of antibody to a larger set of unimportant antigens.

The present study also determined that there was little evidence for an association between MHC polymorphism and recognition of specific parasite antigens. Fisher's exact test was used to determine whether there was any significant association between expression of each allele and recognition of parasite antigens. The results from the deliberate infection in this chapter confirm the results of McCririe *et al.* (1997) who had determined that there was no evidence of any association in the same set of animals using periodate treated parasite antigen samples. The work in this chapter is the first which has investigated for similar associations in a large number of outbred animals under natural infection conditions.

There were only two significant associations between recognition of fourth stage antigens and allele expression at the 1% level, both of which involved recognition of the band at approximately 180 kDa. Seventy eight percent of animals which possessed the A allele recognised the band, while 53 % of the animals which did not possess the A allele did not recognise the band (FET: $P = 0.008$). Additionally, 78% of animals which possessed the L allele did not recognise the band at 180 kDa whereas 59% of the animals which did not possess the L did recognise the same band (FET: $P = 0.005$). However, by chance alone one would expect two of the comparisons made to be significant at the 1% level so it is unclear if these associations are real or a consequence of chance.

More encouraging were the results of the analysis which determined recognition of third stage antigens. The analyses revealed that there were six significant associations between antigen recognition and allele expression at the 1% level. By chance alone one would have expected three significant associations at that level of significance. Although the difference between the observed results and those expected by chance are not significantly different the results do suggest that there may be an effect present. Particularly encouraging is the fact that five of the six associations involved the G2 allele which previous analyses on these animals has been identified as the most resistant allele (Schwaiger *et al.*, 1995). The associations between the G2 allele and recognition of the five bands was positive, or in other words, animals which possessed the G2 allele were significantly more likely to recognise any one of the five bands than animals which did not express the G2 allele. Additionally, animals which possessed the D allele were significantly less likely to recognise the band at approximately 40 kDa than animals that did not recognise the band (FET: $P = 0.009$). None of the bands which

gave a significant association with MHC polymorphism gave significant associations with any markers of resistance in the previous chapters.

While there was no obvious relationship between MHC polymorphism and antigen recognition in these sheep, there is a clear correlation between seen between MHC alleles and antigen recognition in inbred rodents (Kennedy, 1990). McCririe *et al.* (1997) suggested that in outbred animals, a simple relationship between MHC alleles and the immune repertoire may be concealed by polymorphisms in other genes leading to modulation of the immune repertoire. The influence of background genes could also explain why, for example, the response patterns of F_1 hybrid rats *to N. brasiliensis* were not merely summations of parental responses (Kennedy, 1990). For example, polymorphisms of the IFN-γ gene have shown significant associations with resistance. Increased or decreased production of IFN-γ may affect efficient MHC, T-cell or B-cell priming and development and subsequent antigen presentation.

Polymorphisms of the T-cell receptor (TCR) may also be important. Sheep possess a large proportion of γδ T-cells which express a larger repertoire of variable region genes than other mammals (Hein & Peterhans, 1998). It is therefore possible that the highly variable nature of the sheep TCR plays a role in antigen recognition and processing. The sheep γδ T-cell variable region repertoire is also developmentally regulated and foetal-specific and adult-specific expression of particular variable-genes occurs (Hein & Peterhans, 1998). This might explain why immunity to gastrointestinal parasitism develops with age, and in particular the observation in the study of Schwaiger *et al.* (1995), that the association of the G2 allele with reduced egg counts changed as animals aged. Changes in the expression of TCR variable regions could influence antigen recognition and subsequent antibody production and so could account for the observed changes. Assessing how the expression of TCR variable region genes changes with age and in response to parasitic infection may lead to a greater understanding of the mechanisms which control antigen recognition and disease susceptibility. Identification of specific TCR/MHC combinations which confer resistance or susceptibility, and which potentially recognise specific antigenic targets may improve the development of selective breeding programmes and vaccine candidates.

This chapter has investigated the association between MHC polymorphism and host recognition of parasite antigens and resistance. The results have demonstrated that

MHC heterozygote animals produce significantly more plasma IgA than MHC homozygous animals. Although the effect of heterozygote advantage could not be demonstrated in relation to increased resistance, the small potential effect and the limited number of available animals used appeared to mask the true effect. This chapter has also been unable to demonstrate a relationship between MHC polymorphism and antigen recognition in both challenge and natural infections.

Chapter 7

General discussion

Gastrointestinal parasitism remains a major constraint on production and welfare in domestic animals in all geographical locations on the planet. With the ever increasing incidence of resistance to single and multiple classes of anthelmintic drugs, additional non-chemotherapeutic methods of control must be utilised.

This thesis has expanded previous studies which have attempted to more fully understand the sheep's immune response to infection with the gastrointestinal nematode *T. circumcincta.* Further, this thesis has investigated methods which could be used for the improvement of selective breeding schemes. Specifically, this thesis has investigated the kinetics of the IgA and eosinophil response following a large, single challenge infection of sheep with *T. circumcincta* and how these parameters affect the subsequent development of the parasite. The recognition of parasite larval antigens by host plasma IgA was subsequently studied to determine if preferential recognition of any stage specific antigens conferred resistance or susceptibility. Finally, polymorphisms of the ovine MHC *DRB1* locus were investigated to assess whether there were any significant associations between the expression of particular alleles and the recognition of specific parasite antigens. Additionally, an investigation was conducted to determine whether there was any evidence of MHC heterozygote advantage in this host/parasite system.

The work detailed in chapter three confirmed previous descriptions of the host IgA response following secondary challenge (Smith *et al.*, 1984; Wedrychowicz *et al.*, 1994). Although there were differences in the breeds of animal, methods and parasite strains used, the results were similar showing peak responses 7-10 days after secondary infection.

A detailed study of the kinetics of the host immune response following both deliberate and natural primary and secondary infections would greatly increase our understanding of the host/parasite interaction. Measuring the antibody levels in peripheral blood as well as lymph and mucosa, if possible, at frequent intervals would give us a clear indication of which animals are the most resistant and so would improve selection. Frequent and rigorous testing of the other markers of resistance should also be investigated since the work reported in chapter three demonstrated that the peripheral eosinophil response to challenge infection followed a similar pattern to the IgA response. Additionally the plasma pepsinogen response had similar kinetics following

infection. Strangely though there was little correlation between the peripheral eosinophil concentration and the plasma IgA activity during this challenge infection. Further work is required to assess if the same is true for primary infections and for natural infections such as those described in chapter five.

It is clear that measurements of marker traits taken locally rather than peripherally would be preferred but the main barrier to this is practicality. This thesis has shown there are limitations to using peripheral markers but if selection for resistance based on these markers is to become a reality then this is the only viable option.

These results have implications for selective breeding schemes which utilise these and other similar traits. Each trait is weighted according to the relative gain which each gives to the selection objective whether it be for example an increase in production, or a reduction in nematode worm burden or worm length. If the kinetics of these traits follow a similar pattern but are not correlated as shown here, then the timing of the measurements of each trait and the relative weight given to each will be critical in determining the merits of the selection index. The work from this thesis and previous studies (Strain *et al.*, 2002; Stear *et al.*, 1999a; Stear *et al.*, 2002) have demonstrated that these traits are good markers of resistance in both deliberate and natural infections, but there is clearly a need for further research to determine more closely the interaction between the traits, particularly under commercial farming practice conditions.

Clarification of which antibody isotypes and subclasses predominate in these infections is also required. It may be for example that different IgG subclasses are produced in different sheep breeds and against different parasite species. This may explain the results of the various studies described in chapter three. Sheep are currently accepted to express one class each of IgM, IgE and IgA and two of IgG, but no IgD (Hein, 1998), but this might not be the complete picture (Butler, 1998). Butler suggests that the number of IgG and IgA subclasses may not be fully known, particularly given the larger number of subclasses seen in some other species. For example humans and mice have four subclasses each of IgG and humans two of IgA while pigs have at least 6 subclasses of IgG and rabbits an amazing thirteen different IgA subclasses (Butler, 1998).

The differences between the IgA subclasses are caused by mutations in the hinge regions of the molecules. For example, the human IgA subclasses differ sporadically

throughout their sequence, but are dramatically different in the hinge region where human IgA1 has a 13 amino acid segment lacking in IgA2 (Phillips-Quagliata, 2002). This segment has five O-linked glycosylation sites and a series of recognition motifs for proteases of bacterial origin that render IgA1 susceptible to proteolysis. This susceptibility is thought to diminish IgA1's relative usefulness as a protective antibody at mucosal surfaces compared to IgA2 (reviewed in Mestecky *et al.*, 1999). While Butler (1998) does not suggest how many 'new' immunoglobulin subclasses may exist in sheep, this does offer a possible explanation for the variability in antibody responses seen in these infections. With modern techniques this could hopefully be an area clarified quite quickly.

Additionally, and regardless of whether more subclasses of each immunoglobulin isotype are identified, there may be allotypes of each subclass (Phillips-Quagliata, 2002). For example, humans have three IgA2 allotypes (IgA2 m(1), IgA2 m(2) and IgA2(n)) (Mestecky *et al.*, 1999) while six IgA allotypes are serologically identifiable in inbred mice (Phillips-Quagliata, 2002).

Bacterial IgA proteases have been identified as described above, as have other parasite derived proteases, therefore the existence of parasite derived IgA proteases is not an impossibility, indeed the heavy chain of bovine IgG was completely degraded by an aspartyl proteinase secreted by all three host specific parasitic stages of *O. ostertagi* (Geldhof *et al.*, 2000). Immunoglobulin G degradation by a number of gastrointestinal nematode species has been described previously (Tort *et al.*, 1999), but it has yet to be clarified whether this is a specific immune evasion strategy or a non-specific proteolytic event (Geldhof *et al.*, 2000). Identification of these proteases, presuming they exist, as well as the IgA subclasses or allotypes which they specifically proteolyse could be used as additional markers of resistance for use in selective breeding. Additionally the proteases could be targeted as vaccine candidates or the susceptible IgA molecules might be able to be protected by some kind of chemical or biological intervention.

The production of the different antibody isotypes is regulated by subsets of antigen-specific helper T cells through the production of cytokines (Janeway & Travers, 1994). This hypothesis was proposed after it was shown that T cells in mucosal sites in mice were shown to stimulate IgA production (Roitt *et al.*, 2001). IL-4 preferentially switches activated B cells to the IgG1 or IgE isotype with concomitant suppression of the other

isotypes in mice. Similarly, IL-5 induces a 5- to 10-fold increase in IgA production with no change in the other isotypes. In mice TGF-β induces the switch to IgA or IgG2b but in humans it only induces IgA production (Xu *et al.*, 2000). As stated previously there are currently no published articles on the specific actions of cytokines on antibody isotype switching in sheep. Since the number and type of immunoglobulin subclasses is different in sheep compared to mice and humans, the species which have been most widely studied, the cytokines make have different immunoglobulin switching roles.

Although B cells produce cytokines it was not known whether B cells could differentiate into effector subsets that secrete polarised arrays of cytokines. Harris *et al.* (2000) identified two populations of "effector" B cells (Be1 and Be2) that produced distinct patterns of cytokines depending on the cytokine environment in which the cells were stimulated during their primary encounter with antigen and T cells. These effector B cell subsets subsequently regulated the differentiation of naive $CD4^+$ T cells to T_H1 and T_H2 cells through production of polarising cytokines such as IL-4 and IFN-γ. In addition, Be1 and Be2 cells could be identified in animals that were infected with pathogens that preferentially induce a Type 1 and Type 2 immune response. The authors report that together these results suggest that, in addition to their well defined role in antibody production, B cells may regulate immune responses to infectious pathogens through their production of cytokines.

The identification and manipulation of cytokine responses has had a major impact on the understanding of the immune mechanisms involved in murine helminth infection models (Finkelman *et al.*, 1997) but only a few studies have examined cytokine responses in ruminant gastrointestinal infections mainly using RT-PCR technology to detect cytokine mRNA expression. As more reagents become available (in particular monoclonal antibodies), it is expected that this area will expand dramatically in the next few years (Balic *et al.*, 2000a).

Summarising the information Balic *et al.* (2000a) suggests that while it is apparent that there is increased IL-4 (a T_H2 cytokine) mRNA expression in some models of gastrointestinal infections in ruminants, as expected from rodent studies of gastrointestinal nematode infection, this expression does not always correspond to protection. While some authors suggest that these results indicate that the T_H1/T_H2 paradigm may not apply to gastrointestinal nematode infected ruminants, too little data

is available to support this claim at this point. This hypothesis is unlikely particularly in the light of studies such as those by Ferrick *et al.* (1995) which demonstrated that γδ T cells (of which sheep possess a high proportion) in mice infected with *L. monocytogenes* and *N. brasiliensis* discriminated early in infection between the two pathogens producing cytokines associated with the appropriate T_H response (IL-4 or IFN-γ). The authors claimed that their results demonstrated that γδ T cells are involved in establishing primary immune responses.

In addition, cytokine expression can be rapidly up and down regulated and it is important to examine different time periods, especially when evaluating cytokine responses during larval development which can go from L_3 to the adult stage within ten days, each stage with the potential to induce a different cytokine profile. A detailed investigation into which cytokines are important in ruminant gastrointestinal parasitic infections is required, in particular when these cytokines are produced. Once the importance of these different cytokines is established then they too are potential markers of resistance. Two cytokines which appear to play important roles and need further extensive study are IL-5 and TGF-β and these will be briefly discussed.

Interleukin-5 is an eosinopoietic cytokine which stimulates eosinophil survival, growth and differentiation as well as numerous other aspects of eosinophil metabolism and function (Jones, 1993). IL-5 also induces a switch to IgA in mice (Roitt *et al.*, 2001), while the SC of IgA is also a very potent stimulus of eosinophil degranulation (Lamkhioued *et al.*, 1995). For example, although IL-5 deficient mice cannot elicit a pronounced eosinophilia in response to inflammatory stimulation following parasite infection they still produce basal levels of eosinophils that appear to be morphologically and functionally normal (Matthaei *et al.*, 1997). Further, eosinophils themselves, under physiological stimulation, can release significant amounts of IL-5, which may contribute to local eosinophil recruitment and activation (Dubucquoi *et al.*, 1994). More recently, Rainbird *et al.* (1998) demonstrated that the addition of IL-5 to *H. contortus* larval cultures containing antibody and complement resulted in a significant increase in larval immobilisation with unactivated eosinophils suggesting that eosinophil effector function is enhanced following priming with this cytokine. However, IL-5 levels did not appear to be responsible for most of the variation in eosinophil responses in outbred sheep during natural, mixed gastrointestinal nematode infections (Doligalska *et al.*, 1999). Given the importance of the eosinophil and IgA responses in controlling

infection, it is paramount that we work out how IL-5 fits in to the scheme in different sheep breeds and with different parasites. This will clearly be useful as a marker of resistance when these points are clarified.

There is evidence to suggest that both the host and the parasite gain some kind of advantage from the TGF-β released by the host immune response. B cell activation, IgA isotype switching and the homing of T cells to mucosal surfaces can be stimulated by TGF-β1, at least in mice and humans (van Ginkel *et al.*, 1999). Additionally, mice lacking the TGF-β receptor (TβR) had a virtually complete serum IgA deficiency (Stankiewicz *et al.*, 2000). Estrogen and prolactin are critically involved in upregulation of TGF-β2 levels in uterine and mammary tissues during late pregnancy (Schneider *et al.*, 1996), and studies with *C. elegans* further implicate TGF-β and insulin-like signalling pathways with larval arrest and reactivation (Tissenbaum *et al.*, 2000) A similar pathway has been suggested for the development of *S. stercoralis* larvae (Ashton *et al.*, 1999). Subsequently, Arasu (2001) used an *in vitro* assay to show that neither estrogen, prolactin, nor insulin had a direct effect on the feeding/reactivation response of tissue-arrested *A. caninum* larvae; however, TGF-β isoforms 1 and 2 both had significant stimulatory effects that were comparable to the effects of dog serum. Ashton *et al.* (1999) proposed that a similar signalling system was in operation in resumption of larval development in *H. contortus*. It is therefore possible that this may system acts in the other major gastrointestinal parasites of ruminants. To summarise this information, TGF-β is upregulated during pregnancy, and stimulates class switching to IgA, but also signals arrested larvae to resume development. A proportion of the IgA produced at this time will be transported in the colostrum to the new born so the parasites may have evolved to use TGF-β as a signal for redevelopment in to a safer less hostile environment lacking in IgA. This could explain the massive increase in larval development and subsequent egg output in gastrointestinal nematode infections of sheep during the spring lambing season.

Now that it is technically possible to test for many different antibodies, cytokines and cell types from single biological samples using automated analysers a detailed kinetic study, similar to the one described in this thesis should be conducted. A definitive understanding of the antibody isotypes, cytokines and cells involved in the immune response following infection would greatly increase our ability to control the infections, reduce animal suffering and improve selective breeding.

The work detailed in chapters four and five investigated the recognition of stage specific parasite somatic antigens by plasma IgA from experimentally challenged and naturally infected Scottish Blackface sheep. These chapters aimed to confirm and extend the studies of McCririe *et al.* (1997) and Strain & Stear (1999) which had previously investigated the experimentally challenged animals.

A major difference between the present study and those of McCririe *et al.* (1997) and Strain & Stear (1999) was the method. A revised method of Western blotting was used in the present study as it was noted from preliminary kinetic analyses that the pattern of band recognition was associated with the activity of IgA in the samples being tested. On further investigation it was determined that there was a very strong correlation ($r > 0.9$) between the intensity of staining on the Western blot and the strength of the colour change reaction in the ELISA assay. In the kinetic analyses, in individual animals, this meant that many of the observed differences in band recognition were due to the differences in the quantity of IgA in the plasma samples being tested rather than to changes in the recognition profile as the infection proceeded. Therefore, subsequent assays which aimed to determine whether there was any preferential recognition of parasite antigens in resistant animals used plasma samples which had been diluted to contain approximately the same amount of IgA. The present author is unaware of any previous study which has controlled for antibody quantity in a similar system.

The differences between the techniques might explain some of the differences seen between the present analyses and that of McCririe *et al.* (1997) and Strain & Stear (1999). However, this cannot explain why different band associations were seen in the deliberately challenged animals (chapter four) and the naturally infected animals (chapter five). This has significant implications for using these techniques as the basis of vaccine design and selective breeding schemes. This investigation used only one breed of sheep, Scottish Blackface, and the batch of parasite antigen used to test for recognition was also the same. It was impossible however, to control the intake of larvae since one set of animals was naturally infected. It is possible that the infective L_3 larvae used to infect the animals in chapter four differed slightly from those naturally acquired by the animals in chapter five, particularly since different morphs of *T. circumcincta* do exist (Leignel & Cabaret, 2001). It is unclear however how antigenically different these morphs are. If the morphs are antigenically different, particularly in important immunogenic proteins then vaccines or breeding schemes from

one morph may not work on the other. These morphs can now be identified by DNA sequencing and since pasture larval counting is often conducted during selective breeding, identification of the specific morphs involved might be possible.

The present study and that of Strain & Stear (1999) used SDS-reduced parasite extracts while McCririe *et al.* (1997) used periodate treated and SDS-reduced extracts. The antigens identified as important in the present study may be the same as those identified by McCririe *et al.* (1997) but with the carbohydrate chains still attached. Schallig & van Leeuwen (1996) determined that the proteins of larval and adult *H. contortus* were considerably glycosylated. Western-blot analysis using different types of lectins was used to assess the presence of different types of oligosaccharides and revealed that either α-D-mannose or α-D-glucose epitopes were the dominant carbohydrate moieties on the glyoproteins. Several groups have reported that peptide epitopes are important for the induction of protective immunity in helminth infections and that these epitopes might be masked by carbohydrate epitopes (Jarvis & Pritchard, 1992). With respect to this observation Schallig & van Leeuwen (1996) noted that after periodate treatment, several antigens were identified that were not very apparent before deglycosylation. Apparently these antigens are normally not readily accessible to antibody and, probably, partly protected against the host immune response. This is in line with observations that in *Trichinella spiralis*, carbohydrate epitopes might serve to focus the immune response on irrelevant antigens, allowing immune evasion by the parasite (Denkers *et al.*, 1990). Similarly, shared carbohydrate epitopes on surface and secreted antigens of *Toxocara canis* have also been identified, but their function is also still unknown (Maizels *et al.*, 1987).

This is an area that has not received the attention it deserves since these carbohydrate complexes may play critical roles in facilitating invasion and migration in the host (Maizels *et al.*, 1987). While it is accepted that T cells expressing αβ TCR recognise antigenic peptide bound to MHC molecules there is increasing evidence that non-protein antigens can also be recognised by T cells (reviewed in Porcelli *et al.*, 1996). Corinti *et al.* (1997) recently showed that $CD8^+$ αβ T cells from the blood of atopic and healthy individuals recognised a nonpeptide antigen present in an allergenic extract from pollen independent of MHC. Their results suggest that T cell recognition of carbohydrates may play a role in the normal immune response. If a similar system

operates in parasitic infections then this opens further potential avenues for vaccine development.

There is little known about any of the proteins of *T. circumcincta*. At the time of writing a search with 'circumcincta' on the SWISS-PROT protein knowledge base (http://www.expasy.ch/sprot/) results in zero matches while the corresponding search of the TrEMBL database at the same website results in only thirty five matches. Of these thirty five, the majority are NADH dehydrogenase and other protein fragments. The only full length cDNA sequences of *T. circumcincta* which have been identified and are present in the database are β-tubulin (Elard *et al.*, 1996) and galectin (Newton *et al.*, 1997).

There is therefore a considerable amount of information to be gained from analysis of all the other proteins that are present in the extracts of larval and adult parasite preparations. It is unclear if the antigens of interest identified in the present study and all the other antigens for that matter are single proteins or if each particular band on the SDS-PAGE gel is made up of several proteins with the same molecular weight. To determine if these are single or multiple proteins the next stage is to run two dimensional (2D) electrophoresis gels. The two-dimensional electrophoretic separation of proteins, on the basis of charge in the first dimension (iso-electric focusing-IEF) and mass in the second, was first described by O'Farrell (1975). However, as a technique it lacked reproducibility, largely because of the problem with cathodic drift of carrier ampholytes that leads to degradation of the pH gradient at the basic end. This problem has been overcome with the development of immobilised pH gradients (IPG) which are now the preferred means of separation of the first dimension. This is the first stage of the process of identifying proteins which comes under the broad heading of proteomics.

Proteins are then visualised by staining to produce the 2D 'map' of protein spots. Since 2D electrophoresis is generally very repeatable, and with the advent of sophisticated imaging and analysis software, it is possible to accurately compare different protein maps. It would therefore be possible to compare protein maps from different life cycle stages of a parasite to assess if the antigens in each stage differ. In addition, this would be a useful technique for the identification of the different morphs of each parasite species. This is a very sensitive technique as Coomassie brilliant blue staining can detect proteins present in amounts greater than 100ng while silver staining can detect

proteins in the 2-5ng range (Chambers *et al.*, 2000). Very small amounts, perhaps even single larvae or worms, could therefore be tested.

There are a variety of techniques available for the actual characterisation of the individual proteins. While 2D-gel electrophoresis can provide the isoelectric point and approximate mass of a protein, which can itself be useful, this information alone is currently insufficient to identify the majority of proteins. One of the most widely used methods of identifying proteins is through peptide-mass fingerprinting (Henzel *et al.*, 1993). The protein is digested with a proteolytic enzyme, such as trypsin, to produce a set of tryptic fragments unique to each protein. The masses of these peptides are then determined by mass spectrometry for which the most common method is matrix-assisted laser desorption time-of-flight spectrometry (MALDI-TOF) (Chambers *et al.*, 2000). The tryptic masses are then evaluated by computer to fit a user's mass spectrometry data to a protein sequence in an existing database. Obviously, protein identification will only be successful if the protein being analysed is represented in the database. For proteins which have incomplete sequence information, it is necessary to obtain sequence information for the protein by Edman degradation or by nano-electrospray mass spectrometry (Chambers *et al.*, 2000). This sequence information can then be used along with the mass spectrometry information to interrogate expressed sequence tag (EST) databases for details of the cDNA identified clones. A further method of identifying proteins is to generate monoclonal antibodies to a specific protein and use these antibodies to screen cDNA expression libraries (Newton *et al.*, 1997). The sequences of the positive cDNA clones can then be determined which are then compared to the databases to identify the protein.

These procedures could be used to identify the proteins which the present study has determined are important in resistance. Once the sequence of the proteins are known it may be possible to create identical recombinant proteins which could be used as the capture antigen in the ELISA assay rather than the complex mixture of proteins used currently or as the basis of a vaccine. If these proteins are the most important then using a recombinant protein in the ELISA will make identification of the most resistant and susceptible animals much easier as there would be no interference of the signal by cross-reaction with unimportant antigens.

One problem which this application may face is the three dimensional structure of the protein which will obviously be critical for MHC recognition and immunoglobulin binding. However, protein structure determination is by no means an exact science. There are many computer programs available which attempt to predict the structure of proteins by comparing their amino acid sequences to proteins of known structure. The problem is that all the structure of all the proteins are not known at present so when a new protein is identified there is nothing to compare it with. Jones (2001) suggests that eventually the protein structure data banks will be so full as to render protein structure prediction a more or less academic problem. Eventually there will almost always be a close homologue of known structure for any given target protein. But Jones (2001) predicts that this is unlikely to occur for quite some time (perhaps 15 years or even longer).

The development of recombinant (sub-unit) vaccines has already been achieved, and commercial vaccines produced, for *Taenia ovis* infections in sheep and *B. microplus* infections in sheep (Knox, 2000). Bacterially-expressed recombinant versions of several gastrointestinal nematode antigens have been produced which induce protective immunity (Emery, 1996). Difficulties can be encountered when the protective epitopes are conformational in nature, for example the active region of an enzyme, but these problems can be overcome by expressing the parasite antigens in different vectors (Knox, 2000).

Identification and characterisation of the important protective antigens will also provide an interesting method for the investigation of the MHC since the recognition of peptides by MHC molecules will only proceed if there is a very high affinity between the peptide and the antigen binding region. If the amino acid sequence of a protein and its structure can be determined then the complimentary structure of the MHC molecule which binds it could also be determined if the amino acid sequence of the MHC is also known.

Once the sequences of both parasite and host molecules are known it may be possible to investigate how both have evolved. By sequencing similar parasite antigens from closely and not so closely related nematode species and MHC molecules from various ruminant species it may be possible to determine how the host/parasite system has developed over time and whether one species is evolving faster than the other or if both are evolving at the same rate (co-evolution). This kind of investigation may help to

explain observations such as those of Smith *et al.* (2001a) who reported that an experimental vaccine containing *H. contortus* gut derived proteases did not provide any useful cross-protection against either *T. circumcincta*, *T. axei* or *C. oncophora* but in a reciprocal experiment where sheep were immunised with the equivalent glycoproteins from *T. circumcincta* there was some cross-protection against *Haemonchus*.

House mice prefer mates genetically dissimilar at the MHC. The highly polymorphic MHC genes control immunological self/nonself recognition and therefore, this mating preference may function to provide 'good genes' for an individual's offspring. However, the evidence for MHC-dependent mating preferences is controversial, and its function remains unclear. Penn & Potts (1999) recently reviewed the studies on MHC-dependent mating preferences in mice, sheep, and humans and the possible functions of this behaviour. There are three adaptive hypotheses for MHC-dependent mating preferences. First, MHC-disassortative mating preferences produce MHC-heterozygous offspring that may have enhanced immunocompetence. The authors suggest that although this hypothesis is not supported by tests of single parasites, MHC heterozygotes may be resistant to multiple parasites. This hypothesis has recently been confirmed by the same group (Penn *et al.*, 2002), but could not be demonstrated in the work detailed in this thesis.

Secondly Penn & Potts (1999) proposed that MHC-dependent mating preferences enable hosts to provide a "moving target" against rapidly evolving parasites that escape immune recognition (the Red Queen hypothesis). Such parasites are suspected to drive MHC diversity through rare-allele advantage. Thus, the two forms of parasite-mediated selection thought to drive MHC diversity, heterozygote and rare-allele advantage, will also favour MHC-dependent mating preferences. An investigation of the evolution of the parasite and MHC molecules such as that described above could determine if this hypothesis is indeed true.

Finally, Penn & Potts (1999) suggest that MHC- dependent mating preferences may also function to avoid inbreeding; a hypothesis consistent with other evidence that MHC genes play a role in kin recognition. It has been demonstrated in both mice and humans that females can select males which are MHC dissimilar by odour (Penn, 2002).

MHC heterozygotes had no advantage over MHC homozygotes in the natural predominantly *T. circumcincta* infected sheep described in chapter six when the number

and length of worms, as well as faecal egg counts were compared between the groups. Heterozygotes did however have significantly more parasite specific plasma IgA than homozygotes. Since increased plasma IgA has significant effect on worm lengths it is as described previously unclear why in turn MHC heterozygosity does not show a significant advantage. If the effect of heterozygosity on resistance is small then the number of animals used in this part of the study may have been too small to give a significant difference. Determination of MHC polymorphism is becoming much easier and is now routinely done in our laboratory. With the development of quicker and more sensitive techniques the testing of large numbers of animals will be a reality. If it does turn out that MHC heterozygotes do indeed have a significant advantage over MHC homozygotes then this could be included as an additional selection marker. Several rams are usually selected each year for mating with a much larger group of ewes each year. By testing both rams and ewes for MHC alleles it will be possible to reduce the chance of their offspring from being MHC homozygotes. The problem with this is that some alleles are more resistant than others (Schwaiger *et al.*, 1995). Selecting for the most resistant alleles may lead to an increase in the number of homozygotes and therefore an increase in susceptibility. A compromise between increasing MHC heterozygosity and selecting for specific resistant alleles may be the most appropriate course of action.

The present study also confirmed and extended the results of McCririe *et al.* (1997) which showed that there was no simple relationship between MHC polymorphism and recognition of specific parasite antigens. Because of the large number of antigens recognised and the number of different alleles there is a high probability of associations occurring purely by chance alone. Perhaps with more sensitive techniques and more importantly with increased numbers of animals the kind of experiments described in this thesis may yield significant positive associations. Highly sensitive high throughput DNA and protein microarray technologies may make such investigations feasible in large number of animals. Until such times, we can only speculate as to the exact mechanisms by which MHC polymorphisms are associated with resistance.

Breeding for enhanced disease resistance is a relatively simple and readily available method to improve animal welfare and productivity in a variety of situations. An examination of diseases where resistance has been produced by natural selection strongly suggests that disease resistance is sustainable (Stear *et al.*, 2001a), and

additionally, the observed heritabilities for traits related to disease resistance indicate that selective breeding is also feasible. However, further research is necessary to define any adverse associations between disease and production traits. However, even if unfavourable relationships do exist, they do not present critical flaws in breeding schemes as selection indices that be created which contain these unfavourable traits (Stear *et al.*, 2001a). Breeds could also select for resistance to several disease simultaneously, perhaps by breeding for an overall enhancement in immune responsiveness (Stear *et al.*, 2001a).

In conclusion, the work in this thesis has aimed to extend our knowledge about a very complex host/parasite relationship which has been evolving for millions of years. As more information is gained further manipulations of the system will be possible which will improve animal welfare and productivity. The kinetics of the immune response to infection has been examined and has concluded that the timing of any immunological readings which are to be used for selection is critical. There are however limitations to the use of peripheral rather than local measurements. Associations between different immune responses and resistance exist but these change as time proceeds. A further detailed kinetic investigation of the immunoglobulin classes, immune cells and cytokines which respond to infection is required. The preferential recognition of stage specific parasite proteins was also investigated and revealed that recognition of a different set of parasite antigens was associated with resistance in a natural infection compared to a deliberate challenge infection in the same breed of sheep. This has particular implications for vaccine development and selective breeding. Different morphs of a particular species of parasite may exist in different geographical locations so that protein recognition testing, such as that described in this thesis, with one morph of parasite may not be appropriate if a different morph was present in the location where the animals came from. Molecular diagnostic tools to identify specific parasite species may clarify this situation. Finally, this thesis has determined that there is some evidence of MHC heterozygote advantage, at least in immune response, but the lack of an advantage when comparing worm characteristics may be due to a lack of statistical power. Additionally, this thesis has shown, in a natural infection, previous reports which have demonstrated little association between MHC polymorphism and recognition of specific parasite antigens.

Selective breeding of domestic livestock for disease resistance is sustainable, feasible and is already currently underway using some of the traits described in this thesis. Elucidation of some of the points raised in this study will improve these selection schemes by improving animal welfare and productivity.

Appendices

Appendix A: Recipes for reagents used

All reagents were from Sigma unless otherwise stated.

ELISA reagents

Method for making 0.06M carbonate buffer pH 9.6

Stock solutions: 1M $NaHCO_3$: 84g in 1L dH_2O

1M Na_2CO_3 106g in 1L dH_2O

Mix 45.3ml 1M $NaHCO_3$ with 18.2ml 1M Na_2CO_3.

Make up to 1L with dH_2O.

Check the pH and adjust to 9.6 by adding acid or alkali.

Method for making PBS-T solution (0.01M).

Dissolve 1 sachet of PBS pH 7.4 in 1L dH_2O.

Add 1ml of Tween 20 (BDH) to every 1L (0.01M) PBS.

Method for making PBS-TSM.

Dissolve 4g of dried skimmed milk powder (Safeway) in 100ml of PBS-Tween to make a 4% solution.

Larval preparation

Acid PBS pH 1

Prepare PBS normally using dH_2O and sachets of PBS as described previously. Adjust pH to 1 using acid or alkali as required.

PBS/Antibiotic

Mix 1ml of 5mg/ml Streptomicin/5000i.u. Penicillin, 50 µl of 10mg/ml Gentamycin and 0.5ml of 250µg/ml Amphotericin B, make up to 50ml with sterile PBS pH 7.4.

Protease inhibitors solutions (Maizels et al., 1991)

Make the following stock solutions.

EDTA	2.92g in 10 ml dH_2O	(10 ml of 1 M)
EGTA	3.80g in 10 ml dH_2O	(10 ml of 1 M)
NEM	1.25g in 10 ml dH_2O	(10 ml of 1 M)
Pepstatin	6.85mg in 10ml EtOH	(10 ml of 1 mM)
PMSF	581mg in 10 ml EtOH	(10 ml of 0.33 M)
TPCK	352mg in 10 ml EtOH	(10 ml of 0.1 M)

Each stock solution can be stored in 1ml aliquots at –20°C.

Mix 1 ml of each water soluble inhibitor (EDTA, EGTA, NEM) together and make up to 5 ml with dH_2O. This gives a 200x solution and is designated PI-A (aqueous). Store in aliquots at –20°C.

Mix 3 ml of 0.33 M PMSF with 1ml 0.1 M TPCK then add 1 ml of 1 mM pepstatin. Warm in 65^0C water bath to dissolve. This also gives a 200x solution and is designated PI-B (organic). Store in aliquots at –20°C.

Mix 242.2mg Tris in 200ml dH_2O and pH to 8.3 to give a 1M solution of Tris-HCl.

Mix 2ml Tris-HCl, 1ml PI-A and 1ml PI-B then make up to 200ml using H_2O. This gives the final protease inhibitors solution.

For 1% sodium deoxycholate protease inhibitor solution add 2g sodium deoxycholate to 200ml protease inhibitor solution.

Stock solutions for SDS-PAGE

Acrylamide monomer solution (30.8%T 2.7%C_{bis})

30% w/v acrylamide/bis acrylamide stock solution (Severn Biotech) is purchased ready made and stored at 4°C.

4x Running gel buffer (1.5 M Tris-Cl, pH 8.8)

36.3g Tris (FW 121.1g)

Add 150ml ddH_20

Adjust to pH 8.8 with HCl

Add ddH_2O to 200ml

4x Stacking gel buffer (0.5 M Tris-Cl, pH 6.8)

6.0g Tris (FW 121.1g)

Add 80ml ddH_20

Adjust to pH 6.8 with HCl

Add ddH_2O to 100ml

10% sodium dodecylsulphate (SDS)

10g SDS

Add ddH_20 to 100ml

10% Ammonium Persulphate

0.1g Ammonium persulphate (Biorad)

Add ddH_2O to 1.0ml

Should be made fresh each use.

TEMED (N,N,N',N'-Tetramethylethylenediamine)

Comes ready made

2x Gel loading buffer (0.125M Tris-Cl, 4% SDS, 20% v/v glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8)

2.5ml 4x stacking gel buffer

4.0ml 10% SDS

2.0ml glycerol

2.0mg bromophenol blue

0.31g dithiothreitol (DTT; FW 154.2g)

Add ddH_2O to 10.0ml

Stored in 0.5ml aliquots at –20°C.

Tank Buffer (for running gel)

3g Tris

14.4 g Glycine

1g SDS

Add dH_20 to 1L

Transfer Buffer (for transferring proteins to nitrocellulose)

1.93g Tris

9g Glycine

Add dH_2O to 1L

Ponceau Red Stain:

2g Ponceau S

30g Trichloroacetic acid (TCA)

30g Sulfosalicyic acid

Add dH_2O to 100ml and dilute one part of this stock solution with nine parts water to make working solution

Coomassie Brilliant Blue protein stain

1.25g Coomassie Brilliant Blue R250

450ml methanol/H_2O (1:1 v/v)

50ml glacial acetic acid

Filter through a Whatman No. 1 filter paper to remove particulate matter.

Staining solution is stored at room temperature can be reused multiple times without loss of staining efficiency.

Protein gel destain solution

450ml methanol/H_2O (1:1 v/v)

50ml glacial acetic acid

Store at room temperature but do not re-use.

SDS-PAGE gel recipes (Hoefer Scientific Instruments, 1994)

Single % resolving gels

	7.5% gel	12.5% gel
Monomer solution	3.25ml	6.25ml
4x running gel buffer	3.25ml	3.25ml
10% SDS	150µl	150µl
ddH_2O	7.3ml	4.8ml
10% APS	75µl	75µl
TEMED	5µl	5µl

Stacking gel (4% acrylamide)

Monomer solution	1.33ml
4x stacking gel buffer	2.5ml
10% SDS	100µl
ddH_2O	6.0ml
10% APS	50µl
TEMED	5µl

Gradient gels

For 5-20% linear gradient gels the following solutions were prepared and the gels formed using a Pharmacia gradient mixer and an LKB Bromma 2132 Microperpex peristaltic pump.

	5% solution	20% solution
Monomer solution	1.67ml	6.25ml
4x running gel buffer	2.5ml	3.25ml
10% SDS	100µl	150µl
Sucrose	-	1.5g
ddH_2O	5.7ml	4.8ml
10% APS	33µl	75µl
TEMED	3.3µl	5µl

Appendix B: Natural infection: L_3 adjustments

Optical density indices for pre- and post-adjustment anti-L_3 plasma IgA ELISAs from naturally infected Scottish Blackface sheep. The OD index is shown for each animal before and after adjustment. The average OD Index before adjustment was 0.536 ± 0.031. Following adjustment the average OD Index was 0.205 ± 0.006 (n = 180).

Animal	Pre	Post	Animal	Pre	Post
b47	0.918	0.154	b79	0.085	0.085
b48	1.075	0.247	b80	0.615	0.156
b49	1.044	0.187	b81	0.179	0.171
b50	0.785	0.379	b82	0.382	0.220
b51	0.433	0.205	b83	1.031	0.121
b52	0.891	0.172	b84	0.123	0.180
b53	0.348	0.187	b85	1.053	0.168
b55	0.341	0.201	b87	0.526	0.273
b56	0.281	0.212	b88	0.955	0.080
b57	0.720	0.217	b89	1.087	0.057
b58	0.892	0.261	b90	0.609	0.208
b59	0.259	0.203	b91	0.094	0.178
b60	0.376	0.180	b92	0.792	0.172
b63	0.494	0.272	b93	0.110	0.179
b64	0.869	0.182	b94	0.119	0.131
b65	0.049	0.067	b95	1.059	0.189
b66	0.159	0.131	b96	0.658	0.180
b67	0.652	0.148	b97	0.326	0.221
b68	0.758	0.107	b98	0.285	0.234
b69	0.253	0.186	o1	0.785	0.399
b70	0.205	0.163	o10	0.538	0.326
b71	1.016	0.213	o100	1.114	0.113
b72	0.263	0.199	o11	0.312	0.295
b73	1.104	0.046	o12	0.444	0.292
b74	0.088	0.107	o13	0.114	0.132
b75	2.000	0.167	o14	0.085	0.154
b76	0.295	0.293	o15	–0.014	0.012
b77	1.800	0.144	o16	0.148	0.156
b78	1.900	0.322	o17	0.741	0.243

Animal	Pre	Post	Animal	Pre	Post
o18	0.726	0.376	o54	0.433	0.331
o19	0.296	0.275	o55	0.165	0.159
o2	0.180	0.176	o56	0.248	0.195
o20	0.515	0.304	o57	0.263	0.228
o21	0.365	0.278	o58	0.259	0.234
o22	0.059	0.123	o59	0.291	0.232
o23	0.482	0.244	o6	1.112	0.094
o24	1.042	0.136	o60	0.594	0.338
o25	0.242	0.221	o61	0.681	0.283
o26	0.654	0.357	o62	0.312	0.246
o27	0.146	0.171	o63	0.555	0.325
o28	0.160	0.178	o64	1.200	0.134
o29	0.767	0.321	o65	0.300	0.156
o3	0.225	0.202	o66	0.588	0.236
o30	1.139	0.290	o67	0.520	0.239
o31	0.015	0.013	o68	0.174	0.157
o32	0.328	0.195	o69	0.944	0.207
o33	0.378	0.203	o7	1.770	0.225
o34	1.700	0.120	o70	0.988	0.279
o35	1.112	0.164	o72	1.129	0.253
o36	1.049	0.143	o73	1.600	0.147
o37	0.812	0.141	o74	1.035	0.136
o38	0.596	0.208	o75	0.108	0.038
o39	1.645	0.090	o76	0.104	0.077
o4	0.890	0.198	o77	0.442	0.323
o42	0.196	0.177	o78	1.077	0.103
o43	1.066	0.032	o79	0.109	0.090
o44	0.137	0.161	o8	0.842	0.269
o45	0.550	0.219	o80	0.122	0.108
o46	0.752	0.233	o82	0.436	0.218
o47	0.298	0.267	o83	0.416	0.165
o48	0.595	0.188	o84	0.268	0.183
o5	1.080	0.123	o85	0.556	0.328
o50	0.219	0.185	o86	0.317	0.244
o51	0.615	0.174	o87	0.265	0.237
o52	0.687	0.215	o88	0.480	0.284
o53	0.375	0.260	o9	0.500	0.399

Animal	Pre	Post	Animal	Pre	Post
o90	0.617	0.253	y39	1.008	0.202
o92	0.065	0.072	y4	0.723	0.308
o93	0.088	0.076	y40	0.086	0.126
o94	0.587	0.168	y41	0.269	0.220
o95	0.394	0.236	y42	0.159	0.214
o96	0.660	0.156	y43	0.187	0.196
o97	0.696	0.283	y44	0.013	0.071
o98	0.431	0.275	y45	0.167	0.182
o99	0.238	0.204	y46	0.696	0.385
y1	0.573	0.290	y47	0.223	0.238
y2	0.260	0.230	y48	0.480	0.274
y24	0.102	0.125	y5	0.500	0.265
y26	0.066	0.128	y50	0.162	0.169
y27	0.084	0.213	y51	0.496	0.260
y28	0.284	0.283	y52	0.569	0.330
y3	0.280	0.310	y53	0.556	0.380
y30	0.179	0.227	y54	0.627	0.251
y31	0.398	0.295	y55	0.434	0.339
y32	0.475	0.276	y56	0.932	0.311
y33	0.166	0.234	y57	0.215	0.170
y35	0.171	0.216	y59	0.321	0.232
y36	0.360	0.300	y6	1.155	0.221
y37	0.225	0.224	y60	1.055	0.227
y38	0.016	0.042	y61	0.153	0.134

Appendix C: Natural infection: L_4 adjustments

Optical density indices for pre- and post-adjustment anti-L_4 plasma IgA ELISAs from naturally infected Scottish Blackface sheep. The OD index is shown for each animal before and after adjustment. The average OD Index before adjustment 0.456 ± 0.025. Following adjustment the average OD Index 0.204 ± 0.007 (n = 164).

Animal	Pre	Post	Animal	Pre	Post
b49	0.662	0.171	b82	0.706	0.138
b50	0.865	0.149	b83	1.008	0.187
b51	0.337	0.197	b84	0.153	0.162
b52	0.848	0.100	b85	1.025	0.263
b53	0.611	0.126	b87	0.910	0.071
b55	0.493	0.158	b88	0.868	0.161
b56	0.280	0.187	b89	1.116	0.192
b57	0.519	0.197	b90	0.781	0.103
b58	0.400	0.211	b91	0.276	0.202
b59	0.328	0.252	b92	0.632	0.185
b60	0.436	0.170	b93	0.144	0.173
b63	0.427	0.220	b94	0.084	0.124
b64	0.873	0.103	b95	0.908	0.089
b65	0.131	0.179	b96	0.994	0.025
b66	0.205	0.200	b97	0.377	0.234
b67	0.721	0.077	b98	0.288	0.193
b68	0.648	0.174	o1	1.008	0.017
b69	0.377	0.314	o10	0.391	0.217
b70	0.158	0.136	o100	0.802	0.453
b71	1.004	0.146	o11	0.550	0.228
b72	0.238	0.204	o12	0.712	0.246
b73	1.136	0.381	o13	0.253	0.215
b74	0.115	0.138	o14	0.153	0.208
b75	1.452	0.239	o15	–0.018	0.030
b76	0.339	0.332	o16	0.240	0.217
b77	0.913	0.055	o17	0.456	0.236
b78	1.389	0.703	o18	0.446	0.261
b79	0.175	0.190	o19	0.263	0.209
b80	0.798	0.067	o2	0.174	0.176
b81	0.198	0.192	o20	0.373	0.217

Animal	Pre	Post	Animal	Pre	Post
o21	0.168	0.177	o62	0.375	0.282
o22	0.081	0.133	o63	0.715	0.400
o23	0.525	0.178	o65	0.305	0.234
o25	0.371	0.272	o67	0.353	0.217
o26	0.651	0.302	o68	0.097	0.174
o27	0.235	0.209	o7	1.095	0.136
o28	0.111	0.141	o72	0.834	0.209
o29	0.775	0.167	o75	0.190	0.176
o3	0.326	0.176	o76	0.033	0.112
o31	0.008	0.055	o77	0.464	0.289
o32	0.205	0.188	o79	0.172	0.211
o33	0.349	0.317	o8	0.749	0.142
o35	1.074	0.167	o80	0.046	0.104
o36	0.802	0.325	o82	0.363	0.246
o37	0.538	0.260	o83	0.248	0.254
o38	0.550	0.234	o84	0.210	0.240
o39	1.085	0.472	o85	0.494	0.303
o4	1.008	0.049	o86	0.216	0.255
o42	0.286	0.273	o87	0.256	0.294
o44	0.104	0.184	o88	0.416	0.328
o45	0.584	0.254	o9	0.873	0.198
o46	0.713	0.348	o90	0.486	0.183
o47	0.359	0.258	o92	–0.014	0.030
o5	1.035	0.102	o93	0.207	0.219
o50	0.261	0.221	o94	0.452	0.258
o51	0.603	0.172	o95	0.312	0.249
o52	0.613	0.274	o98	0.254	0.250
o53	0.415	0.233	o99	0.204	0.186
o54	0.494	0.309	y1	0.548	0.218
o55	0.322	0.187	y10	0.374	0.265
o56	0.265	0.224	y13	1.123	0.568
o57	0.309	0.334	y2	0.250	0.231
o58	0.379	0.257	y24	0.055	0.109
o59	0.316	0.298	y26	0.085	0.111
o6	0.798	0.127	y27	0.350	0.184
o60	1.059	0.130	y28	0.177	0.183
o61	0.893	0.309	y3	0.330	0.238

Animal	Pre	Post	Animal	Pre	Post
y30	0.167	0.166	y46	0.768	0.258
y31	0.841	0.052	y47	0.101	0.175
y32	0.366	0.247	y48	0.486	0.188
y33	0.107	0.130	y5	0.342	0.231
y35	0.252	0.185	y50	0.216	0.199
y36	0.329	0.237	y51	0.464	0.209
y37	0.256	0.199	y52	0.630	0.192
y38	0.012	0.059	y54	0.467	0.224
y4	0.702	0.211	y55	0.474	0.322
y40	0.033	0.094	y57	0.207	0.164
y41	0.207	0.198	y59	0.202	0.192
y42	0.242	0.222	y61	0.119	0.124
y43	0.121	0.163	y7	0.412	0.271
y44	0.056	0.092	y8	0.037	0.080
y45	0.117	0.180	y9	0.513	0.241

List of References

Abbott, E. M., Parkins, J. J. & Holmes, P. H. (1985). Influence of dietary protein on parasite establishment and pathogenesis in Finn Dorset and Scottish Blackface lambs given a single moderate infection of *Haemonchus contortus*. *Research in Veterinary Science* **38**, 6-13.

Abbott, E. M., Parkins, J. J. & Holmes, P. H. (1986). The effect of dietary protein on the pathogenesis of acute ovine haemonchosis. *Veterinary Parasitology* **20**, 275-289.

Acuna-Soto, R., Maguire, J. H. & Wirth, D. F. (2000). Gender distribution in asymptomatic and invasive amebiasis. *American Journal of Gastroenterology* **95**, 1277-1283.

Adams, D. B. (1982). Time of onset and target of immune reactions in sheep with acquired immunity against *Haemonchus contortus*. *International Journal for Parasitology* **12**, 439-443.

Adams, D. B. (1989). A preliminary evaluation of factors affecting an experimental system for vaccination-and-challenge with *Haemonchus contortus* in sheep. *International Journal for Parasitology* **19**, 169-175.

Aidoo, M., Terlouw, D. J., Kolczak, M. S., McElroy, P. D., ter Kuile, F. O., Kariuki, S., Nahlen, B. L., Lal, A. A. & Udhayakumar, V. (2002). Protective effects of the sickle cell gene against malaria morbidity and mortality. *Lancet* **359**, 1311-1312.

Akpavie, S. O. & Pirie, H. M. (1989). The globule leucocyte: morphology, origin, function and fate, a review. *Anatomia, Histologia, Embryologia: Journal of Veterinary Medicine Series C* **18**, 87-95.

Al Saqur, I., Bairden, K. & Armour, J. (1982). Population study of bovine *Ostertagia* spp. infective on herbage and in soil. *Research in Veterinary Science* **32**, 332-337.

Albers, G. A., Gray, G. D., Piper, L. R., Barker, J. S., Le Jambre, L. F. & Barger, I. A. (1987). The genetics of resistance and resilience to *Haemonchus contortus* infection in young merino sheep. *International Journal for Parasitology* **17**, 1355-1363.

Alexander, N. D. & Grenfell, B. T. (1999). The effect of pregnancy on *Wuchereria bancrofti* microfilarial load in humans. *Parasitology* **119**, 151-156.

Almeria, S., Canals, A., Zarlenga, D. S. & Gasbarre, L. C. (1997). Isolation and phenotypic characterization of abomasal mucosal lymphocytes in the course of a primary *Ostertagia ostertagi* infection in calves. *Veterinary Immunology and Immunopathology*. **57**, 87-98.

Altaif, K. I. & Dargie, J. D. (1978a). Genetic resistance to helminths. The influence of breed and haemoglobin type on the response of sheep to primary infections with *Haemonchus contortus*. *Parasitology* **77**, 161-175.

Altaif, K. I. & Dargie, J. D. (1978b). Genetic resistance to helminths. Comparison of the development of *Ostertagia circumcincta* infections in Scottish Blackface sheep of different haemoglobin type. *Research in Veterinary Science* **24**, 391-393.

Anderson, N., Hansky, J. & Titchen, D. A. (1985). Effects on plasma pepsinogen, gastrin and pancreatic-polypeptide of *Ostertagia* spp. transferred directly into the abomasum of sheep. *International Journal for Parasitology* **15**, 159-165.

Anderson, R. C. (2000). Nematode parasites of vertebrates. Their development and transmission. CABI Publishing, Oxon, UK.

Anderson, R. M. (1993). Epidemiology. In: Modern parasitology. A textbook of parasitology. Cox, F. E. G. (ed). Blackwell Scientific Publications. Oxford, UK. pp. 75-116.

Anderson, R. M. & Schad, G. A. (1985). Hookworm burdens and faecal egg counts: an analysis of the biological basis of variation. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **79**, 812-825.

Angyalosi, G., Neveu, R., Wolowczuk, I., Delanoye, A., Herno, J., Auriault, C. & Pancré, V. (2001). HLA class II polymorphism influences onset and severity of pathology in *Schistosoma mansoni*-infected transgenic mice. *Infection and Immunity* **69**, 5874-5882.

Anuradha, Pal, R. & Katiyar, J. C. (1990). Sex-influenced population kinetics of *Leishmania donovani* in hamsters. *Indian Journal of Experimental Biology* **28**, 876-879.

Arasu, P. (2001). *In vitro* reactivation of *Ancylostoma caninum* tissue-arrested third-stage larvae by transforming growth factor-β. *Journal of Parasitology* **87**, 733-738.

Arasu, P. & Kwak, D. (1999). Developmental arrest and pregnancy-induced transmammary transmission of *Ancylostoma caninum* larvae in the murine model. *Journal of Parasitology* **85**, 779-784.

Armour, J., Jarrett, W. F. H. & Jennings, F. W. (1966). Experimental *Ostertagia circumcincta* infections in sheep: development and pathogenesis of a single infection. *American Journal of Veterinary Research* **27**, 1267-1278.

Ashton, F. T., Li, J. & Schad, G. A. (1999). Chemo- and thermosensory neurons: structure and function in animal parasitic nematodes. *Veterinary Parasitology* **84**, 297-316.

Athanasiadou, S., Kyriazakis, I., Jackson, F. & Coop, R. L. (2000). Consequences of long-term feeding with condensed tannins on sheep parasitised with *Trichostrongylus colubriformis*. *International Journal for Parasitology* **30**, 1025-1033.

Atkins, N. S., Conway, D. J., Lindo, J. F., Bailey, J. W. & Bundy, D. A. (1999). L_3 antigen-specific antibody isotype responses in human strongyloidiasis: correlations with larval output. *Parasite Immunology* **21**, 517-526.

Atkins, N. S., Lindo, J. F., Lee, M. G., Conway, D. J., Bailey, J. W., Robinson, R. D. & Bundy, D. A. (1997). Humoral responses in human strongyloidiasis: correlations with infection chronicity. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **91**, 609-613.

Baker, D. G. & Gershwin, L. J. (1993). Inverse relationship between IgE and worm burdens in cattle infected with *Ostertagia ostertagi*. *Veterinary Parasitology* **47**, 87-97.

Baldwin, R. L. (2000). Sheep gastrointestinal development in response to different dietary treatments. *Small Ruminant Research* **35**, 39-47.

Balic, A., Bowles, V. M. & Meeusen, E. N. (2000a). The immunobiology of gastrointestinal nematode infections in ruminants. *Advances in Parasitology* **45**, 181-241.

Balic, A., Bowles, V. M. & Meeusen, E. N. (2000b). Cellular profiles in the abomasal mucosa and lymph node during primary infection with *Haemonchus contortus* in sheep. *Veterinary Immunology and Immunopathology* **75**, 109-120.

Balic, A., Bowles, V. M. & Meeusen, E. N. (2002). Mechanisms of immunity to *Haemonchus contortus* infection in sheep. *Parasite Immunology* **24**, 39-46.

Bang, K. S., Familton, A. S. & Sykes, A. R. (1990). Effect of copper oxide wire particle treatment on establishment of major gastrointestinal nematodes in lambs. *Research in Veterinary Science* **49**, 132-137.

Barger, I. A. (1993). Influence of sex and reproductive status on susceptibility of ruminants to nematode parasitism. *International Journal for Parasitology* **23**, 463-469.

Barger, I. A. (1996). Prospects for integration of novel parasite control options into grazing systems. *International Journal for Parasitology* **26**, 1001-1007.

Barger, I. A. (1997). Control by management. *Veterinary Parasitology* **72**, 493-506.

Barnes, E. H., Dobson, R. J. & Barger, I. A. (1995). Worm control and anthelmintic resistance: adventures with a model. *Parasitology Today* **11**, 56-63.

Bath, G. F., Malan, F. S. & van Wyk, J. A. (1997). The use of clinical anemia as an aid in the control of haemonchosis in sheep. *Proceedings of the 4th International Congress for Sheep Veterinarians*, 381-385.

Bawden, R. J. (1969). The relationships between *Oesophagostomum columbianum* infection and the nutritional status of sheep. *Australian Journal of Agricultural Research* **20**, 589-599.

Beach, R. S., Gershwin, M. E. & Hurley, L. S. (1983). Persistent immunological consequences of gestation zinc deprivation. *American Journal of Clinical Nutrition* **38**, 579-590.

Beh, K. J. & Maddox, J. F. (1996). Prospects for development of genetic markers for resistance to gastrointestinal parasite infection in sheep. *International Journal for Parasitology* **26**, 879-897.

Behm, C. A. & Ovington, K. S. (2000). The role of eosinophils in parasitic helminth infections: insights from genetically modified mice. *Parasitology Today* **16**, 202-209.

Behnke, J. M., De Clercq, D., Sacko, M., Gilbert, F. S., Ouattara, D. B. & Vercruysse, J. (2000). The epidemiology of human hookworm infections in the southern region of Mali. *Tropical Medicine and International Health* **5**, 343-354.

Bell, B. (1997). Mineral nutrition in sheep. *[Online]* Available from: *http://www.gov.on.ca/OMAFRA/english/livestock/sheep/facts/minernut.htm* [Accessed 4th Sepember 2002].

Benitez-Usher, C., Armour, J., Duncan, J. L., Urquhart, G. M. & Gettinby, G. (1977). A study of some factors influencing the immunization of sheep against *Haemonchus contortus* using attenuated larvae. *Veterinary Parasitology* **3**, 327-342.

Bernstein, R. E. (1987). Nonenzymatically glycosylated proteins. *Advances in Clinical Chemistry* **26**, 1-78.

Bird, J., Shulaw, W. P., Pope, W. F. & Bremer, C. A. (2001). Control of anthelmintic resistant endoparasites in a commercial sheep flock through parasite community replacement. *Veterinary Parasitology* **97**, 219-225.

Bisset, S. A. & Morris, C. A. (1996). Feasibility and implications of breeding sheep for resilience to nematode challenge. *International Journal for Parasitology* **26**, 857-868.

Bizzell, W. E. & Ciordia, H. (1965). Dissemination of infective larvae of trichostrongylid parasites of ruminants from feces to pasture by the fungus, *Pilobolus* spp.. *Journal of Parasitology* **51**, 184.

Blattman, A. N., Hulme, D. J., Kinghorn, B. P., Woolaston, R. R., Gray, G. D. & Beh, K. J. (1993). A search for associations between major histocompatibility complex restriction fragment length polymorphism bands and resistance to *Haemonchus contortus* infection in sheep. *Animal Genetics* **24**, 277-282.

Boag, B. & Thomas, R. J. (1975). The population dynamics of nematode parasites of sheep in northern England. *Research in Veterinary Science* **19**, 293-295.

Bodmer, J. G., Marsh, S. G. & Albert, E. (1990). Nomenclature for factors of the HLA system, 1989. *Immunology Today* **11**, 3-10.

Boersema, J. H. (1985). Chemotherapy of gastrointestinal nematodiasis in ruminants. In: Chemotherapy of gastrointestinal helminths. Vanden Bossche, H., Thienpont, D. & Janssens, P. G. (eds). Springer-Verlag. Berlin, Germany, pp. 407-442.

Boissier, J. & Moné, H. (2000). Experimental observations on the sex ratio of adult *Schistosoma mansoni*, with comments on the natural male bias. *Parasitology* **121**, 379-383.

Bown, M. D., Poppi, D. P. & Sykes, A. R. (1989). The effects of a concurrent infection of *Trichostrongylus colubriformis* and *Ostertagia circumcincta* on calcium, phosphorus and magnesium transactions along the digestive tract of lambs. *Journal of Comparitive Pathology* **101**, 11-20.

Box, G. E. & Cox, D. R. (1964). An analysis of transformations. *Journal of the Royal Statistical Society Series B (Methodological)* **26**, 211-252.

Britt, D. P. (1982). Benzimidazole-resistant nematodes in Britain. The *Veterinary Record* **110**, 343-344.

Bryan, R. P. & Kerr, J. D. (1989). Factors affecting the survival and migration of the free-living stages of gastrointestinal nematode parasites of cattle in central Queensland. *Veterinary Parasitology* **30**, 315-326.

Buddle, B. M., Jowett, G., Green, R. S., Douch, P. G. & Risdon, P. L. (1992). Association of blood eosinophilia with the expression of resistance in Romney lambs to nematodes. *International Journal for Parasitology* **22**, 955-960.

Bundy, D. A., Cooper, E. S., Thompson, D. E., Anderson, R. M. & Didier, J. M. (1987). Age-related prevalence and intensity of *Trichuris trichiura* infection in a St. Lucian community. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **81**, 85-94.

Butler, J. E. (1998). Immunoglobulin diversity, B-cell and antibody repertoire development in large farm animals. *Revue Scientifique et Technique de l'Office International des Epizooties* **17**, 43-70.

Callinan, A. P. (1978). The ecology of the free-living stages of *Ostertagia circumcincta*. *International Journal for Parasitology* **8**, 233-237.

Capron, A., Riveau, G., Grzych, J. M., Boulanger, D., Capron, M. & Pierce, R. (1994). Development of a vaccine strategy against human and bovine schistosomiasis. Background and update. *Tropical Geographical Medicine* **46**, 242-246.

Carrington, M., Nelson, G. W., Martin, M. P., Kissner, T., Vlahov, D., Goedert, J. J., Kaslow, R., Buchbinder, S., Hoots, K. & O'Brien, S. J. (1999). *HLA* and HIV-1: heterozygote advantage and *B*35-Cw*04* disadvantage. *Science* **283**, 1748-1752.

Chambers, G., Lawrie, L., Cash, P. & Murray, G. I. (2000). Proteomics: a new approach to the study of disease. *Journal of Pathology* **192**, 280-288.

Charley-Poulain, J., Luffau, G. & Pery, P. (1984). Serum and abomasal antibody response of sheep to infections with *Haemonchus contortus*. *Veterinary Parasitology* **14**, 129-141.

Ciordia, H. & Bizzell, W. E. (1963). The effects of various constant temperatures on the development of free-living stages of some nematode parasites of cattle. *Journal of Parasitology* **49**, 60-63.

Colditz, I. G., Watson, D. L., Gray, G. D. & Eady, S. J. (1996). Some relationships between age, immune responsiveness and resistance to parasites in ruminants. *International Journal for Parasitology* **26**, 869-877.

Cole, R. K. (1968). Studies on genetic resistance to Marek's disease. *Avian Diseases* **12**, 9-28.

Coles, G. C. (1998). Drug-resistant parasites of sheep: An emerging problem in Britain? *Parasitology Today* **14**, 86-88.

Coles, G. C., Warner, A. K. & Best, J. R. (1996). Triple resistant *Ostertagia* from angora goats. *The Veterinary Record* **139**, 299-300.

Coltman, D. W., Wilson, K., Pilkington, J., Stear, M. J. & Pemberton, J. M. (2001). A microsatellite polymorphism in the gamma interferon gene is associated with resistance

to gastrointestinal nematodes in a naturally-parasitized population of Soay sheep. *Parasitology* **122**, 571-582.

Coop, R. L. & Field, A. C. (1983). Effect of phosphorus intake on growth rate, food intake and quality of the skeleton of growing lambs infected with the intestinal nematode *Trichostrongylus vitrinus*. *Research in Veterinary Science* **35**, 175-181.

Coop, R. L. & Holmes, P. H. (1996). Nutrition and parasite interaction. *International Journal for Parasitology* **26**, 951-962.

Coop, R. L., Huntley, J. F. & Smith, W. D. (1995). Effect of dietary protein supplementation on the development of immunity to *Ostertagia circumcincta* in growing lambs. *Research in Veterinary Science* **59**, 24-29.

Coop, R. L. & Kyriazakis, I. (1999). Nutrition-parasite interaction. *Veterinary Parasitology* **84**, 187-204.

Coop, R. L. & Kyriazakis, I. (2001). Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. *Trends in Parasitology* **17**, 325-330.

Corinti, S., De Palma, R. D., Fontana, A., Gagliardi, C., Pini, C. & Sallusto, F. (1997). Major histocompatibility complex-independent recognition of a distinctive pollen antigen, most likely a carbohydrate, by human $CD8^+$ α/β T cells. *Journal of Experimental Medicine* **186**, 899-908.

Coyne, C. P. & Brake, D. (2001). Characterisation of *Haemonchus contortus*-derived cell populations propagated *in vitro* in a tissue culture environment and their potential to induce protective immunity in sheep. *International Journal for Parasitology* **31**, 359-376.

Crofton, H. D. (1965). Ecology and biological plasticity of sheep nematodes. I. The effect of temperature on the hatching of eggs of some nematode parasites of sheep. *The Cornell Veterinarian* **55**, 242-250.

Crofton, H. D. & Whitlock, J. H. (1965). Ecology and biological plasticity of sheep nematodes. III. Studies on *Ostertagia circumcincta* (Stadelmann 1894). *The Cornell Veterinarian* **55**, 259-262.

Crompton, D. W. (1984). Influence of parasitic infection on food intake. *Federation Proceedings* **43**, 239-245.

Cunningham-Rundles, C. (2001). Physiology of IgA and IgA deficiency. *Journal of Clinical Immunology* **21**, 303-309.

Cuquerella, M., Gómez-Muñoz, M. T. & Alunda, J. M. (1991). Serum IgG response of Manchego lambs to infections with *Haemonchus contortus* and preliminary characterization of adult antigens. *Veterinary Parasitology* **38**, 131-143.

Cuquerella, M., Gómez-Muñoz, M. T., Mendez, S. & Alunda, J. M. (1994). Partial protection of Manchego sheep against *Haemonchus contortus* after a 6-month postpriming period. *Journal of Veterinary Medicine Series B* **41**, 399-406.

Cury, M. C., Guimarães, M. P., Lima, W. S. & Vitor, R. W. (2002). Western blot analysis of the humoral response of dogs experimentally infected with *Angiostrongylus vasorum* (Baillet, 1866). *Veterinary Parasitology* **106**, 83-87.

D'Agostino, R. B. S. (1986). Goodness-of-fit techniques. M. Dekker. New York, USA.

Dacie, J. V. & Lewis, S. M. (1979). Practical Haematology. Churchill, J. & A. London, U.K.

Dakkak, A., Fioramonti, J. & Bueno, L. (1981). *Haemonchus contortus* third stage larvae in sheep; kinetics of arrival into the abomasum and transformation during rumino-omasal transit. *Research in Veterinary Science* **31**, 384-385.

Dalton, J. P. & Mulcahy, G. (2001). Parasite vaccines - a reality? *Veterinary Parasitology* **98**, 149-167.

Datta, F. U., Nolan, J. V., Rowe, J. B., Gray, G. D. & Crook, B. J. (1999). Long-term effects of short-term provision of protein-enriched diets on resistance to nematode infection, and live-weight gain and wool growth in sheep. *International Journal for Parasitology* **29**, 479-488.

Dawkins, H. J., Windon, R. G. & Eagleson, G. K. (1989). Eosinophil responses in sheep selected for high and low responsiveness to *Trichostrongylus colubriformis*. *International Journal for Parasitology* **19**, 199-205.

Day, K. P., Gregory, W. F. & Maizels, R. M. (1991). Age-specific acquisition of immunity to infective larvae in a bancroftian filariasis endemic area of Papua New Guinea. *Parasite Immunology* **13**, 277-290.

De Graaf, D. C., Berghen, P., Moens, L., de Marez, T. M., Raes, S., Blaxter, M. L. & Vercruysse, J. (1996). Isolation, characterization and immunolocalization of a globin-like antigen from *Ostertagia ostertagi* adults. *Parasitology* **113**, 63-69.

De La Concha, E. G., Fernandez-Arquero, M., Martinez, A., Vidal, F., Vigil, P., Conejero, L., Garcia-Rodriguez, M. C. & Fontan, G. (1999). HLA class II homozygosity confers susceptibility to common variable immunodeficiency (CVID). *Clinical Experimental Immunology* **116**, 516-520.

Denkers, E. Y., Wassom, D. L., Krco, C. J. & Hayes, C. E. (1990). The mouse antibody response to *Trichinella spiralis* defines a single, immunodominant epitope shared by multiple antigens. *Journal of Immunology* **144**, 3152-3159.

Dineen, J. K., Gregg, P. & Lascelles, A. K. (1978). The response of lambs to vaccination at weaning with irradiated *Trichostrongylus colubriformis* larvae: segregation into 'responders' and 'non-responders'. *International Journal for Parasitology* **8**, 59-63.

Doherty, P. C. & Zinkernagel, R. M. (1975). Enhanced immunological surveillance in mice heterozygous at the H-2 gene complex. *Nature* **256**, 50-52.

Doligalska, M., Moskwa, B. & Stear, M. J. (1999). Relationships among peripheral eosinophilia, eosinophil peroxidase activity, interleukin-5 concentration and faecal nematode egg count during natural, mixed gastrointestinal nematode infection. *Veterinary Immunology and Immunopathology* **70**, 299-308.

Donald, A. D. (1968). Ecology of the free-living stages of nematode parasites of sheep. *Australian Veterinary Journal* **44**, 139-144.

Donaldson, J., van Houtert, M. F. & Sykes, A. R. (1998). The effect of nutrition on the periparturient parasite status of mature ewes. *Animal Science* **67**, 523-533.

Donaldson, J., van Houtert, M. F. & Sykes, A. R. (2001). The effect of dietary fish-meal supplementation on parasite burdens of periparturient sheep. *Animal Science* **72**, 149-158.

Douch, P. G., Green, R. S., Morris, C. A., Bisset, S. A., Vlassoff, A., Baker, R. L., Watson, T. G., Hurford, A. P. & Wheeler, M. (1995). Genetic and phenotypic relationships among anti-*Trichostrongylus colubriformis* antibody level, fecal egg count and body weight traits in grazing Romney sheep. *Livestock Production Science* **41**, 121-132.

Douch, P. G., Green, R. S., Morris, C. A., McEwan, J. C. & Windon, R. G. (1996a). Phenotypic markers for selection of nematode-resistant sheep. *International Journal for Parasitology* **26**, 899-911.

Douch, P. G., Green, R. S. & Risdon, P. L. (1994). Antibody responses of sheep to challenge with *Trichostrongylus colubriformis* and the effect of dexamethasone treatment. *International Journal for Parasitology* **24**, 921-928.

Douch, P. G., Harrison, G. B., Elliott, D. C., Buchanan, L. L. & Greer, K. S. (1986). Relationship of gastrointestinal histology and mucus antiparasite activity with the development of resistance to trichostrongyle infections in sheep. *Veterinary Parasitology* **20**, 315-331.

Douch, P. G. & Morum, P. E. (1993). The effect of age on the response of Romney sheep to gastrointestinal nematodes during grazing. *International Journal for Parasitology* **23**, 651-655.

Douch, P. G., Morum, P. E. & Rabel, B. (1996b). Secretion of anti-parasite substances and leukotrienes from ovine gastrointestinal tissues and isolated mucosal mast cells. *International Journal for Parasitology* **26**, 205-211.

Douch, P. G. & Outteridge, P. M. (1989). The relationship betwen ovine lymphocyte antigens and parasitological and production parameters in Romney sheep. *International Journal for Parasitology* **19**, 35-41.

Drudge, J. H., Szanto, J., Wyant, Z. N. & Elam, G. W. (1964). Field studies on parasite control of sheep: Comparison of thiabendazole, ruelene and phenothiazine. *American Journal of Veterinary Research* **25**, 1512-1518.

Dubucquoi, S., Desreumaux, P., Janin, A., Klein, O., Goldman, M., Tavernier, J., Capron, A. & Capron, M. (1994). Interleukin 5 synthesis by eosinophils: association with granules and immunoglobulin-dependent secretion. *Journal of Experimental Medicine* **179**, 703-708.

Dunne, D. W., Jones, F. M., Cook, L. & Moloney, N. A. (1994). Passively transferable protection against *Schistosoma japonicum* induced in the mouse by multiple vaccination with attenuated larvae: the development of immunity, antibody isotype responses and antigen recognition. *Parasite Immunology* **16**, 655-668.

Dunsmore, J. D. (1965). *Ostertagia* spp. in lambs and pregnant ewes. *Journal of Helminthology* **39**, 159-184.

Durie, P. H. (1961). Parasite gastro-enteritis of cattle: the distribution and survival of infective strongyle larvae on pasture. *Australian Journal of Agricultural Research* **12**, 1200-1211.

Dynes, R. A., Poppi, D. P., Barrell, G. K. & Sykes, A. R. (1998). Elevation of feed intake in parasite-infected lambs by central administration of a cholecystokinin receptor antagonist. *British Journal Nutrition* **79**, 47-54.

Edwards, K. E., Jepson, R. P. & Eagleson, G. K. (1960). Value of plasma pepsinogen estimation. *British Medical Journal* **1**, 30-32.

Elard, L., Comes, A. M. & Humbert, J. F. (1996). Sequences of beta-tubulin cDNA from benzimidazole-susceptible and -resistant strains of *Teladorsagia circumcincta*, a nematode parasite of small ruminants. *Molecular and Biochemical Parasitology* **79**, 249-253.

Eloi-Santos, S., Olsen, N. J., Correa-Oliveira, R. & Colley, D. G. (1992). *Schistosoma mansoni*: mortality, pathophysiology, and susceptibility differences in male and female mice. *Experimental Parasitology* **75**, 168-175.

Emery, D. L. (1996). Vaccination against worm parasites of animals. *Veterinary Parasitology* **64**, 31-45.

Errouissi, F., Alvinerie, M., Galtier, P., Kerboeuf, D. & Lumaret, J. P. (2001). The negative effects of the residues of ivermectin in cattle dung using a sustained-release bolus on *Aphodius constans* (Duft.) (Coleoptera: Aphodiidae). *Veterinary Research* **32**, 421-427.

Eysker, M. (1991). Direct measurement of dispersal of *Dictyocaulus viviparus* in sporangia of *Pilobolus* species. *Research in Veterinary Science* **50**, 29-32.

Faedo, M., Larsen, M. & Waller, P. J. (1997). The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: comparison between Australian isolates of *Arthrobotrys* spp. and *Duddingtonia flagrans*. *Veterinary Parasitology* **72**, 149-155.

Ferguson, E. G., Mitchell, G. B. & MacPherson, A. (1989). Cobalt deficiency and *Ostertagia circumcincta* infection in lambs. *The Veterinary Record* **124**, 20.

Fernández, S., Šarkunas, M. & Roepstorff, A. (2001). Survival of infective *Ostertagia ostertagi* larvae on pasture plots under different simulated grazing conditions. *Veterinary Parasitology* **96**, 291-299.

Ferre, I., Brusa, C. M., Manzanera, E., Rojo-Vázquez, F. A., Buratovich, O. F. & Mantecón, A. R. (1995). Effect of supplementary feeding on the gastrointestinal strongylid eggs shedding in grazing pregnant Merino ewes. *Journal of Animal Feed Science* **4**, 237-245.

Ferrick, D. A., Schrenzel, M. D., Mulvania, T., Hsieh, B., Ferlin, W. G. & Lepper, H. (1995). Differential production of interferon-gamma and interleukin-4 in response to

T_H1- and T_H2-stimulating pathogens by gamma delta T cells *in vivo*. *Nature* **373**, 255-257.

Fincher, G. T. (1973). Dung beetles as biological control agents for gastrointestinal parasites of livestock. *Journal of Parasitology* **59**, 396-399.

Finkelman, F. D., Shea-Donohue, T., Goldhill, J., Sullivan, C. A., Morris, S. C., Madden, K. B., Gause, W. C. & Urban, J. F. Jr. (1997). Cytokine regulation of host defense against parasitic gastrointestinal nematodes: Lessons from studies with rodent models. *Annual Review of Immunology* **15**, 505-533.

Forbes, T. D. A. & Hodgson, J. (1985). The reaction of grazing sheep and cattle to the presence of dung from the same or the other species. *Grass and Forage Science* **40**, 177-182.

Foundation for Research, S. &. T. (2000). Green kit a money saver. *[Online]* Available from: *http://www.scoop.co.nz/stories/SC0003/S00012.htm* [Accessed 4th Sepember 2002]

Fox, M. T. (1993). Pathophysiology of infection with *Ostertagia ostertagi* in cattle. *Veterinary Parasitology* **46**, 143-158.

Fox, M. T. (1997). Pathophysiology of infection with gastrointestinal nematodes in domestic ruminants: recent developments. *Veterinary Parasitology* **72**, 285-308.

Fox, M. T., Gerrelli, D., Pitt, S. R., Jacobs, D. E., Gill, M. & Gale, D. L. (1989a). *Ostertagia ostertagi* infection in the calf: effects of a trickle challenge on appetite, digestibility, rate of passage of digesta and liveweight gain. *Research in Veterinary Science* **47**, 294-298.

Fox, M. T., Gerrelli, D., Shivalkar, P. & Jacobs, D. E. (1989b). Effect of omeprazole treatment on feed intake and blood gastrin and pepsinogen levels in the calf. *Research in Veterinary Science* **46**, 280-282.

Gabriel, S. E., Brigman, K. N., Koller, B. H., Boucher, R. C. & Stutts, M. J. (1994). Cystic fibrosis heterozygote resistance to cholera toxin in the cystic fibrosis mouse model. *Science* **266**, 107-109.

Ganley, L. & Rajan, T. V. (2001). Endogenous testosterone levels do not affect filarial worm burdens in mice. *Experimental Parasitology* **98**, 29-34.

Gasbarre, L. C., Leighton, E. A. & Sonstegard, T. (2001). Role of the bovine immune system and genome in resistance to gastrointestinal nematodes. *Veterinary Parasitology* **98**, 51-64.

Geldhof, P., Claerebout, E., Knox, D. P., Agneessens, J. & Vercruysse, J. (2000). Proteinases released *in vitro* by the parasitic stages of the bovine abomasal nematode *Ostertagia ostertagi. Parasitology* **121** , 639-647.

Gibbs, J., Young, R. C. & Smith, G. P. (1973). Cholecystokinin decreases food intake in rats. *Journal of Comparitive and Physiological Psychology* **84**, 488-495.

Gibson, T. E. & Everett, G. (1972). The ecology of the free-living stages of *Ostertagia circumcincta. Parasitology* **64**, 451-460.

Gibson, T. E. & Parfitt, J. W. (1972). The effect of age on the development by sheep of resistance to *Trichostrongylus colubriformis*. *Research in Veterinary Science* **13**, 529-535.

Gill, H. S. (1991). Genetic-control of acquired resistance to haemonchosis in Merino lambs. *Parasite Immunology* **13**, 617-628.

Gill, H. S., Gray, G. D., Watson, D. L. & Husband, A. J. (1993a). Isotype-specific antibody responses to *Haemonchus contortus* in genetically resistant sheep. *Parasite Immunology* **15**, 61-67.

Gill, H. S., Watson, D. L. & Brandon, M. R. (1993b). Monoclonal antibody to $CD4^+$ T cells abrogates genetic resistance to *Haemonchus contortus* in sheep. *Immunology* **78**, 43-49.

Githigia, S. M., Thamsborg, S. M. & Larsen, M. (2001). Effectiveness of grazing management in controlling gastrointestinal nematodes in weaner lambs on pasture in Denmark. *Veterinary Parasitology* **99**, 15-27.

Githigia, S. M., Thamsborg, S. M., Larsen, M., Kyvsgaard, N. C. & Nansen, P. (1997). The preventive effect of the fungus *Duddingtonia flagrans* on trichostrongyle infections of lambs on pasture. *International Journal for Parasitology* **27**, 931-939.

Gómez-Muñoz, M. T., Cuquerella, M., Gómez-Iglesias, L. A., Méndez, S., Fernández-Pérez, F. J., de la Fuente, C. & Alunda, J. M. (1999). Serum antibody response of Castellana sheep to *Haemonchus contortus* infection and challenge: relationship to abomasal worm burdens. *Veterinary Parasitology* **81**, 281-293.

Gomez, K. A. & Gomez, A. A. (1984). Statistical procedures for agricultural research. John Wiley & Sons, Inc. New York, USA.

Gordon, H. M. & Whitlock, H. V. (1939). A new technique for counting nematode eggs in sheep faeces. *Journal of the Council for Scientific and Industrial Research, Australia* **12**, 50-50.

Gray, G. D. (1991). Breeding for resistance to trichostrongyle nematodes in sheep. In: Breeding for disease resistance in farm animals. Owen, J. B. & Axford, R. F. E. (eds). CAB International. Oxon, UK, pp. 139-161.

Gray, G. D. (1997). The use of genetically resistant sheep to control nematode parasitism. *Veterinary Parasitology* **72**, 345-366.

Gregg, P., Dineen, J. K., Rothwell, T. L. & Kelly, J. D. (1978). The effect of age on the response of sheep to vaccination with irradiated *Trichostrongylus colubriformis* larvae. *Veterinary Parasitology* **4**, 35-48.

Griebel, P. J. (1998). Sheep immunology and goat peculiarities. In: Handbook of vertebrate immunology. Pastoret, P. P., Griebel, P., Bazin, H. & Govaerts, A. (eds). Academic Press. London, UK, pp. 485-533.

Grønvold, J. (1984). Rain splash dispersal of third-stage larvae of *Cooperia* spp. (Trichostrongylidae). *Journal of Parasitology* **70**, 924-926.

Grønvold, J. (1987). Field experiment on the ability of earthworms (Lumbricidae) to reduce the transmission of infective larvae of *Cooperia oncophora* (Trichostrongylidae) from cow pats to grass. *Journal of Parasitology* **73**, 1133-1137.

Grønvold, J. & Høgh-Schmidt, K. (1989). Factors influencing rain splash dispersal of infective larvae of *Ostertagia ostertagi* (Trichostrongylidae) from cow pats to the surroundings. *Veterinary Parasitology* **31**, 57-70.

Gruner, L., Bouix, J., Cabaret, J., Boulard, C., Cortet, J., Sauve, C., Molenat, G. & Calamel, M. (1992). Effect of genetic type, lactation and management on helminth infection of ewes in an intensive grazing system on irrigated pasture. *International Journal for Parasitology.* **22**, 919-925.

Gruner, L., Cabaret, J., Sauve, C. & Pailhories, R. (1986). Comparative susceptibility of Romanov and Lacaune sheep to gastrointestinal nematodes and small lungworms. *Veterinary Parasitology* **19**, 85-93.

Gruner, L., Mandonnet, N., Bouix, J., Vu Tien Khang, T., Cabaret, J., Hoste, H., Kerboeuf, D. & Barnouin, J. (1994). Worm population characteristics and pathological changes in lambs after a single or trickle infection with *Teladorsagia circumcincta*. *International Journal for Parasitology* **24**, 347-356.

Grzych, J. M., Grezel, D., Xu, C. B., Neyrinck, J. L., Capron, M., Ouma, J. H., Butterworth, A. E. & Capron, A. (1993). IgA antibodies to a protective antigen in human *Schistosomiasis mansoni*. *Journal of Immunology* **150**, 527-535.

Gulland, F. M. (1992). The role of nematode parasites in Soay sheep (*Ovis aries L.*) mortality during a population crash. *Parasitology* **105**, 493-503.

Gulland, F. M. & Fox, M. (1992). Epidemiology of nematode infections of Soay sheep (*Ovis aries L.*) on St Kilda. *Parasitology* **105**, 481-492.

Haley, A. J. (1961). Biology of the rat nematode, *Nippostrongylus brasiliensis* (Travassos, 1914). II. Preparasitic stages and development in the laboratory rat. *The Journal of Parasitology* **48**, 13-23.

Harris, D. P., Haynes, L., Sayles, P. C., Duso, D. K., Eaton, S. M., Lepak, N. M., Johnson, L. L., Swain, S. L. & Lund, F. E. (2000). Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nature Immunology* **1**, 475-482.

Haukisalmi, V., Henttonen, H. & Vikman, P. (1996). Variability of sex ratio, mating probability and egg production in an intestinal nematode in its fluctuating host population. *International Journal for Parasitology* **26**, 755-764.

Heath, M. F. & Connan, R. M. (1991). Interaction of *Ostertagia* and *Nematodirus* species in sheep and the potential of serum fructosamine determination in monitoring gastrointestinal parasitism. *Research in Veterinary Science* **51**, 322-326.

Hein, W. R. (1998). Immunoglobulins. In: Handbook of vertebrate immunology. Pastoret, P. P., Griebel, P., Bazin, H. & Govaerts, A. (eds). Academic Press. London, UK.

Hein, W. R. & Peterhans, E. (1998). T-cell receptors. In: Handbook of vertebrate immunology. Pastoret, P. P., Griebel, P., Bazin, H. & Govaerts, A. (eds). Academic Press. London, UK, pp. 500-502.

Helmy, H., Weil, G. J., Faris, R., Gad, A. M., Chandrashekar, R., Ashour, A. & Ramzy, R. M. (2000). Human antibody responses to *Wuchereria bancrofti* infective larvae. *Parasite Immunology* **22**, 89-96.

Henzel, W. J., Billeci, T. M., Stults, J. T., Wong, S. C., Grimley, C. & Watanabe, C. (1993). Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. *Proceedings of the National Academy of Sciences of the USA* **90**, 5011-5015.

Heringstad, B., Klemetsdal, G. & Ruane, J. (2000). Selection for mastitis resistance in dairy cattle: a review with focus on the situation in the Nordic countries. *Livestock Production Science* **64**, 95-106.

Herlich, H. (1956). A digestion method for post-mortem recovery of nematodes from ruminants. *Proceedings of the Helminthological Society of Washington* **23**, 102-103.

Hernandez, M. G., Hafalla, J. C., Acosta, L. P., Aligui, F. F., Aligui, G. D., Ramirez, B. L., Dunne, D. W. & Santiago, M. L. (1999). Paramyosin is a major target of the human IgA response against *Schistosoma japonicum*. *Parasite Immunology* **21**, 641-647.

Hertzberg, H., Schallig, H. D. & Deplazes, P. (1999). Development of a protective immunity against *Ostertagia leptospicularis* in trickle-infected sheep and parallel changes of serum gastrin, pepsinogen and antibody levels. *Veterinary Journal* **157**, 148-159.

Hilderson, H., De Graaf, D. C., Vercruysse, J. & Berghen, P. (1993). Characterisation of *Ostertagia ostertagi* antigens by the different bovine immunoglobulin isotypes. *Research in Veterinary Science* **55**, 203-208.

Hill, A. V. S., Allsopp, C. E. M., Kwiatowski, D., Anstey, N. M., Twumasi, P., Rowe, P. A., Bennett, S., Brewster, D., McMichael, A. J. & Greenwood, B. M. (1991). Common West African HLA antigens are associated with protection from severe malaria. *Nature* **352** , 595-600.

Hoefer Scientific Instruments (1994). Protein electrophoesis. Applications guide. Hoefer Scientific Instruments San Fransico, USA.

Hohenhaus, M. A. & Outteridge, P. M. (1995). The immunogenetics of resistance to *Trichostrongylus colubriformis* and *Haemonchus contortus* parasites in sheep. *British Veterinary Journal* **151**, 119-140.

Höhler, T., Gerken, G., Notghi, A., Knolle, P., Lubjuhn, R., Taheri, H., Schneider, P. M., Meyer zum Büschenfelde, K. H. & Rittner, C. (1997). MHC class II genes influence the susceptibility to chronic active hepatitis C. *Journal of Hepatology* **27**, 259-264.

Holmes, P. H. (1987). Pathophysiology of nematode infections. *International Journal for Parasitology* **17**, 443-451.

Holter, P. (1979). Effect of dung-beetles (*Aphodius* spp.) and earthworms on the disappearance of cattle dung. *Oikos* **32**, 393-402.

Hong, C., Hunt, K. R. & Coles, G. C. (1996). Occurrence of anthelmintic resistant nematodes on sheep farms in England and goat farms in England and Wales. *The Veterinary Record* **139**, 83-86.

Horbury, S. R., Mercer, J. G. & Chappell, L. H. (1995). Anorexia induced by the parasitic nematode, *Nippostrongylus brasiliensis*: effects on NPY and CRF gene expression in the rat hypothalamus. *Journal of Neuroendocrinology* **7**, 867-873.

Houdijk, J. G., Kyriazakis, I., Jackson, F., Huntley, J. F. & Coop, R. L. (2000). Can an increased intake of metabolizable protein affect the periparturient relaxation in immunity against *Teladorsagia circumcincta* in sheep? *Veterinary Parasitology* **91**, 43-62.

Huntley, J. F., Gibson, S., Brown, D., Smith, W. D., Jackson, F. & Miller, H. R. (1987). Systemic release of a mast cell proteinase following nematode infections in sheep. *Parasite Immunology* **9**, 603-614.

Huntley, J. F., Newlands, G. & Miller, H. R. (1984). The isolation and characterization of globule leukocytes: their derivation from mucosal mast cells in parasitized sheep. *Parasite Immunology* **6**, 371-390.

Huntley, J. F., Redmond, J., Welfare, W., Brennan, G., Jackson, F., Kooyman, F. & Vervelde, L. (2001). Studies on the immunoglobulin E responses to *Teladorsagia circumcincta* in sheep: purification of a major high molecular weight allergen. *Parasite Immunology* **23**, 227-235.

Huntley, J. F., Schallig, H. D., Kooyman, F. N., MacKellar, A., Jackson, F. & Smith, W. D. (1998a). IgE antibody during infection with the ovine abomasal nematode, *Teladorsagia circumcincta*: primary and secondary responses in serum and gastric lymph of sheep. *Parasite Immunology* **20**, 565-571.

Huntley, J. F., Schallig, H. D., Kooyman, F. N., MacKellar, A., Millership, J. & Smith, W. D. (1998b). IgE responses in the serum and gastric lymph of sheep infected with *Teladorsagia circumcincta*. *Parasite Immunology* **20**, 163-168.

Hutchings, M. R., Kyriazakis, I., Anderson, D. H., Gordon, I. J. & Coop, R. L. (1998). Behavioural strategies used by parasitized and non-parasitized sheep to avoid ingestion of gastro-intestinal nematodes associated with faeces. *Animal Science* **67**, 97-106.

Hutchings, M. R., Kyriazakis, I., Gordon, I. J. & Jackson, F. (1999). Trade-offs between nutrient intake and faecal avoidance in herbivore foraging decisions: the effect of animal parasitic status, level of feeding motivation and sward nitrogen content. *Journal of Animal Ecology* **68**, 310-323.

Hutchings, M. R., Kyriazakis, I., Papachristou, T. G., Gordon, I. J. & Jackson, F. (2000). The herbivores' dilemma: trade-offs between nutrition and parasitism in foraging decisions. *Oecologia* **124**, 242-251.

Israf, D. A., Jackson, F., Stevenson, L. M., Jones, D. G., Jackson, E., Huntley, J. F. & Coop, R. L. (1997). Persistence of immunity to *Nematodirus battus* infection in lambs. *Veterinary Parasitology* **71**, 39-52.

Jackson, F. & Coop, R. L. (2000). The development of anthelmintic resistance in sheep nematodes. *Parasitology* **120**, S95-S107.

Jackson, F., Jackson, E. & Coop, R. L. (1992). Evidence of multiple anthelmintic resistance in a strain of *Teladorsagia circumcincta* (*Ostertagia circumcincta*) isolated from goats in Scotland. *Research in Veterinary Science* **53**, 371-374.

Janeway, C. A. Jr. & Travers, P. (1994). Immunobiology. The immune system in health and disease. Garland Publishing Inc. London, UK.

Jarvis, L. M. & Pritchard, D. I. (1992). An evaluation of the role of carbohydrate epitopes in immunity to *Trichinella spiralis*. *Parasite Immunology* **14**, 489-501.

Jeffcoate, I. A., Fishwick, G., Bairden, K., Armour, J. & Holmes, P. H. (1990). Pathophysiology of the periparturient egg rise in sheep: the role of prolactin. *Research in Veterinary Science* **48**, 295-300.

Jones, D. G. (1993). The eosinophil. *Journal of Comparative Pathology* **108**, 317-335.

Jones, D. T. (2001). Critically assessing the state-of-the-art in protein structure prediction. *Pharmacogenomics Journal* **1**, 126-134.

Jones, W. O., Emery, D. L., McClure, S. J. & Wagland, B. M. (1994). Changes in inflammatory mediators and larval inhibitory activity in intestinal contents and mucus during primary and challenge infections of sheep with *Trichostrongylus colubriformis*. *International Journal for Parasitology* **24**, 519-525.

Jones, W. O. & Symons, L. E. (1982). Protein synthesis in the whole body, liver, skeletal muscle and kidney cortex of lambs infected by the nematode *Trichostrongylus colubriformis*. *International Journal for Parasitology* **12**, 295-301.

Kamiya, M., Oku, Y., Fukumoto, S. & Ooi, H. K. (1983). Preliminary observation on the absence of globule leucocytes in mast cell-deficient W/Wv anemic mice after *Trichinella spiralis* infection. *Japanese Journal of Veterinary Research* **31**, 133-140.

Kao, R. R., Leathwick, D. M., Roberts, M. G. & Sutherland, I. A. (2000). Nematode parasites of sheep: a survey of epidemiological parameters and their application in a simple model. *Parasitology* **121**, 85-103.

Kassai, T., Fesus, L., Hendrikx, W. M., Takats, C., Fok, E., Redl, P., Takacs, E., Nilsson, P. R., Van Leeuwen, M. A., Jansen, J. & . (1990). Is there a relationship between haemoglobin genotype and the innate resistance to experimental *Haemonchus contortus* infection in Merino lambs? *Veterinary Parasitology* **37**, 61-77.

Kates, K. C. (1950). Survival on pasture of free-living stages of some common gastro-intestinal nematodes of sheep. *Proceedings of the Helminthological Society of Washington* **17**, 39-58.

Katunguka-Rwakishaya, E., Murray, M. & Holmes, P. H. (1992). The pathophysiology of ovine trypanosomosis: haematological and blood biochemical changes. *Veterinary Parasitology* **45**, 17-32.

Kennedy, M. W. (1990). Resistance to parasitic nematodes-how is the MHC involved? *Parasitology Today* **6**, 374-375.

Kimambo, A. E., Macrae, J. C. & Dewey, P. J. (1988). The effect of daily challenge with *Trichostrongylus colubriformis* larvae on the nutrition and performance of immunologically-resistant sheep. *Veterinary Parasitology* **28**, 205-212.

Knox, D. P. (2000). Development of vaccines against gastrointestinal nematodes. *Parasitology* **120**, S43-S61.

Knox, D. P. & Smith, W. D. (2001). Vaccination against gastrointestinal nematode parasites of ruminants using gut-expressed antigens. *Veterinary Parasitology* **100**, 21-32.

Knox, M. & Steel, J. (1996). Nutritional enhancement of parasite control in small ruminant production systems in developing countries of south-east Asia and the Pacific. *International Journal for Parasitology* **26**, 963-970.

Knox, M. R. & Faedo, M. (2001). Biological control of field infections of nematode parasites of young sheep with *Duddingtonia flagrans* and effects of spore intake on efficacy. *Veterinary Parasitology* **101**, 155-160.

Koski, K. G. & Scott, M. E. (2001). Gastrointestinal nematodes, nutrition and immunity: breaking the negative spiral. *Annual Review of Nutrition* **21**, 297-321.

Koski, K. G., Su, Z. & Scott, M. E. (1999). Energy deficits suppress both systemic and gut immunity during infection. *Biochemical and Biophysical Research Communications* **264**, 796-801.

Krecek, R. C. & Murrell, K. D. (1988). Observations on the ability of larval *Ostertagia ostertagi* to migrate through pasture soil. *Proceedings of the Helminthological Society of Washington* **55**, 24-27.

Krecek, R. C., Murrell, K. D. & Douglass, L. W. (1990). Effects of microclimatic variables on the availability and movement of third-stage larvae of *Ostertagia ostertagi* on herbage. *Onderstepoort Journal of Veterinary Research* **57**, 133-135.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lamkhioued, B., Gounni, A. S., Gruart, V., Pierce, A., Capron, A. & Capron, M. (1995). Human eosinophils express a receptor for secretory component. Role in secretory IgA-dependent activation. *European Journal of Immunology* **25**, 117-125.

Larsen, M. (2000). Prospects for controlling animal parasitic nematodes by predacious micro fungi. *Parasitology* **120**, S121-S131.

Lascelles, A. K., Beh, K. J., Kerlin, R. L., Watson, D. L. & Mukkur, T. K. (1985). The generation of antibody responses of diferent isotype specificity in relation to mucosal defence in ruminants. In: Immunology of the sheep. Morris, B. & Miyasaka, M. (eds). Hoffman-La-Roche and Company. Basle, Switzerland, pp. 410-435.

Laubach, H. E. (1989). Effect of dietary magnesium on *Ascaris suum* infections of mice. *Biochemical Medicine and Metabolic Biology* **42**, 95-104.

Leignel, V. & Cabaret, J. (2001). Are *Teladorsagia circumcincta* (Nematoda) morphs equally able to survive under anthelmintic treatment in sheep on pastures? *Parasitology Research* **87**, 687-692.

Levine, N. D. (1980). Nematode parasites of domestic animals and man. Burgess Publishing Corp. Minneapolis, USA.

Levine, N. D. & Andersen, F. L. (1973). Development and survival of *Trichostrongylus colubriformis* on pasture. *Journal of Parasitology* **59**, 147-165.

Lunn, P. G. & Northrop-Clewes, C. A. (1993). The impact of gastrointestinal parasites on protein-energy malnutrition in man. *Proceedings of the Nutrition Society* **52**, 101-111.

Madden, K. B., Urban, J. F., Jr., Ziltener, H. J., Schrader, J. W., Finkelman, F. D. & Katona, I. M. (1991). Antibodies to IL-3 and IL-4 suppress helminth-induced intestinal mastocytosis. *Journal of Immunology* **147**, 1387-1391.

Maizels, R. M., Blaxter, M. L., Robertson, B. D. & Selkirk, M. E. (1991). Parasite antigens, parasite genes. A laboratory manual for molecular parasitology. Cambridge University Press. Cambridge, UK.

Maizels, R. M., Kennedy, M. W., Meghji, M., Robertson, B. D. & Smith, H. V. (1987). Shared carbohydrate epitopes on distinct surface and secreted antigens of the parasitic nematode *Toxocara canis*. *Journal of Immunology* **139**, 207-214.

Mallard, B. A., Wilkie, B. N., Kennedy, B. W., Gibson, J. P. & Quinton, M. (1998). Immune responsiveness in swine: Eight generations of selection for high and low immune response in Yorkshire pigs. *Proceedings of the 6th World Congress on Genetics Applied to Livestock Production* **27**, 257-264.

Mansfield, L. S. & Schad, G. A. (1992). *Strongyloides stercoralis* infection in IgA-deficient dogs. *American Journal of Tropical Medicine and Hygiene* **47**, 830-836.

Manton, V. J., Peacock, R., Poynter, D., Silverman, P. H. & Terry, R. J. (1962). The influence of age on naturally acquired resistance to *Haemochus contortus* in lambs. *Research in Veterinary Science* **3**, 308-314.

Matthaei, K. I., Foster, P. & Young, I. G. (1997). The role of interleukin-5 (IL-5) *in vivo*: studies with IL-5 deficient mice. *Memórias do Instituto Oswaldo Cruz* **92 Suppl 2**, 63-68.

McClure, S. J., Emery, D. L., Wagland, B. M. & Jones, W. O. (1992). A serial study of rejection of *Trichostrongylus colubriformis* by immune sheep. *International Journal for Parasitology* **22**, 227-234.

McCririe, L., Bairden, K., Britton, C., Buitkamp, J., McKeand, J. B. & Stear, M. J. (1997). Heterogeneity in the recognition of *Ostertagia circumcincta* antigens by serum antibody from mature, infected sheep. *Parasite Immunology* **19**, 235-242.

McDonald, J. W., Overend, D. J. & Paynter, D. I. (1989). Influence of selenium status in Merino weaners on resistance to trichostrongylid infection. *Research in Veterinary Science* **47**, 319-322.

McEwan, J. C., Dodds, K. G., Greer, G. J., Bain, W. E., Duncan, S. J., Wheeler, R., Knowler, K. J., Reid, P. J., Green, R. S. & Douch, P. G. (1995). Genetic estimates for parasite resistance traits in sheep and their correlations with production traits. *New Zealand Journal of Zoology* **22**, 177-177.

McGillivery, D. J., Yong, W. K., Adler, B. & Riffkin, G. G. (1992). A purified stage-specific 31 kDa antigen as a potential protective antigen against *Ostertagia circumcincta* infection in lambs. *Vaccine* **10**, 607-613.

McGillivery, D. J., Yong, W. K., Riffkin, G. G. & Adler, B. (1989). Extraction and identification of a 31,000 mol-wt glycoprotein antigen of *Ostertagia circumcincta* by sera from resistant sheep. *International Journal for Parasitology* **19**, 473-478.

McGillivery, D. J., Yong, W. K., Riffkin, G. G. & Adler, B. (1990). The distribution and localization of the stage-specific gp31 antigen from infective *Ostertagia circumcincta* larvae. *International Journal for Parasitology* **20**, 87-93.

McKellar, Q. A. (1993). Interactions of *Ostertagia* species with their bovine and ovine hosts. *International Journal for Parasitology* **23**, 451-462.

McManus, D. P., Ross, A. G., Williams, G. M., Sleigh, A. C., Wiest, P., Erlich, H., Trachtenberg, E., Guanling, W., McGarvey, S. T., Li, Y. S. & Waine, G. J. (2001a). HLA class II antigens positively and negatively associated with hepatosplenic schistosomiasis in a Chinese population. *International Journal for Parasitology* **31**, 674-680.

McManus, D. P., Wong, J. Y., Zhou, J., Cai, C., Zeng, Q., Smyth, D., Li, Y., Kalinna, B. H., Duke, M. J. & Yi, X. (2001b). Recombinant paramyosin (rec-Sj-97) tested for immunogenicity and vaccine efficacy against *Schistosoma japonicum* in mice and water buffaloes. *Vaccine* **20**, 870-878.

Meeusen, E. N. & Balic, A. (2000). Do eosinophils have a role in the killing of helminth parasites? *Parasitology Today* **16**, 95-101.

Mestecky, J., Moro, I. & Underdown, B. J. (1999). Mucosal immunoglobulins. In: Mucosal immunology. Ogra, P. L., Mestecky, J., Lamm, M. E., Strober, W., Bienenstock, J. & McGhee, J. R. (eds). Academic Press. San Diego, USA. pp 133-152.

Miller, H. R. (1984). The protective mucosal response against gastrointestinal nematodes in ruminants and laboratory animals. *Veterinary Immunology and Immunopathology* **6**, 167-259.

Mock, B. A. & Nacy, C. A. (1988). Hormonal modulation of sex differences in resistance to *Leishmania major* systemic infections. *Infection and Immunity* **56**, 3316-3319.

Morton, R. E., Yong, W. K., Riffkin, G. G., Bozas, S. E., Spithill, T. W., Adler, B. & Parsons, J. C. (1995). Inability to reproduce protection against *Teladorsagia circumcincta* in sheep with a purified stage-specific 31 kDa antigen complex. *Vaccine* **13**, 1482.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of Immunology* **136**, 2348-2357.

Murray, J. & Smith, W. D. (1994). Ingestion of host immunoglobulin by three non-blood-feeding nematode parasites of ruminants. *Research in Veterinary Science* **57**, 387-389.

Murray, M., Miller, H. R. & Jarrett, W. F. (1968). The globule leukocyte and its derivation from the subepithelial mast cell. *Laboratory Investigation* **19**, 222-234.

Murray, M., Stear, M. J., Trail, J. C., d'Ieteren, G. D., Agyemang, K. & Dwinger, R. H. (1991). Trypanosomiasis in cattle: prospects for control. In: Breeding for disease resistance in farm animals. Owen, J. B. & Axford, R. F. (eds). CAB International. Oxon, UK, pp. 203-223.

Newlands, G. F., Skuce, P. J., Knox, D. P., Smith, S. K. & Smith, W. D. (1999). Cloning and characterization of a β-galactoside-binding protein (galectin) from the gut of the gastrointestinal nematode parasite *Haemonchus contortus*. *Parasitology* **119**, 483-490.

Newton, S. E., Monti, J. R., Greenhalgh, C. J., Ashman, K. & Meeusen, E. N. (1997). cDNA cloning of galectins from third stage larvae of the parasitic nematode *Teladorsagia circumcincta*. *Molecular and Biochemical Parasitology* **86**, 143-153.

Niezen, J. H., Charleston, W. A., Hodgson, J., Miller, C. M., Waghorn, T. S. & Robertson, H. A. (1998). Effect of plant species on the larvae of gastrointestinal nematodes which parasitise sheep. *International Journal for Parasitology* **28**, 791-803.

Niezen, J. H., Charleston, W. A., Robertson, H. A., Shelton, D., Waghorn, G. C. & Green, R. (2002a). The effect of feeding sulla (*Hedysarum coronarium*) or lucerne (*Medicago sativa*) on lamb parasite burdens and development of immunity to gastrointestinal nematodes. *Veterinary Parasitology* **105**, 229-245.

Niezen, J. H., Waghorn, T. S., Charleston, W. A. & Waghorn, G. C. (1995). Growth and gastrointestinal nematode parasitism in lambs grazing either lucerne (*Medicago sativa*) or sulla (*Hedysarum coronarium*) which contains condensed tannins. *Journal of Agricultural Science (Cambridge)* **125**, 289.

Niezen, J. H., Waghorn, G. C., Graham, T., Carter, J. L. & Leathwick, D. M. (2002b). The effect of diet fed to lambs on subsequent development of *Trichostrongylus colubriformis* larvae *in vitro* and on pasture. *Veterinary Parasitology* **105**, 269-283.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry* **250**, 4007-4021.

O'Sullivan, B. M. & Donald, A. D. (1970). A field study of nematode parasite populations in the lactating ewe. *Parasitology* **61**, 301-315.

Ortega-Mora, L. M. & Wright, S. E. (1994). Age-related resistance in ovine cryptosporidiosis: patterns of infection and humoral immune response. *Infection and Immunity* **62**, 5003-5009.

Ortiz, P. L., Claxton, J. R., Clarkson, M. J., McGarry, J. & Williams, D. J. (2000). The specificity of antibody responses in cattle naturally exposed to *Fasciola hepatica*. *Veterinary Parasitology* **93**, 121-134.

Outteridge, P. M., Stewart, D. J., Skerman, T. M., Dufty, J. H., Egerton, J. R., Ferrier, G. & Marshall, D. J. (1989). A positive association between resistance to ovine footrot and particular lymphocyte antigen types. *Australian Veterinary Journal* **66**, 175-179.

Ovington, K. S., Bacarese-Hamilton, A. J. & Bloom, S. R. (1985). *Nippostrongylus brasiliensis*: changes in plasma levels of gastrointestinal hormones in the infected rat. *Experimental Parasitology* **60**, 276-284.

Pandey, V. S., Chaer, A. & Dakkak, A. (1993). Effect of temperature and relative humidity on survival of eggs and infective larvae of *Ostertagia circumcincta*. *Veterinary Parasitology* **49**, 219-227.

Paterson, S., Wilson, K. & Pemberton, J. M. (1998). Major histocompatibility complex variation associated with juvenile survival and parasite resistance in a large unmanaged ungulate population (*Ovis aries L.*). *Proceedings of the National Academy of Sciences of the USA* **95**, 3714-3719.

Paynter, D. I. (1992). Pepsinogen activity: Determination in serum. Australian Standard Diagnostic Techniques for Animal Diseases. Standing Committee of Agriculture, CSIRO. Australia.

Peña, M. T., Miller, J. E., Fontenot, M. E., Gillespie, A. & Larsen, M. (2002). Evaluation of *Duddingtonia flagrans* in reducing infective larvae of *Haemonchus contortus* in feces of sheep. *Veterinary Parasitology* **103**, 259-265.

Peña, M. T., Miller, J. E., Wyatt, W. & Kearney, M. T. (2000). Differences in susceptibility to gastrointestinal nematode infection between Angus and Brangus cattle in south Louisiana. *Veterinary Parasitology* **89**, 51-61.

Penn, D. J. (2002). The scent of genetic compatibility: sexual selection and the major histocompatibility complex. *Ethology* **108**, 1-21.

Penn, D. J., Damjanovich, K. & Potts, W. K. (2002). MHC heterozygosity confers a selective advantage against multiple-strain infections. *Proceedings of the National Academy of Sciences of the USA* **99**, 11260-11264.

Penn, D. J. & Potts, W. K. (1999). The evolution of mating preferences and major histocompatibility complex genes. *American Naturalist* **153**, 145-164.

Pernthaner, A., Stankiewicz, M., Bisset, S. A., Jonas, W. E., Cabaj, W. & Pulford, H. D. (1995). The immune responsiveness of Romney sheep selected for resistance or susceptibility to gastrointestinal nematodes: lymphocyte blastogenic activity, eosinophilia and total white blood cell counts. *International Journal for Parasitology* **25**, 523-529.

Pfeffer, A., Douch, P. G., Shaw, R. J., Gatehouse, T. K., Rabel, B., Green, R. S., Shirer, C. L., Jonas, W. E. & Bisset, S. (1996). Sequential cellular and humoral responses in the abomasal mucosa and blood of Romney sheep dosed with *Trichostrongylus axei*. *International Journal for Parasitology* **26**, 765-773.

Phillips-Quagliata, J. M. (2002). Mouse IgA allotypes have major differences in their hinge regions. *Immunogenetics* **53**, 1033-1038.

Pienaar, J. G., Basson, P. A., du Plessis, J. L., Collins, H. M., Naude, T. W., Boyazoglu, P. A., Boomker, J., Reyers, F. & Pienaar, W. L. (1999). Experimental studies with *Stronglyloides papillosus* in goats. *Onderstepoort Journal of Veterinary Research* **66**, 191-235.

Porcelli, S. A., Morita, C. T. & Modlin, R. L. (1996). T-cell recognition of non-peptide antigens. *Current Opinions in Immunology* **8**, 510-516.

Potts, W. K. (2002). Wayne Potts laboratory research summary. *[Online]* Available from: *http://stormy.biology.utah.edu/people/wayne.html* Accessed 4th Sepember 2002.

Potts, W. K., Manning, C.J. & Wakeland, E.K. (1994). The role of infectious disease, inbreeding and mating preferences in maintaining MHC genetic diversity: an experimental test. *Philosophical Transactions of the Royal Society of London : Series B. Biological sciences* **346**, 369-378.

Preston, J. M. & Allonby, E. W. (1979). The influence of haemoglobin phenotype on the susceptibility of sheep to *Haemonchus contortus* infection in Kenya. *Research in Veterinary Science* **26**, 140-144.

Quinnell, R. J., Griffin, J., Nowell, M. A., Raiko, A. & Pritchard, D. I. (2001). Predisposition to hookworm infection in Papua New Guinea. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **95**, 139-142.

Rainbird, M. A., MacMillan, D. & Meeusen, E. N. (1998). Eosinophil-mediated killing of *Haemonchus contortus* larvae: effect of eosinophil activation and role of antibody, complement and interleukin-5. *Parasite Immunology* **20**, 93-103.

Rees, G. (1950). Observations on the vertical migrations of the third-stage larva of *Haemonchus contortus* (Rud.) on experimental plots of *Lolium perenne* S24, in relation to meterological and micrometerological factors. *Parasitology* **40**, 127-143.

Remoué, F., Mani, J. C., Pugniere, M., Schacht, A. M., Capron, A. & Riveau, G. (2002). Functional specific binding of testosterone to *Schistosoma haematobium* 28-kilodalton glutathione S-transferase. *Infection and Immunity* **70**, 601-605.

Ren, P., Lim, C. S., Johnsen, R., Albert, P. S., Pilgrim, D. & Riddle, D. L. (1996). Control of *C. elegans* larval development by neuronal expression of a TGF-beta homolog. *Science* **274**, 1389-1391.

Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J. L., Drumm. M. L., Iannuzzi, M. C., Collins, F. S. & Tsui, L. (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**, 1066-1073.

Roitt, I., Brostoff, J. & Male, D. (2001). Immunology. Mosby. London, UK.

Romjali, E., Dorny, P., Batubara, A., Pandey, V. S. & Gatenby, R. M. (1997). Peri-parturient rise in faecal strongyle egg counts of different genotypes of sheep in North Sumatra, Indonesia. *Veterinary Parasitology* **68**, 191-196.

Rose, J. H. (1961). Some observations on the free-living stages of *Ostertagia ostertagi*, astomach worm of cattle. *Parasitology* **51**, 295-307.

Rothwell, T. L. W. (1989). Immune expulsion of parasitic nematodes from the alimentary tract. *International Journal for Parasitology* **19**, 139-168.

Rulicke, T., Chapuisat, M., Homberger, F. R., Macas, E. & Wedekind, C. (1998). MHC-genotype of progeny influenced by parental infection. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **265**, 711-716.

Sabatinelli, G., Romi, R., Ralamboranto, L. & Majori, G. (1996). Age-related prevalence of *Plasmodium falciparum* circumsporozoite antibody in an hyperendemic area of Madagascar, and its relationship with parasite prevalence. *American Journal of Tropical Medicine and Hygiene* **55**, 278-281.

Salman, S. K. & Duncan, J. L. (1984). The abomasal histology of worm-free sheep given primary and challenge infections of *Haemonchus contortus*. *Veterinary Parasitology* **16**, 43-54.

Šarkunas, M., Larsen, M., Nansen, P. & Hansen, J. W. (2000). Biological control of trichostrongylid infections in calves on pasture in Lithuania using *Duddingtonia flagrans*, a nematode-trapping fungus. *Journal of Helminthology* **74**, 355-359.

Sauermann, U., Stahl-Hennig, C., Stolte, N., Muhl, T., Krawczak, M., Spring, M., Fuchs, D., Kaup, F. J., Hunsmann, G. & Sopper, S. (2000). Homozygosity for a conserved Mhc class II DQ-DRB haplotype is associated with rapid disease progression in simian immunodeficiency virus-infected macaques: results from a prospective study. *Journal of Infectious Disease* **182**, 716-724.

Schallig, H. D. (2000). Immunological responses of sheep to *Haemonchus contortus*. *Parasitology* **120**, S63-S72.

Schallig, H. D. & van Leeuwen, M. A. (1996). Carbohydrate epitopes on *Haemonchus contortus* antigens. *Parasitology Research* **82**, 38-42.

Schallig, H. D., van Leeuwen, M. A. & Cornelissen, A. W. (1997). Protective immunity induced by vaccination with two *Haemonchus contortus* excretory secretory proteins in sheep. *Parasite Immunology* **19**, 447-453.

Schallig, H. D., van Leeuwen, M. A. & Hendrikx, W. M. (1995). Isotype-specific serum antibody-responses of sheep to *Haemonchus contortus* antigens. *Veterinary Parasitology* **56**, 149-162.

Schleiermacher, D., Rogier, C., Spiegel, A., Tall, A., Trape, J. F. & Mercereau-Puijalon, O. (2001). Increased multiplicity of *Plasmodium falciparum* infections and skewed distribution of individual *msp1* and *msp2* alleles during pregnancy in Ndiop, a Senegalese village with seasonal, mesoendemic malaria. *American Journal of Tropical Medicine and Hygiene* **64**, 303-309.

Schneider, S. L., Gollnick, S. O., Grande, C., Pazik, J. E. & Tomasi, T. B. (1996). Differential regulation of TGF-β2 by hormones in rat uterus and mammary gland. *Journal of Reproductive Immunology* **32**, 125-144.

Schwaiger, F. W., Gostomski, D., Stear, M. J., Duncan, J. L., McKellar, Q. A., Epplen, J. T. & Buitkamp, J. (1995). An ovine *major histocompatibility complex DRB1* allele is associated with low faecal egg counts following natural, predominantly *Ostertagia circumcincta* infection. *International Journal for Parasitology* **25**, 815-822.

Scott, I. & McKellar, Q. A. (1998). The effects of excretions/secretions of *Ostertagia circumcincta* on ovine abomasal tissues *in vitro*. *International Journal for Parasitology* **28**, 451-460.

Seaton, D. S., Jackson, F., Smith, W. D. & Angus, K. W. (1989). Development of immunity to incoming radiolabelled larvae in lambs continuously infected with *Ostertagia circumcincta*. *Research in Veterinary Science* **46**, 241-246.

Seigler, D. S. (1979). Toxic seed lipids. In: Herbivores: Their interaction with secondary plant metabolites. Rosenthal, G. A. & Janzen, D. H. (eds). Academic Press. New York, USA. pp. 449-470.

Sharp, P. J., Smith, D. R., Bach, W., Wagland, B. M. & Cobon, G. S. (1991). Purified glutathione S-transferases from parasites as candidate protective antigens. *International Journal for Parasitology* **21**, 839-846.

Shaw, R. J., Gatehouse, T. K. & McNeill, M. M. (1998). Serum IgE responses during primary and challenge infections of sheep with *Trichostrongylus colubriformis*. *International Journal for Parasitology* **28**, 293-302.

Silangwa, S. M. & Todd, A. C. (1964). Vertical migration of trichostrongylid larvae on grassses. *Journal of Parasitology* **56**, 278-285.

Silvestre, A. & Humbert, J. F. (2000). A molecular tool for species identification and benzimidazole resistance diagnosis in larval communities of small ruminant parasites. *Experimental Parasitology* **95**, 271-276.

Simpson, H. V., Simpson, B. H., Simcock, D. C., Reynolds, G. W. & Pomroy, W. E. (1999). Abomasal secretion in sheep receiving adult *Ostertagia circumcincta* that are prevented from contact with the mucosa. *New Zealand Veterinary Journal* **47**, 20-24.

Sinski, E., Bairden, K., Duncan, J. L., Eisler, M. C., Holmes, P. H., McKellar, Q. A., Murray, M. & Stear, M. J. (1995). Local and plasma antibody responses to the parasitic larval stages of the abomasal nematode *Ostertagia circumcincta*. *Veterinary Parasitology* **59**, 107-118.

Sitte, K., Brinkworth, R., East, I. J. & Jazwinska, E. C. (2002). A single amino acid deletion in the antigen binding site of *BoLA-DRB3* is predicted to affect peptide binding. *Veterinary Immunology and Immunopathology* **85**, 129-135.

Skinner, W. D. & Todd, K. S., Jr. (1980). Lateral migration of *Haemonchus contortus* larvae on pasture. *American Journal of Veterinary Research* **41**, 395-398.

Smith, J. L. (1999). Foodborne infections during pregnancy. *Journal of Food Protection* **62**, 818-829.

Smith, W. D. (1999). Prospects for vaccines of helminth parasites of grazing ruminants. *International Journal for Parasitology* **29**, 17-24.

Smith, W. D., Dawson, A. M., Wells, P. W. & Burrells, C. (1975). Immunoglobulin concentrations in ovine body fluids. *Res. Vet.Sci.* **19**, 189-194.

Smith, W. D., Jackson, F., Jackson, E. & Williams, J. (1983). Local immunity and *Ostertagia circumcincta*: changes in the gastric lymph of immune sheep after a challenge infection. *Journal of Comparative Pathology* **93**, 479-488.

Smith, W. D., Jackson, F., Jackson, E. & Williams, J. (1985a). Age immunity to *Ostertagia circumcincta*: comparison of the local immune responses of 4 1/2- and 10-month-old lambs. *Journal of Comparative Pathology* **95**, 235-245.

Smith, W. D., Jackson, F., Jackson, E. & Williams, J. (1985b). Ovine ostertagiasis: a natural host parasite interaction for studying protective local immune responses in the gut. In: Immunology of the sheep. Morris, B. & Miyasaka, M. (eds). Hoffman-La-Roche and Company. Basle, Switzerland, pp. 483-498.

Smith, W. D., Jackson, F., Jackson, E., Williams, J. & Miller, H. R. (1984). Manifestations of resistance to ovine ostertagiasis associated with immunological responses in the gastric lymph. *Journal of Comparative Pathology* **94**, 591-601.

Smith, W. D., Pettit, D. & Smith, S. K. (2001a). Cross-protection studies with gut membrane glycoprotein antigens from *Haemonchus contortus* and *Teladorsagia circumcincta*. *Parasite Immunology* **23**, 203-211.

Smith, W. D., van Wyk, J. A. & van Strijp, M. F. (2001b). Preliminary observations on the potential of gut membrane proteins of *Haemonchus contortus* as candidate vaccine antigens in sheep on naturally infected pasture. *Veterinary Parasitology* **98**, 285-297.

Smyth, J. D. (1994). Introduction to animal parasitology. Cambridge University Press. Cambridge, UK.

Sokal, R. R. & Rohlf, F. J. (1995). Biometry: the principles and practice of statistics in biological research. W.H. Freeman and Company. New York, USA.

Stankiewicz, M., Hadas, E., Shaw, R. & Green, R. (2000). Immunisation of lambs with drug-abbreviated *Haemonchus contortus* infections: protection against homologous and heterologous challenge. *Parasitology Research* **86**, 758-761.

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., Strain, S. & Murray, M. (1997). The genetic basis of resistance to *Ostertagia circumcincta* in lambs. *The Veterinary Journal* **154**, 111-119.

Stear, M. J., Bairden, K., Bishop, S. C., Buitkamp, J., Epplen, J. T., Gostomski, D., McKellar, Q. A., Schwaiger, F. W. & Wallace, D. S. (1996a). An ovine lymphocyte antigen is associated with reduced faecal egg counts in four-month-old lambs following natural, predominantly *Ostertagia circumcincta* infection. *International Journal for Parasitology* **26**, 423-428.

Stear, M. J., Bairden, K., Bishop, S. C., Duncan, J. L., Karimi, S. K., McKellar, Q. A. & Murray, M. (1995a). Different patterns of faecal egg output following infection of Scottish Blackface lambs with *Ostertagia circumcincta*. *Veterinary Parasitology* **59**, 29-38.

Stear, M. J., Bairden, K., Bishop, S. C., Gettinby, G., McKellar, Q. A., Park, M., Strain, S. & Wallace, D. S. (1998). The processes influencing the distribution of parasitic nematodes among naturally infected lambs. *Parasitology* **117**, 165-171.

Stear, M. J., Bairden, K., Duncan, J. L., Eckersall, P. D., Fishwick, G., Graham, P. A., Holmes, P. H., McKellar, Q. A., Mitchell, S., Murray, M., Parkins, J. J. & Wallace, D. S. (2000a). The influence of relative resistance and urea-supplementation on deliberate infection with *Teladorsagia circumcincta* during winter. *Veterinary Parasitology* **94**, 45-54.

Stear, M. J., Bairden, K., Duncan, J. L., Gettinby, G., McKellar, Q. A., Murray, M. & Wallace, D. S. (1995b). The distribution of faecal nematode egg counts in Scottish Blackface lambs following natural, predominantly *Ostertagia circumcincta* infection. *Parasitology* **110**, 573-581.

Stear, M. J., Bairden, K., McKellar, Q. A., Scott, I. & Strain, S. (1999a). The relationship between the number and size of nematodes in the abomasum and the concentration of pepsinogen in ovine plasma. *Research in Veterinary Science* **67**, 89-92.

Stear, M. J., Baldock, F. C., Brown, S. C., Gershwin, L. J., Hetzel, D. J. S., Miller, J. E., Nicholas, F. W., Rudder, T. H. & Tierney, T. J. (1990). The genetic control of nematode infections in ruminants. *Fourth world congress on genetics applied to livestock production* **XVI**, 449-452.

Stear, M. J., Bishop, S. C., Doligalska, M., Duncan, J. L., Holmes, P. H., Irvine, J., McCririe, L., McKellar, Q. A., Sinski, E. & Murray, M. (1995c). Regulation of egg production, worm burden, worm length and worm fecundity by host responses in sheep infected with *Ostertagia circumcincta*. *Parasite Immunology* **17**, 643-652.

Stear, M. J., Bishop, S. C., Duncan, J. L., McKellar, Q. A. & Murray, M. (1995d). The repeatability of faecal egg counts, peripheral eosinophil counts, and plasma pepsinogen concentrations during deliberate infections with *Ostertagia circumcincta*. *International Journal for Parasitology* **25**, 375-380.

Stear, M. J., Bishop, S. C., Mallard, B. A. & Raadsma, H. (2001a). The sustainability, feasibility and desirability of breeding livestock for disease resistance. *Research in Veterinary Science* **71**, 1-7.

Stear, M. J., Eckersall, P. D., Graham, P. A., McKellar, Q. A., Mitchell, S. & Bishop, S. C. (2001b). Fructosamine concentration and resistance to natural, predominantly *Teladorsagia circumcincta* infection. *Parasitology* **123**, 211-218.

Stear, M. J., Henderson, N. G., Kerr, A., McKellar, Q. A., Mitchell, S., Seeley, C. & Bishop, S. C. (2002). Eosinophilia as a marker of resistance to *Teladorsagia circumcincta* in Scottish Blackface lambs. *Parasitology* **124**, 553-560.

Stear, M. J., Mitchell, S., Strain, S., Bishop, S. C. & McKellar, Q. A. (2000b). The influence of age on the variation among sheep in susceptibility to natural nematode infection. *Veterinary Parasitology* **89**, 31-36.

Stear, M. J., Park, M. & Bishop, S. C. (1996b). The key components of resistance to *Ostertagia circumcincta* in lambs. *Parasitology Today* **12**, 438-441.

Stear, M. J., Strain, S. & Bishop, S. C. (1999b). How lambs control infection with *Ostertagia circumcincta*. *Veterinary Immunology and Immunopathology* **72** , 213-218.

Steinberg, M. H. & Mitchell, T. E. (1999). Novel approaches to treatment of sickle cell anaemia. *Expert Opinions on Investigational Drugs* **8**, 1823-1836.

Stevenson, L. A., Gasser, R. B. & Chilton, N. B. (1996). The ITS-2 rDNA of *Teladorsagia circumcincta, T. trifurcata* and *T. davtiani* (Nematoda: Trichostrongylidae) indicates that these taxa are one species. *International Journal for Parasitology* **26**, 1123-1126.

Stevenson, L. M., Huntley, J. F., Smith, W. D. & Jones, D. G. (1994). Local eosinophil- and mast cell-related responses in abomasal nematode infections of lambs. *FEMS Immunology and Medical Microbiology* **8**, 167-174.

Strain, S. A., Bishop, S. C., Henderson, N. G., Kerr, A., McKellar, Q. A., Mitchell, S. & Stear, M. J. (2002). The genetic control of IgA activity against *Teladorsagia circumcincta* and its association with parasite resistance in naturally infected sheep. *Parasitology* **124**, 545-552.

Strain, S. A. & Stear, M. J. (1999). The recognition of molecules from fourth-stage larvae of *Ostertagia circumcincta* by IgA from infected sheep. *Parasite Immunology* **21**, 163-168.

Stromberg, B. E. (1997). Environmental factors influencing transmission. *Veterinary Parasitology* **72**, 247-256.

Stromberg, B. E., Schlotthauer, J. C., Haggard, D. L., Vatthauer, R. J., Hanke, H. & Myers, G. H. (1991). Epizootiology of helminth parasitism in a beef cow/calf herd in Minnesota. *American Journal of Veterinary Research* **52**, 1712-1716.

Sutherland, I. A., Brown, A. E., Green, R. S., Miller, C. M. & Leathwick, D. M. (1999). The immune response of sheep to larval challenge with *Ostertagia circumcincta* and *O. ostertagi*. *Veterinary Parasitology* **84**, 125-135.

Suttle, N. F., Knox, D. P., Angus, K. W., Jackson, F. & Coop, R. L. (1992a). Effects of dietary molybdenum on nematode and host during *Haemonchus contortus* infection in lambs. *Research in Veterinary Science* **52**, 230-235.

Suttle, N. F., Knox, D. P., Jackson, F., Coop, R. L. & Angus, K. W. (1992b). Effects of dietary molybdenum on nematode and host during *Trichostrongylus vitrinus* infection in lambs. *Research in Veterinary Science* **52**, 224-229.

Swain, T. (1979). Tannins and lignins. In: Herbivores: Their interaction with secondary plant metabolites. Rosenthal, G. A. & Janzen, D. H. (eds). Academic Press. New York, USA. pp. 657-682.

Sykes, A. R. & Coop, R. L. (1977). Intake and utilization of food by growing sheep with abomasal damage caused by daily dosing with *O. circuminata* larvae. *Journal of Agricultural Science (Cambridge)* **88**, 671-677.

Sykes, A. R., Coop, R. L. & Angus, K. W. (1975). Experimental production of osteoporosis in growing lambs by continuous dosing with *Trichostrongylus colubriformis* larvae. *Journal of Comparative Pathology* **85**, 549-559.

Symons, L. E. & Hennessy, D. R. (1981). Cholecystokinin and anorexia in sheep infected by the intestinal nematode *Trichostrongylus colubriformis*. *International Journal for Parasitology* **11**, 55-58.

Symons, L. E. & Jones, W. O. (1975). Skeletal muscle, liver and wool protein synthesis by sheep infected by the nematode *Trichostrongylus colubriformis*. *Australian Journal of Agricultural Research* **26**, 1063-1072.

Symons, L. E. (1985). Anorexia: occurrence, pathophysiology, and possible causes in parasitic infections. *Advances in Parasitology* **24**, 103-133.

Theis, J. H. & Schwab, R. G. (1992). Seasonal prevalence of *Taenia taeniaeformis*: relationship to age, sex, reproduction and abundance of an intermediate host (*Peromyscus maniculatus*). *Journal of Wildlife Diseases* **28**, 42-50.

Thio, C. L., Carrington, M., Marti, D., O'Brien, S. J., Vlahov, D., Nelson, K. E., Astemborski, J. & Thomas, D. L. (1999). Class II HLA alleles and hepatitis B virus persistence in African Americans. *Journal of Infectious Disease* **179**, 1004-1006.

Thouvenin, M., Candolfi, E., Villard, O., Klein, J. P. & Kien, T. (1997). Immune response in a murine model of congenital toxoplasmosis: increased susceptibility of pregnant mice and transplacental passage of *Toxoplasma gondii* are type 2-dependent. *Parassitologia* **39**, 279-283.

Threlkeld, W. L. (1934). The life history of *Ostertagia circumcincta*. *Virginia Agricultural Experimental Station Technical Bulletin* **52**, 1-24.

Threlkeld, W. L. (1946). The life history of *Ostertagia ostertagi*. *Virginia Agricultural Experimental Station Technical Bulletin* **100**, 3-14.

Tissenbaum, H. A., Hawdon, J., Perregaux, M., Hotez, P., Guarente, L. & Ruvkun, G. (2000). A common muscarinic pathway for diapause recovery in the distantly related nematode species *Caenorhabditis elegans* and *Ancylostoma caninum*. *Proceedings of the National Academy of Sciences of the USA* **97**, 460-465.

Tort, J., Brindley, P. J., Knox, D., Wolfe, K. H. & Dalton, J. P. (1999). Proteinases and associated genes of parasitic helminths. *Advances in Parasitology* **43**, 161-266.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the USA* **76**, 4350-4354.

Urquhart, G. M., Armour, J., Duncan, J. L., Dunn, A. M. & Jennings, F. W. (1996). Veterinary Parasitology. Longman Scientific and Technical Harlow, Essex.

Valcarcel, F. & Garcia, C. C. (1999). Prevalence and seasonal pattern of caprine trichostrongyles in a dry area of central Spain. *Journal of Veterinary Medicine Series B* **46**, 673-681.

Valderrabano, J., Delfa, R. & Uriarte, J. (2002). Effect of level of feed intake on the development of gastrointestinal parasitism in growing lambs. *Veterinary Parasitology* **104**, 327-338.

van Ginkel, F. W., Wahl, S. M., Kearney, J. F., Kweon, M. N., Fujihashi, K., Burrows, P. D., Kiyono, H. & McGhee, J. R. (1999). Partial IgA-deficiency with increased Th2-type cytokines in TGF-β1 knockout mice. *Journal of Immunology* **163**, 1951-1957.

van Houtert, M. F. & Sykes, A. R. (1996). Implications of nutrition for the ability of ruminants to withstand gastrointestinal nematode infections. *International Journal for Parasitology* **26**, 1151-1168.

Vassella, E., Reuner, B., Yutzy, B. & Boshart, M. (1997). Differentiation of African trypanosomes is controlled by a density sensing mechanism which signals cell cycle arrest via the cAMP pathway. *Journal of Cell Science* **110**, 2661-2671.

Vatta, A. F., Krecek, R. C., Letty, B. A., van der Linde, M. J., Grimbeek, R. J., de Villiers, J. F., Motswatswe, P. W., Molebiemang, G. S., Boshoff, H. M. & Hansen, J. W. (2002). Incidence of *Haemonchus* spp. and effect on haematocrit and eye colour in goats farmed under resource-poor conditions in South Africa. *Veterinary Parasitology* **103**, 119-131.

Vercruysse, J. & Claerebout, E. (2001). Treatment vs non-treatment of helminth infections in cattle: defining the threshold. *Veterinary Parasitology* **98**, 195-214.

Vervelde, L., Kooyman, F. N., Van Leeuwen, M. A., Schallig, H. D., MacKellar, A., Huntley, J. F. & Cornelissen, A. W. (2001). Age-related protective immunity after vaccination with *Haemonchus contortus* excretory/secretory proteins. *Parasite Immunology* **23**, 419-426.

Vipond, J. (1998). Stapledon report: The control of worms in sheep. *[Online]* Available from: *http://www.sac.ac.uk/animal/external/publications/stapledon%20rep/default.htm* [Accessed 4th Sepember 2002].

Waghorn, T. S., Leathwick, D. M., Chen, L.-Y., Gray, R. A. & Skipp, R. A. (2002). Influence of nematophagous fungi, earthworms and dung burial on development of the free-living stages of *Ostertagia* (*Teladorsagia*) *circumcincta* in New Zealand. *Veterinary Parasitology* **104**, 119-129.

Walker, W., Roberts, C. W., Ferguson, D. J., Jebbari, H. & Alexander, J. (1997). Innate immunity to *Toxoplasma gondii* is influenced by gender and is associated with differences in interleukin-12 and gamma interferon production. *Infection and Immunity* **65**, 1119-1121.

Waller, P. J. & Faedo, M. (1996). The prospects for biological control of the free-living stages of nematode parasites of livestock. *International Journal for Parasitology* **26**, 915-925.

Watson, D. L., Colditz, I. G., Andrew, M., Gill, H. S. & Altmann, K. G. (1994). Age-dependent immune response in Merino sheep. *Research in Veterinary Science* **57**, 152-158.

Watson, D. L. & Gill, H. S. (1991a). Post natal ontogeny of immunological responsiveness in Merino sheep. *Research in Veterinary Science* **51**, 88-93.

Watson, D. L. & Gill, H. S. (1991b). Effect of weaning on antibody responses and nematode parasitism in Merino lambs. *Research in Veterinary Science* **51**, 128-132.

Wedrychowicz, H., Bairden, K., Dunlop, E. M., Holmes, P. H. & Tait, A. (1995). Immune-response of lambs to vaccination with *Ostertagia circumcincta* surface antigens eliciting bile antibody-responses. *International Journal for Parasitology* **25**, 1111-1121.

Wedrychowicz, H., Bairden, K., Tait, A. & Holmes, P. H. (1992). Immune responses of sheep to surface antigens of infective larvae of *Ostertagia circumcincta*. *Parasite Immunology* **14**, 249-266.

Wedrychowicz, H., Holmes, P. H., Bairden, K. & Tait, A. (1994). Surface and excretory/secretory antigens of 4th-stage larvae and adult *Ostertagia circumcincta*. *Veterinary Parasitology* **53**, 117-132.

Williams, J. C. & Bilkovich, F. R. (1973). Distribution of *Ostertagia ostertagi* infective larvae on pasture herbage. *American Journal of Veterinary Research* **34**, 1337-1344.

Wilson, W. D. & Field, A. C. (1983). Absorption and secretion of calcium and phosphorus in the alimentary tract of lambs infected with daily doses of *Trichostrongylus colubriformis* or *Ostertagia circumcincta* larvae. *Journal of Comparative Pathology* **93**, 61-71.

Windon, R. G. & Dineen, J. K. (1981). The effect of selection of both sire and dam on the response of F1 generation lambs to vaccination with irradiated *Trichostrongylus colubriformis* larvae. *International Journal for Parasitology* **11**, 11-18.

Winter, M. D., Wright, C. & Lee, D. L. (1997). The mast cell and eosinophil response of young lambs to a primary infection with *Nematodirus battus*. *Parasitology* **114**, 189-193.

Woolaston, R. R. & Baker, R. L. (1996). Prospects of breeding small ruminants for resistance to internal parasites. *International Journal for Parasitology* **26**, 845-855.

Woolaston, R. R., Manueli, P., Eady, S. J., Barger, I. A., Le Jambre, L. F., Banks, D. J. D. & Windon, R. G. (1996). The value of circulating eosinophil count as a selection criterion for resistance of sheep to trichostrongyle parasites. *International Journal for Parasitology* **26**, 123-126.

Xu, C. B., Verwaerde, C., Gras-Masse, H., Fontaine, J., Bossus, M., Trottein, F., Wolowczuk, I., Tartar, A. & Capron, A. (1993). *Schistosoma mansoni* 28-kDa glutathione S-transferase and immunity against parasite fecundity and egg viability. *Journal of Immunology* **150**, 940-949.

Xu, C. B., Verwaerde, C., Grzych, J. M., Fontaine, J. & Capron, A. (1991). A monoclonal antibody blocking the *Schistosoma mansoni* 28-kDa glutathione S-transferase activity reduces female worm fecundity and egg viability. *European Journal of Immunology* **21**, 1801-1807.

Xu, J., Ahmad, A., Jones, J. F., Dolcetti, R., Vaccher, E., Prasad, U. & Menezes, J. (2000). Elevated serum transforming growth factor β1 levels in Epstein-Barr virus-associated diseases and their correlation with virus-specific immunoglobulin A (IgA) and IgM. *Journal of Virology* **74**, 2443-2446.

Yong, W. K., Saunders, M. J., Morton, R. E., Hermon, M., McGillivery, D. J., Hooper, I. J., Morgan, P., Riffkin, G. G., Cummins, L. J. & Thompson, R. M. (1991). Immunology of *Ostertagia* resistance. In: Breeding for disease resistance in sheep. Gray, G. D. & Woolaston, R. R. (eds). Australian Wool Corporation. Melbourne, Australia pp. 87-93.

Young, A. J. (1998). Leukocyte migration. In: Handbook of vertebrate immunology. Pastoret, P. P., Griebel, P., Bazin, H. & Govaerts, A. (eds). Academic Press. London, UK, pp. 493-496.

Zarlenga, D. S., Chute, M. B., Gasbarre, L. C. & Boyd, P. C. (2001). A multiplex PCR assay for differentiating economically important gastrointestinal nematodes of cattle. *Veterinary Parasitology* 97, 199-209.

Zhang, S., Li, B. & Weil, G. J. (1999). Human antibody responses to *Brugia malayi* antigens in brugian filariasis. *Internationl Journal for Parasitology* **29**, 429-436.