Validation of genetic and phenotypic markers and the difference between the sexes on nematode infection in Scottish Blackface lambs

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Abstract

Sheep farming plays an important role in the economies in many countries, and is considered an important source of meat, milk, and wool for humans. Livestock are threatened by gastrointestinal parasites, especially nematode infection, which are one of the greatest causes of disease and lost productivity. Nematodes are traditionally controlled by anthelmintic compounds, but with reports of complete multi-drug resistance, other modalities of prevention and treatment are urgently needed. One of the additional methods for controlling nematode infection in domestic sheep is deliberate selection for parasite resistance. Hence, this thesis has investigated, firstly, the general distribution of faecal egg counts among and between Scottish Blackface lamb populations, which provides a better understanding of host-parasite relationship. Secondly, it has identified the genetic markers that have significant association with resistance to nematode infection. This provides more through understanding of the genetic mechanism underlying nematode resistance. These results facilitate the selection of resistant animals. A longitudinal study of the mean and distribution of faecal egg counts was made in Scottish Blackface lambs following natural infection with gastrointestinal nematodes over three years at monthly intervals between August to October. This study has shown that there was no discernible pattern to faecal egg counts within each year and mean faecal egg counts in October were lower than mean faecal egg counts in September in each year. In addition faecal egg counts in male lambs were consistently higher than female lambs at 6-months of age. Mean egg counts vary among different populations and among the same population sampled at different time, and high means are not necessarily due to high intensities of infection but probably due to the contribution of species other than *T. circumcincta*. Older lambs showed significant association between faecal egg counts and polymorphisms in the IFN-γ gene. Two genotypes at this microsatellite appear to be responsible for moderate and high faecal egg output, while the other genotype was associated with low faecal egg counts. Older male lambs showed significant association with the IFN-γ loci, while female lambs did not.

Declaration

This is to declare that the research reported in this thesis is my own original work and has not been submitted for any other degree.

The quantitative trait loci studies in chapter six were carried out in conjunction with Miss G. Davies, Roslin institute, Edinburgh. Any other collaborations or assistance have been stated in the acknowledgement section.

Omry Milad Abuargob

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

Variation among faecal egg counts following natural nematode infection in Scottish Blackface lambs. Stear, M.J; Abuargob, O; Benothman, M; Bishop, S.C; Innocent, G.T; Kerr, A; Mitchell, S. Parasitology (In Press)

Quantitative trait loci associated with parasitic infection in Scottish Blackface sheep. Davies, G; Stear, M.J; Benothman, M; Abuargob, O; Kerr, A; Mitchell, S; Bishop, S.C. Heredity (In Press)

Dedication

To the soul of my father, I wish you were still here…

Milad Abuargob

Acknowledgements

First of all I would like to thank and praise almighty Allah, who gave me the strength and patience to complete this thesis.

All thanks should go to my supervisor Professor Michael Stear for giving me the opportunity to participate in this fascinating research area and for his help, support, advice and encouragement. I am especially indebted to him for his patience in helping me with statistics and data analysis throughout my study and when he just being there when things went wrong. All thanks to my second supervisor Dr. Lesley Fitton for her advice and help working through this thesis.

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Abbreviation

A. suum	*Ascaris suum*
B. indicus	*Bos indicus*
B. Taurus	*Bos taurus*
C. oncophora	*Cooperia oncophora*
C. punctata	*Cooperia punctata*
Ca++	calcium
Cl	chloride
°C	degrees Celsius
cm	centimetre
cM	centimorgan
CO_2	carbon dioxide
CP	crude protein
Cu	copper
DF	Degree of freedom
D. flagrans	*Duddingtonia flagrans*
dH_20	distilled water
E. coli	*Escherichia coli*
EDTA	disodium ethylene diamine tetracetic acid
ELISA	enzyme linked immunosorbent assay
epg	eggs per gram
Fe	iron
g	grams
H. contortus	*Haemonchus contortus*
hr	hours
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_1	first stage larvae
L_2	second stage larvae

L_3	third stage larvae
L_4	fourth stage larvae
log10	logarithm to the base 10
M	molar
Mg++	magnesium
mg	milligrams
MHC	major histocompatibility complex
min	minutes
µg	micrograms
µM	micromolar
µm	micrometre
ml	millilitres
mM	millimolar
Mn++	manganese
mRNA	messenger RNA
N. americanus	*Necator americanus*
N. battus	*Nematodirus battus*
N. brasiliensis	*Nippostrongylus brasiliensis*
N. filicollis	*Nematodirus filicollis*
N. spathiger	*Nematodirus spathiger*
Na	sodium
no.	number
O. circumcincta	*Ostertagia circumcincta*
O. ostertagi	*Ostertagia ostertagi*
OD	Optical Density
%	percent
P++	phosphorous
P	probability
PBS	phosphate buffered saline
PCV	packed cell volume
PR	Probability
rpm	revolutions per minute
S. japonicum	*Schistosoma japonicum*
S. mansoni	*Schistosoma mansoni*
SDS	sodium dodecylsulphate

Se++	selenium
S.E.	standard error of the mean
spp.	species
T. axei	*Trichostrongylus axei*
T. circumcincta	*Teladorsagia circumcincta*
T. colubriformis	*Trichostrongylus colubriformis*
T. gondii	*Toxoplasma gondii*
T. trifurcata	*Teladorsagia trifurcata*
T. vitrinus	*Trichostrongylus vitrinus*
TCR	T cell receptor
T_H1/T_H2	T helper cells, subtypes 1 and 2
UK	United Kingdom
USA	United State of America
V	Volts
W. bancrofti	*Wuchereria bancrofti*

CHAPTER ONE

GENERAL INTRODUCTION

1-1 INTRODUCTION

Livestock farming plays an important role in the economies of many countries. In Mediterranean and northern European countries, sheep farming is an important component of livestock farming, perhaps because management is easier and the reproductive cycle is shorter than the other livestock species. In many parts of the world sheep are the only suitable livestock therefore they are the main source of income to farmers and breeders. In addition sheep are considered as an important source of meat, milk and wool for humans.

The Food and Agriculture Organisation of the United Nations (FAO-STAT 2003), has estimated that the sheep population over the world in the year 2002 is slightly over 1,000 million. Figure 1-1 shows the total world sheep population for recent decades. These figures have not changed greatly from year to year, but there are slight changes between developed countries and developing countries, where it appears from Figure 1-1 that developing countries in the last 20 years have experienced a better growth in sheep populations than the developed countries.

Figure 1-1: Sheep population / sheep stocks (Head)- (FAO-STAT 2003)

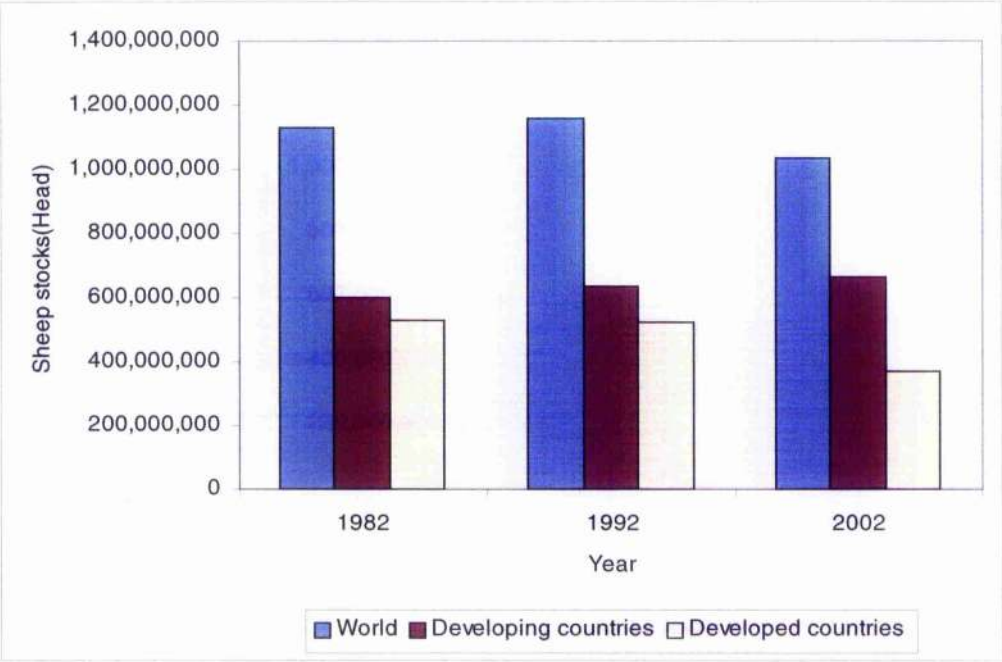
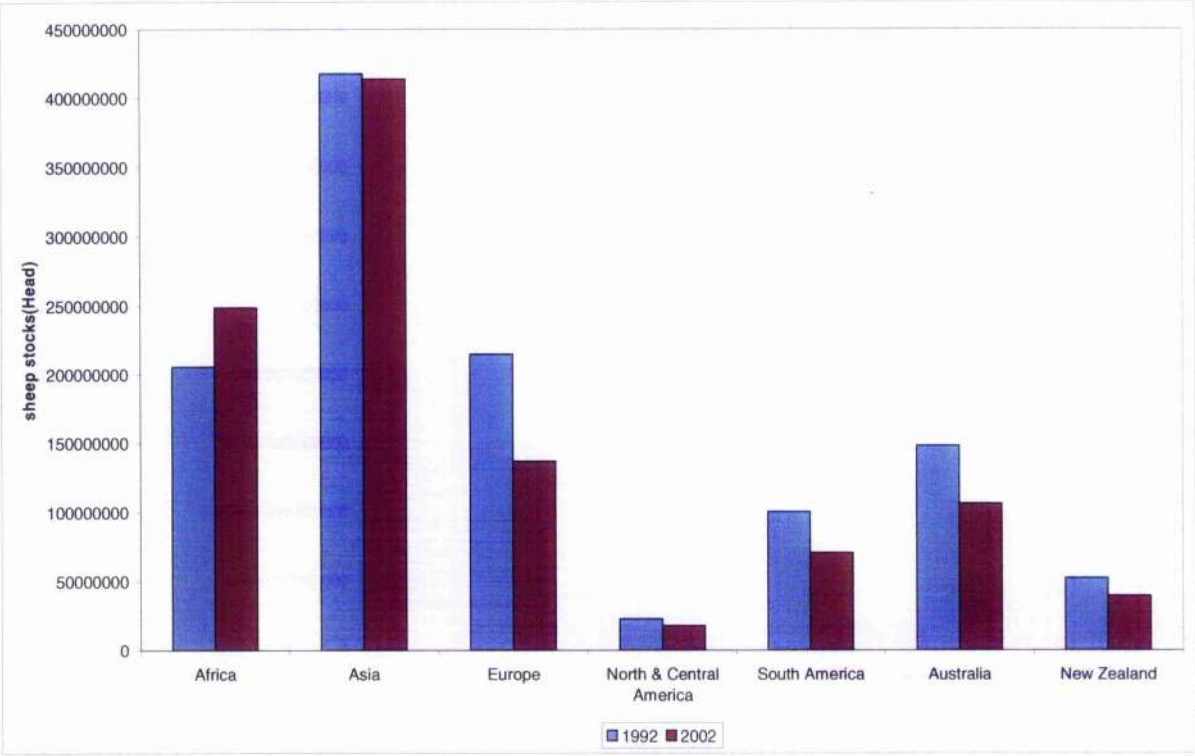


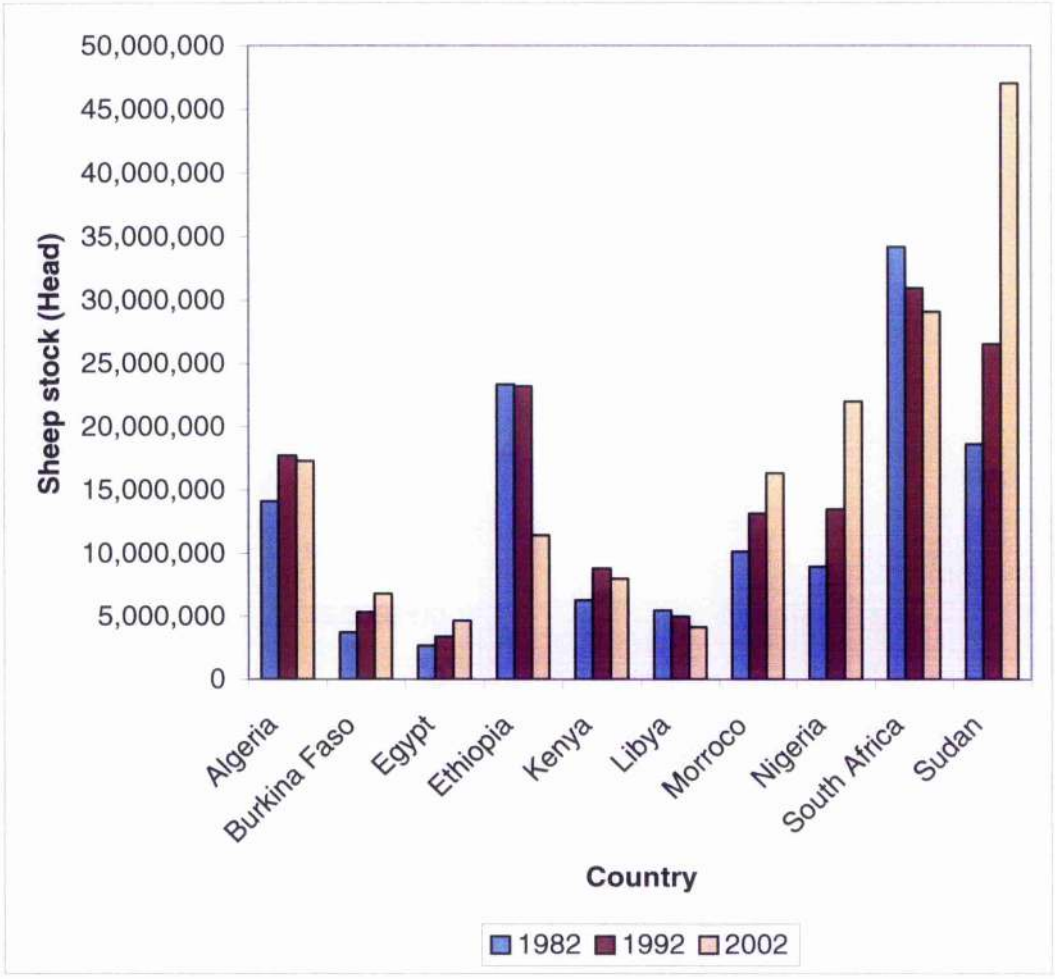
Figure 1-2 shows that in Africa there has been an increase of 16.4% in the sheep population over the last 12 years, and decreases of 2.4% in Asia, 34% in Europe and 22% in Australia &New Zealand.

Figure 1-2: Changes in the distribution of sheep over the continent-(FAO-STAT 2003)



There has been greater growth in some individual countries, especially in Africa, where it is clear from Figure 1-3 that a country like Sudan has a greatly increased sheep population over the last 20 years (from 18.6 million to 47 million).

Figure1-3: Sheep population distribution in African countries- (FAOSTAT-2003)



Livestock farming is threatened by the presence of parasitic infection, especially nematode infections which are a major constraint on livestock productivity. Parasitism mainly affects young animals and weaners. Yearlings are the most susceptible (Uhlinger and Di Pietro 1996; Radostits et al. 1994). Table 1-1 shows the wide range of nematode species that infect sheep and their anatomical location (Soulsby 1986).

Table.1-1 Gastro-intestinal Nematode of Sheep- (Soulsby 1986)

Site	Parasite
Rumen	*Gongylonema verrucosum* *Gongylonema monnigi*
Abomasum	*Teladorsagia circumcincta* *Haemonchus contortus* *Haemonchus placei* *Trichostrongylus axei* *Ostertagia trifurcata* *Marshallagia marshalli*
Small Intestine	*Trichostrongylus colubriformis* *Trichostrongylus capricola* *Trichostrongylus rugatus* *Trichostrongylus vitrinus* *Cooperia curticei* *Cooperia spatulata* *Nematodirus spathiger* *Nematodirus battus* *Nematodirus filicollis* *Nematodirus abnormalis* *Bunostomum trigonocephalum* *Gaigeria pachyscelis*
Large Intestine	*Chabertia ovina* *Trichuris ovis* *Skrjabinema ovina*

Different species of nematodes may occur together in the abomasum and small intestine of sheep (Radostits et al. 1994; Armour and Coop 1991). *T. circumcincta* is the most common species in temperate areas and *H. contortus* in tropical and sub-tropical areas (Bouix et al. 1998). These species are the most economically important (Allonby and Urquhart 1975).

1-2 EFFECT ON PRODUCTIVITY

Nematode parasitism affects animal productivity by:

1-2-1 Reduction in live weight gain:

This is the most characteristic feature of nematode infection. This effect varies with the nutritional and immunological status of the host, age, level of infection and species of parasite (Holmes 1987). Gastrointestinal parasites may depress growth rate by as much as one third (Coop et al. 1985). Reduction in live weight gain is about 50% in lambs infected with *T. colubriformis* and *T. circumcincta* (Sykes and Coop 1977; Sykes and Coop 1976), and about 38% in lambs infected with *H. contortus* (Albers et al. 1989; Abbott et al. 1986)

1-2-2 Adverse effect on the quality and quantity of wool production:

Numerous studies have shown these effects (Albers and Gray 1987; Albers and Le Jambre 2000; Barger 1984; Barger and Southcott 1975; Barger et al. 1973).

1-2-3 Changes in body composition:

These include the following (Holmes 1987):

- Decrease in fat deposition.
- Decrease in protein deposition.
- Decrease in skeletal calcium.

- Decrease in body water.

1-2-4 Effect on milk production:

Thomas and Ali (1983) reported that lactating infected ewes with *H. contortus* showed an overall reduction in milk production of about 23%.

1-2-5 Overall effect of parasitism

Acute parasitic infection leads to a high mortality rate whilst various levels of morbidity and premature culling characterise chronic parasitic infection in infected animals. In addition, economic losses in the U.S.A to internal and external parasites are estimated at more than $ 3 billion per year. Parasites are economically costly at much lower levels than previously believed (Smith 2002). Another study has estimated that, the total annual cost of $ 222 million Australian Dollar to the Australian sheep industry was caused by parasitic infection (McLeod 1995).

1-3 LIFE CYCLE

The life cycles of several parasitic nematodes have been described by Urquhart et al. (1996) and Soulsby (1986).

1-3-1 Basic life cycle of parasitic nematode:

Sexes are separate, and males are generally smaller than females. Adults mate in the infected host and females lay eggs or larvae in faeces. Eggs either hatch inside or outside the body of the final host. In the complete life cycle there are four moults, the successive larval stages being designated L1, L2, L3, L4 and finally L5, which is the immature adult. Temperature,

moisture and even the larva itself are factors influencing hatching processes, and the survival of eggs and infective larvae, (Urquhart et al. 1996; Pandey et al. 1993).

The life cycle is divided into two forms:

Direct life cycle:

This is the common form of nematode life cycle, in which firstly, the hatching eggs moult twice (L1, L2) in the faeces, and infection of the final host takes place by ingestion of contaminated food with L3 larvae.

Indirect life cycle:

After the eggs hatch, larvae moult twice in an intermediate host, and the final host gets infected either by:

a) Ingestion of the intermediate host.
b) Inoculation of the infective stage (L3), during feeding by the intermediate host (blood sucking insects).

The above is a general basic of nematode life cycle, and there are differences and variation between species.

1-3-2 General considerations in nematode life cycle:

1-3-2-1 Arrested larval development, (Hypobiosis):

A proportion of the larvae population can discontinue their parasitic development for number of reasons:

1. Seasonal stimulus, due to unsuitable environmental conditions for larval development before ingestion. Larvae can resume development when better conditions are available (Urquhart et al. 1996; Connan 1971)

II. Host immunity, due to acquired resistance to nematode infection, where it is positively associated with the presence of parasite specific IgA and IgG1 (Stear et al. 1995b).

III. In heavily infected hosts (Dunsmore 1960).

IV. Strain variation where, some nematode strains have a tendency to arrest development, while it is rare in other strains.

1-3-2-2 Periparturient rise in egg count:

This phenomenon is defined as a temporal rise in the number of nematode eggs in faeces at around the time of parturition and during early lactation. It occurs especially in ewes, goats and sows (Urquhart et al. 1996). It is possibly due to:

- Resumption of the development of arrested larvae.
- Loss of acquired immunity around the time of parturition (Barger 1993; Jeffcoate et al. 1992).
- Nutritional stress, in the late stages of pregnancy and early lactation.

1-3-3 *Teladorsagia circumcincta*

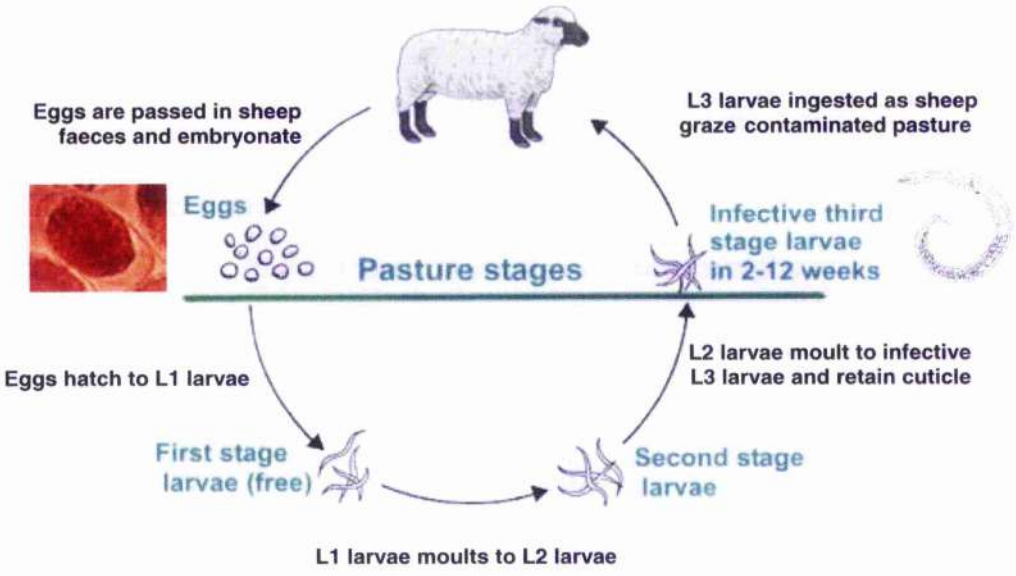
1-3-3-1 Life cycle

The life cycle of this species is direct (Figure 1-4). Adults mate in the abomasum of the infected host, and eggs are passed in the faeces onto pasture. Eggs hatch in the faeces and moult twice, first into L1, and then to L2, which feed on bacteria. Under optimal conditions the infective L3 larval stage develops. L3 larvae are prevented from feeding due to the presence of the retained cuticle of L2, which protects them from unsuitable environmental conditions. The period from egg maturation to L3 larvae is about two weeks. L3 larvae migrate to the grass blades, where grazing sheep eat them.

After ingestion of the infective L3 larvae by the host, they shed the remaining sheath of L2 in the rumen of the host. Exsheathment occurs when the host produces unionised component, which in turn stimuli the larvae to secrete exsheathment fluid, containig an enzyme leucine aminopeptidase, which dissolves the sheath (Rogers and Sommerville 1957; Soulsby 1986; Urquhart et al. 1996).

Larvae migrate to the gastric glands in the abomasal mucosa where they moult twice and adult parasites emerge within two weeks of infection.

Fig. 1-4: *T. circumcincta* life cycle



1-3-3-2 Epidemiology

The rate of development and the survival of the eggs and free-living stages of *T.circumcincta* are strongly influenced by various environmental factors. Temperature and humidity are the two major factors, which affect their rate of development and survival in the external

environment. In temperate areas 95% of eggs hatch to first stage larvae at 4°C (Pandey et al. 1993). Gibson and Everett (1972) reported that the eggs of *T.circumcincta* developed at all times of the year at weekly mean soil temperatures of 2°C-23°C. The ideal temperature for the development of eggs to infective larvae is 16°C (Pandey et al. 1993). The L3 (infective stage) is more resistant to low temperature and survives better at lower relative humidity of 30-50% (Pandey et al. 1993).

The numbers of L3 in herbage increase sharply from mid-summer onwards and are derived mainly from eggs passed in the faeces of ewes during the periparturient rise in faecal egg count and eggs passed by lambs following the ingestion of over-wintered larvae (Urquhart et al. 1996; Soulsby 1986; Boag and Thomas 1970). This results in an accumulation of large numbers of L3 larvae and can lead to severe pasture contamination from July to October. Ingestion of considerable numbers of these larvae can result in (Type 1) Teladorsagiasis in lambs in their first grazing season (Armour and Coop 1991).

If L3 are ingested thereafter they may become arrested in development as fourth-stage larvae in the gastric gland of the host for several months (Urquhart et al. 1996). Large numbers of these larvae can resume development in late winter/ early spring, and give rise to (Type2) Teladorsagiasis (Armour and Coop 1991).

1-3-3-3 Pathogenesis

The morphological changes and functional consequences of infection with *T. circumcincta* are due to the presence of sufficient numbers of the larvae in the abomasum of the infected host. Pathological and biochemical changes and severe clinical signs are maximal when the fourth stage larvae emerge from the gastric gland (Armour et al. 1966; Urquhart et al. 1996). Murray et al. (1970), reported that the major pathological feature of the infection is a

reduction in the function of the gastric glands and may in addition, affect many parts of the gastric mucosa.

In the parasitized gastric glands, the developing parasites reduce proteolytic acid secretion from the mature differentiated cells especially parietal cells, which are replaced rapidly by undifferentiated non-acid secreting cells. Reduction in the acidity of the abomasal fluid leads to an increase in the pH of the abomasum, resulting in firstly; failure to convert any pepsinogen produced to pepsin and consequent reduction in digestive efficiency, and secondly to loss of bacteriostatic effect. The parasitized gland becomes enlarged by the growing worm, which stretches the neighbouring non-parasitized glands and results in marked hyperplasia and frequent thickening and oedema of the abomasal mucosa. The junction complex between many of the cells appears to be incompletely formed, resulting in leakage of macromolecules (pepsinogen) into the circulation, leading to increased plasma pepsinogen levels. Plasma protein losses result in, hypoalbuminaemia, and the regional lymph nodes are swollen (Urquhart et al. 1996; Soulsby 1986). Grossly the lesion is a raised circular nodule with a visible central orifice, which, in a heavy infection, may aggregate together to form the characteristic morocco leather appearance.

1-3-3-4 Clinical signs

Acute Teladorsagiasis (Type1) disease occurs in lambs in their first grazing season at pasture from mid-summer onwards and the signs are; watery diarrhoea, ill thrift, weight loss, failure to gain weight, anorexia, and, in severe cases, dehydration develops (Urquhart et al. 1996; Armour and Coop 1991; Radostits et al.1994).

Sub-acute or chronic Teladorsagiasis (Type 2), is caused by the emergence of large numbers of arrested larvae in late winter or early spring, where the animals occasionally exhibit intermittent diarrhoea, loss of appetite and anaemia (Armour and Coop 1991).

1-3-3-5 Diagnosis

This is based on the grazing history, clinical signs and seasonality of the infection (Urquhart et al., 1996; Soulsby 1986). Faecal egg counts are not always proportional to the size of larval challenge or worm burden (Coop et al. 1977; Callinan and Arundel 1982; Gibson and Everett 1978). Low or negative faecal egg counts may be a false negative result in Type I and Type II teladorsagiasis. Elevated levels of plasma pepsinogen during infection are a valuable diagnostic aid (Stear et al. 1999a).

1-3-4 *Haemonchus contortus*

1-3-4-1 Life cycle

Parasitic developments are similar to those of *Teladorsagia* spp. The life cycle is direct and eggs are passed in the faeces and hatch within 15-20 hours to produce the first free-living larvae L1, which in turn moults to the second (L2) and third (L3) respectively. L3 is the infective stage, and development from egg to L3 can be as short as 5 days (Urquhart et al., 1996). Infection takes place by ingestion of the infective L3 stage. Exsheathment takes place in the rumen of the host, and free L3 develop between the abomasal gastric epithelial cells to L4 and subsequently into adults on the abomasal surface. They have a lancet that enables them to obtain blood from the mucosal vessels (Urquhart et al., 1996; Soulsby 1986). The pre-patent period is three weeks.

1-3-4-2 Epidemiology

In tropical and sub-tropical regions, *H. contortus* develop maximally at relatively warm temperatures, therefore Haemonchosis is basically a disease of sheep in warm climate regions. High humidity is essential for larval development and survival and so the severity of outbreaks of this disease greatly depends on the level of rainfall. Severe pasture contaminations of L3 can occur very quickly due to the high faecal worm egg output of between 2000 and 20 000 eggs per gram (Urquhart et al., 1996). In prolonged dry seasons in some areas, this parasite is able to arrest development (hypobiosis), where faecal eggs and larvae are unable to survive. It resumes development when environmental conditions are more suitable (Soulsby, 1986). In other areas where there is more frequent rainfall no significant degree of hypobiosis has been observed (Urquhart et al., 1996). In temperate regions, there is usually a single annual life cycle, where the majority of the infective larvae ingested by ewes and lambs in early summer undergo hypobiosis in the abomasum, and resume development next spring (Urquhart et al., 1996).

1-3-4-3 Pathogenesis

The main consequence of the blood feeding of *H. contortus* is anaemia (Urquhart et al., 1996; Armour and Coop 1991; Soulsby, 1986). The average daily blood loss has been calculated at 0.05 ml/parasite/day (Clark et al. 1962).

Development of anaemia in sheep heavily infected by *H. contortus* can be categorised into three stages (Dargie and Allonby 1975). In the first stage, one to four weeks after infection, there is a progressive and dramatic fall in the packed cell volume (PCV) while serum iron remains normal. In the second stage, six to 14 weeks after infection, the PCV stays steady but below the normal levels despite the continual loss of blood, as the infected sheep compensate for these losses by increasing erythrocyte production. Infected sheep shows

severe iron depletion due to the increase in plasma iron turnover. The third stage is manifested by a sharp drop in PCV that results from a dyshaemopoiesis due to iron deficiency. Animals infected by *H. contortus* also lose a huge quantity of serum proteins into the gut (Dargie and Allonby 1975).

1-3-4-4 Clinical signs

The disease may be divided into three syndromes; hyper acute, acute and chronic, depending on the degree of parasitism (Urquhart et al., 1996; Armour and Coop 1991; Soulsby, 1986). Hyper acute haemonchosis occurs when a susceptible animal ingests a large number of parasites, leading rapidly to severe anaemia, dark faeces and sudden death from haemorrhagic gastritis. The acute syndrome is characterized by anaemia with pallor of mucous membrane and submandibular oedema (bottle jaw). Diarrhoea does not normally occur. In chronic Haemonchosis, which is possibly more characteristic of the naturally occurring disease (Allonby and Urquhart 1975), the infected sheep became lethargic, unthrifty and emaciated.

1-3-4-5 Diagnosis

The clinical signs and history supported by high faecal worm egg counts often establish diagnosis (Urquhart et al., 1996; Soulsby, 1986)

1-4 CONTROL

The most commonly used method to control gastro-intestinal nematodes of sheep involves the strategic treatment with anthelmentics (Armour and Coop 1991; Mitchell and Fitzsimons 1983; Armour and Bogan 1982) and, where practical, grazing management (Uriarte and Valderrabano 1990; Herd 1988).

1-4-1 Anthelmintic

The first broad spectrum anthelmintics, which were introduced early in the 1960s, were Thiabendazole and Levamisol (reviewed by Armour and Bogan 1982). They have had a revolutionary effect on sheep husbandry. Since then a wide range of these drugs has been available. Sheep are commonly infected by a variety of gut nematodes and more than ten different species can occur in one infected sheep (Radostits et al. 1994; Armour and Coop 1991; Armour and Bogan 1982), therefore anthelmintic drugs are measured by their efficacy against a wide range of both arrested and developing larvae and adult worms.

Anthelmintic drugs use are now so widespread and conventional that it is believed that without these drugs the sheep industry could not exist in its current form (Stear et al. 2000). Anthelmintic drugs have proved successful for helminth control for three specific reasons. They are effective against a number of co-infecting seasonally different nematodes. They are reasonably priced relative to the cost of production losses and their integration with farm management practices is relatively easy.

The successful control of any helminth disease is based on the knowledge of its epidemiology (Armour and Coop 1991) and to obtain maximum benefits, animals should be treated at the time of maximal risk of exposure. Strategic dosing involves treatment of the ewes at lambing to reduce pasture contamination, followed by dosing the lambs during the grazing period to minimise the effects of the disease.

1-4-2 Grazing management

The main aim of controlled grazing is to reduce the chances of the host coming into contact with the infective stages of the parasite, especially the susceptible animals, which include pregnant ewes and young animals (Armour and Coop 1991), and this can be achieved by

grazing sheep and lambs on pasture that has not carried young sheep or pre-parturient ewes during the last 12 months, allowing firstly the highly susceptible lambs to graze followed by the older and relatively immune animals (Uriarte and Valderrabano 1990). Niven et al. (2002) compared two strategies for grazing management system during winter and spring for Merino weaners. A 'standard' strategy, comprising set-stocked wethers given anthelmintic treatments in late October and early February, was compared with a 'new' one in which intensive grazing for 1 month after each treatment and destocking for 1 or 2 months was integrated with the anthelmintic treatments. Results from these studies indicated that contamination of pastures prepared according to the new strategy was substantially reduced.

The numbers of trichostrongylid larvae, except Nematodirus spp, were reduced by 50 to 95% and weaners grew significantly more clean wool and had greater weight gain compared to those of the standard strategy. Grazing management in the UK may use the Rutter system of clean grazing which involves administration of anthelmintic treatment of the ewes before they are moved onto a clean pasture. Another study has demonstrated that weaning lambs at the beginning of July and moving them before the expected mid-summer rise in herbage infection to a clean pasture will prevent parasitic gastroenteritis and achieve good production whether the move is accompanied by anthelmintic treatment or not. The effects will be subject to prevailing nematode species, local climatic conditions and length of the grazing season (Githigia et al. 2001).

Niezen et al. (1998) investigated the effect of several plant species effect on the ability of larvae to parasitise sheep. They concluded that herbage species had a highly significant effect on the number of larvae recovered during investigation, with greatest numbers recovered from Yorkshire fog, ryegrass and cocksfoot while white clover and Lucerne yielded lowest numbers. Furthermore, studies in this area suggest that the herbage species

such as white clover can reduce the production losses due to nematode infection (Niezen et al. 2002b). There is some evidence that changing the type of forage fed to lambs affects the worm burdens and faecal egg counts of lambs suffering from parasitic gastroenteritis. Lambs with naturally acquired gastrointestinal nematode burden, and which grazed sulla *(Hedysarum coronarium),* had lower faecal egg counts and gastrointestinal nematode burdens than those which grazed Lucerne *(Medicago sativa)* (Niezen et al. 1995). This may be attributed primarily to the increased post-ruminal availability of protein, resulting from decreased protein degradation in the rumen due to the presence of condensed tannins in the sulla (Waghorn et al. 1987). It appears to be an immune response to sulla which is associated with higher antibody titres against secretory-excretory antigens from adult *T.circumcincta* and to adult and larval *T. colubriformis* (Niezen et al. 2002a).

1-4-3 Anthelmintic resistance

The gastrointestinal nematodes of sheep can be controlled by anthelmintic treatment and planned rotational regimes, but these control schemes are threatened by the emergence of nematodes which are resistant to the most commonly used anthelmintic treatment (Jackson 1993). Drudge et al. (1957) first reported anthelmintic resistance in *H.contortus* to phenothiazine. Resistance has been reported in many others parasitic strains to anthelmintic treatment worldwide (Sutherland et al. 2003; Hughes et al. 2004; Hughes et al. 2005; Kaplan 2004; Sargison et al. 2004; Coles 2005; Le Jambre et al. 2005; Hong et al. 1996; Waller et al. 1995; Hong et al. 1994; Jackson et al. 1992; Drudge et al. 1964). Such increase in reporting of nematode resistance is strong evidence to a problem that is intensifying, with reports of cases of multiple anthelmintic resistances (Bartley et al. 2004) and other reports of complete multi-drug resistance to anthelmintics now emerging (Chandrawathani et al. 2004b).

Despite attempts for new broad-spectrum anthelmintics developments in this area are slow. Recently, cyclooctadepsipeptides, have been described as effective against nematodes (von Samson-Himmelstjerna et al. 2005), also Paraherquamides are being investigated for anthelmintic properties (Zinser et al. 2002). Nevertheless, continual reliance on the use of drugs even with the development of new anthelmintics is not sustainable in the long term, as resistance to any of these drugs is likely to develop. Also increasing consumer concerns about drug residues in meat and the undesirable environmental effects of chemicals, suggest that alternative control methods are needed.

1-4-4 Protein supplementation

Coop and Kyriazakis (2001) reported that protein supplementation of the sheep diet influences the degree of expression of immunity and results in an accompanying reduction in establishment of incoming larvae and reduced survival of established larvae. A combination of relatively resistant sheep and nutritional supplementation is an effective method of controlling parasite infection (Stear et al. 2000).

Datta et al. (1999) demonstrated that short-term provision of protein-enriched diets leads to long-term beneficial effects of higher live-weight gain, higher wool production, higher antibody responses and lower FEC. Another study was conducted to assess the effect of providing daily copper oxide wire particle (COWP) capsules, on established or incoming mixed nematode infections in young sheep, and results from this study showed that lambs with established (6 week old) infections, resulted in 97% and 56% reduction of the adult and early L4 stages of *H. contortus*, respectively, compared with controls and there was a 74% reduction in *Teladorsagia circumcincta* infections in the COWP lambs compared with controls (Waller et al. 2004a). In addition Burke et al. (2005) determined the effectiveness of

copper oxide wire particles in pregnant Katahdin ewes and demonstrated significant decrease in FEC in comparison to control ewes.

1-4-5 Vaccination

Vaccines give useful protection against a wide range of microorganisms that infect both human and animals. Antiparasite vaccines of animals have recently been reviewed, and much of the main work on the development of vaccines to gastrointestinal nematodes has been discussed (Emery 1996). While there are some vaccines available against toxoplasmosis in sheep and the tick *Boophilus microplus*, the only commercially available vaccine for the control of helminth infections in ruminants is that for the bovine lungworm, *Dictyocaulus viviparus* (Smith 1999).

An ideal vaccine would have to be effective against a number of different nematodes, be cost effective and not required repeat vaccination within one season (Knox 2000; Knox et al. 2003). Two types of vaccines have been studied and used. The first type is based on antigens that are accessible to the host's immune system during natural infection. The advantage of such a vaccine is that continual natural exposure acts as a booster to the primary vaccine. However, many of the antigens to which the host mounts an immune response are non-protective and there is considerable variation among animals in the identification of parasite molecules (McCririe et al. 1997; Haswell-Elkins et al. 1989).

The second method has been to look for antigens that are hidden from the afferent immune system during normal infection (Munn 1997). The target antigens must be inaccessible to the host's immune system but must be accessible to antibodies and other immune components induced by the vaccination. This approach has been elegantly demonstrated for a *H. contortus* vaccine which gives protection greater than 90% (Tavernor et al. 1992).

Vaccination with a membrane-bound thiol Sepharose-binding fraction (TSBP) of adult *H. cotortus* with enriched cystein proteinase activity has led to a significant reduction in worm burden but not FEC (Redmond and Knox 2004). Dominguez-Torano et al. (2000) reported that vaccination of Manchego lambs with a di-peptide fraction of *H. contortus* led to lengthening of prepatent periods, significant reduction in FEC, reduced variations in packed cell volume values and significantly lower worm burden at necropsy in comparison to unvaccinated animals. Sheep vaccinated with Di-dithiothreitol fractionated excretory/secretory products of adult *H. contortus*, showed a high level of protection in which egg output and worm burden were reduced by 52% and 50%, respectively (Bakker et al. 2004).

Results from a vaccine based on a *T. circumcincta* L3 excretory/secretory antigen that was recognised by resistant sheep were encouraging, but recent attempts have failed to replicate the success (Morton et al. 1995). Trials using excretory/secretory antigens against *T.colubriformis* show promising results (Emery 1996)

Although a considerable amount of research has been conducted, it is highly unlikely that a vaccine will be available any time in the near future and, until a vaccine can be demonstrated to produce adequate protection, other methods of control are needed.

1-4-6 Biological control

A new non-chemotherapeutic method to control gastrointestinal nematodes of animals is biological control. Despite experiments to employ various species against plant and animal parasitic nematodes in the 1930's, it was not until the 1990's, when selection by simulating passage through the gastro-intestinal tract of cattle led to isolation of the fungus *Duddingtonia flagrans,* that a major breakthrough was achieved (Hertzberg et al. 2002).

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 and Faedo 1996). Two means of biological control that have provoked interest are the use of nematophagous fungi and different species of grass.

Duddingtonia flagrans fungus produces a sticky three-dimensional network and produces high numbers of thick-walled resting spores, *chlamydospores*. These spores survive passage through the gastro-intestinal tract of grazing livestock and are capable of growing and subsequently traping nematodes, including larval stages of parasitic nematodes (Waller and Faedo 1996; Larsen 2000; Pena et al. 2002; Chandrawathani et al. 2004a). Field trials evaluating this method of control under a natural commercial environment demonstrated its suitability at reducing nematode infection (Waller et al. 2004b), with no negative environmental impacts (Knox et al. 2002) . In contrast, an other study reported no significant differences in overall FEC, packed cell volume or animal weight between animals' administrated fungus and control groups (Fontenot et al. 2003).

Duddingtonia flagrans appears to survive passage through the ruminant gut more efficiently than other nematophagous fungi (Faedo et al. 1998). Githigia et al. (1997) demonstrated that feeding lambs predominantly infected with *T.circumcincta* with *D. flagrans* led to a reduction in newly acquired worm burdens of 62%.

Other species of nematophagous fungi have been investigated for their nematode reducing capacity including *Dactylaria* species, *Arthrobotrys oligspora conidia*, but *D. flagrans* remains the most likely candidate for this type of disease control (Flores-Crespo et al. 2003).

1-4-7 Breeding for resistance to gastrointestinal nematodes of sheep

For many years domestic animals have been selectively bred to satisfy human needs. It is becoming more common to breed selectively for enhanced disease resistance. In nematode infection in sheep, several investigations have shown that sheep can be bred for resistance to gastrointestinal parasites and that individual breeds appear to be more resistant to infection than others (Gray and Woolaston 1991). For example the Red Massai breed appears to be more resistant to *H. contortus* infection than European breeds (Mugambi et al. 1996; Mugambi 1994) and breeds resistant to infection can be substituted for those that are more susceptible (Baker 1995). Recently, sheep breeders in Australia, New Zealand and the UK have started to breed for resistance to gastrointestinal nematode infection (Bisset and Morris 1996; Woolaston and Baker 1996).

Genetic variation in resistance of sheep to gastric nematode infection is well documented and it comes from both between-breed and within-breed comparisons (Albers and Gray 1987; Barger 1989; Gray et al. 1987). Most of the work on genetic resistance to infection has been selection from within-population breeding programmes in sheep. Nematodes in ruminants have a negative binomial distribution (Barger 1985), with a small proportion of the hosts carrying large numbers of the parasites.

Resistance to nematode infection has been measured by faecal egg counts, PCV following infection, erythrocyte potassium content or serum iron concentration (Piper 1987), and there has been great success in breeding resistant lines of sheep (Bisset et al. 1992; Albers et al. 1987; Windon and Dineen 1981), but McEwan et al. (1995) have reported unfavourable correlations between egg count and productivity in lines of selected sheep on the basis of low faecal egg count following natural exposure to a mixed infection of *Trichostrongylus* and *Ostertagia* spp, where the lambs with low faecal egg counts have a poorer live-weight

gain and a lighter fleece. Programmes are now established to breed for resilience (refers to the ability of sheep to maintain production levels in presence of gastric nematode infection), and initial results show that although heritability is low, resilience is correlated with increased productivity (Bisset and Morris 1996).

1-5 GENERAL INFLUENCES ON SHEEP SUSCEPTIBILITY TO NEMATODE INFECTION

1-5-1 Host age

It is well established that the age of the host influences its ability to develop an effective immune response to a wide variety of pathogens. Older hosts appear to be more resistant than younger ones to a wide range of parasitic infections such as *Toxocara canis* (Bundy et al. 1987b), *Trichuris muris* (Bundy et al. 1987a), *Plasmodium falciparum* (Sabatinelli et al. 1996) and hookworm infections caused by *Necator americanus* (Quinnell et al. 2001). Ruminants less than six month of age are generally more susceptible to many infectious microorganisms and macroparasites (Colditz et al. 1996). Pienaar et al. (1999) reported that young goats up to 12 month of age are more susceptible to the common threadworm, *Strongyloides papillosus*. A marked decrease in the severity of clinical signs was observed as the age at infection increased following infection of lambs with *Cryptosporodium parvum oocysts* (Ortega-Mora and Wright 1994).

This age variation in susceptibility to infection can readily be explained, as younger animals have not been previously exposed to the agent and so have no active immunity, the suppressive effects of passively acquired maternal antibody or stress associated with early stages such as weaning. However even when these factors are taken into account there still

appears to be a constitutive immunological hypo-responsiveness to infection (Watson and Gill 1991).

Younger lambs have significantly lower proportions of CD4+ and CD8+ lymphocytes and greater proportions of B cells and T19+ lymphocytes than mature sheep (Colditz et al. 1996; Watson et al. 1994). Generally young lambs lese than a year old mount smaller antibody and T cell responses than older sheep (Watson et al. 1994; Watson and Gill 1991). Young lambs, naturally infected by grazing have significantly fewer globule leucocytes/mucosal mast cells than older animals (Douch and Morum 1993).

1-5-2 Host breed

Evidence for breed variation in the resistance of sheep to nematode infection is well documented and this variation comes from both between-breed and within-breed comparisons (see above).

1-5-2-1 Between-breed variation in resistance to nematode infection

Numerous studies have investigated the differences between breeds in resistance to nematode infection. In Kenya, the indigenous Red Maasai was more resistant to *H.contortus* infection than other breeds (Preston and Allonby 1979; Preston and Allonby 1978; Mugambi et al. 1996; Mugambi et al. 1997). The Gulf Coast Native sheep are more resistant than Suffolk sheep (Miller et al. 1998). The Texel breed is more resistant than Suffolks to natural helminth challenge based on FEC and nematode burden (Good et al. 2005). The native Horro lambs has a significantly lower FEC than the Menz lambs (Haile et al. 2002). The mature Droper crossbred, Katahdin breed and St. Croix breed ewes were more resistant than Hampshire breed (Burke and Miller 2002). The Sabi breed has a lower FEC than the Dorper breed (Matika et al. 2003). The Black Belly sheep are more resistant than the French INRA

410 lambs following deliberate infection with *H contortus* (Gruner et al. 2003). Dorset/ Rambouillet sheep showed higher faecal egg counts and greater alterations in haematological parameters than the St. Croix or Florida Native sheep (Zajac et al. 1990). Radhakrishnan et al. (1972) demonstrated that Florida Native lambs are more resistant to *H. contortus* infection than Rambouillet lambs. Stewart et al. (1937) reported that breeds of sheep vary in susceptibility to infection with *T. circumcincta* in the following ascending order: Romney, cross-bred, Southdown, Rambouillet, Shropshire and Hampshire. In addition, crossbreds have significantly lower faecal egg counts than purebred lambs naturally infected with *T. circumcincta*. Dorset lambs have shown a greater resistance to infection with *T. axei* than Blackface lambs (Ross 1970); the Caribbean Hair and the Katahdin breed are more resistant to the crossbred Dorper breed (Vanimisetti et al. 2004); the Santa Ines young male sheep have superior capabilities to control infection over the Suffolk and Ile de France breeds (Amarante et al. 2004).

1-5-2-2 Within-breed variation in resistance to nematode infection

Differences in resistance to nematode infection within-breed have been reported (Whitlock 1958). Ile examined offspring from parents selected for increased resistance to *H. contortus*. The offspring from parents selected for increased resistance had lower faecal egg counts and higher PCVs following infection than others. In a comparative study, the offspring from a resistant ram were compared with those from two susceptible rams and resistance to *T. circumcincta* was shown to be heritable (Scrivner 1967). Differences within the Merino breed to *H. contortus* infection has been reported (Allonby and Urquhart 1976), and similar findings were reported for the Scottish-Blackface and the Finnish-Dorset breeds (Altaif and Dargie 1978a). Sheep that were homozygous for haemoglobin type A had lower worm burdens after infection than their homozygous B or heterozygous counterparts. In addition, Scottish-Blackface sheep with haemoglobin type A infected with *T.circumcincta* showed

milder biochemical and pathophysiological changes than their haemoglobin type B counterparts (Altaif and Dargie 1978b). Also evidence of within-breed variation was reported by (Schwaiger et al. 1995), describing FEC variation within the Blackface breed. Selection has produced lines of resistant and susceptible ewes to *H. contortus* in the Merino breed (Sreter et al. 1994; Woolaston et al. 1996; Kahn et al. 2003). Variation in resistance to infection has been described in feral Soay sheep (Coltman et al. 2001), and in the Texel breed (Sayers et al. 2005).

1-5-3 Host sex

Sex differences in parasitic infections have been of interest to parasitologists for a long time, virtually since the study of animal parasites became well established near the beginning of the 20th century.

Studies in human infections with trematode parasites reported different sex responses. Males were found to have higher IgG1, IgG4 and IgE to *Schistosoma mansoni* and *Schistosoma japonicum* adult worm when human antibody isotype responses were examined. Webster et al. (1997) and Remoue et al. (2000), observed that the specific IgG3 response was predominant in male populations with a low intensity of infection and was associated with maximal response to glutathione s-transferase (*S. mansoni* 28GST antigen) in infected human populations. The specific IgA response and production of TGF-β and IL-10 were found to be significantly higher in females compared to males in a Senegalese population chronically infected with *S.haematobium* (Remoue et al. 2001). In human filariasis, the intensity of infection with *Wuchereria bancrofti* is reduced in females compared to males (Alexander and Grenfell 1999) and males have significantly heavier infections of the human hookworm *Necator americanus* than females (Behnke et al. 2000). These differences are

usually attributed to ecological (sociological) causes, which include differential exposure to pathogens because of sex-specific behaviour or morphology (Zuk and McKean 1996).

Differences in the sex susceptibility to infection in rodents do not appear to be very clear. However Charniga et al. (1981) reported that enteritis in female mice infected with *Trichinella spiralis* was greater than that in male mice, but other studies demonstrated that males were more sensitive to *T.spiralis* and *T.pseudospiralis* infections than the females (Figallova and Prokopic 1988; Reddington et al. 1981).

Other studies showed that, female mice were generally more susceptible to infection than males, although worm expulsion occurred sooner in female rats infected with *Nippostrongylus brasiliensis* than in males (Waddell et al. 1971; Murray et al. 1971), and the same was true in *S.mansoni* infection in mice (Nakazawa et al. 1997; Eloi-Santos et al. 1992). Male mice are more resistant than female mice to infection with *Toxoplasma gondii* and this difference correlates with enhanced innate immune responses in these animals. Male mice exhibited longer survival times, lower parasite burdens, and less severe pathological changes post-infection. These observed differences were attributed to the fact that male mice rapidly produced high levels of IL-12, interferon gamma and tumour necrosis factor (TNF)-alfa compared to the female mice (Walker et al. 1997). More interestingly, no sex differences were observed in the susceptibility to infection with *Taenia taeniaeformis* of wild deermice (Theis and Schwab 1992).

Studies in ruminants have noted differences between the host sexes. Females were more frequently infected than males in traditionally reared goats originating from a dry area of central Spain and naturally infected with a range of gastrointestinal nematodes (Valcarcel

and Garcia 1999), but another study found no differences in susceptibility to *H.contortus* infection in castrated male and female merino lambs (Albers et al. 1987).

There is increasing evidence that male sheep with gastrointestinal nematodes infection show generally greater intensity and high prevalence than females. This has been demonstrated in natural, predominantly *T. circumcincta* infections (Stear et al. 1995a; Gulland and Fox 1992), experimental infections with *H.contortus* (Adams 1989), deliberate infection with *T. colubriformis* (Windon and Dineen 1981) and with *O.columbianum* (Dobson 1964).

These differences are usually attributed to a physiological cause, usually hormonal in origin, as in some of the studies cited above, there may be an association between testesterone and the immune system (Barger 1993). It must be noted that the majority of male sheep in the farming industry are castrated and therefore, sex hormones could not be the main cause of differences between males and females. The more ecological view usually postulated that gender differences in parasitic infection were due to differences in the life histories of males and females, with one sex perhaps eating more or different food, and thus ingesting more infective stages, or perhaps inhabiting an area with a greater tendency to harbour parasites, such as a stream margin (Herd et al. 1992).

1-6 OBJECTIVE OF THIS STUDY

The general aim of this thesis is to investigate some of the factors influencing host parasite interactions involving sheep naturally infected with gastrointestinal nematodes, predominantly *T.circumcincta*.

Male sheep are more susceptible to nematode infection than female. Most of the previous studies have not given a satisfactory explanation for this variation; and the mechanism underlying this variation among the sex remains unclear.

The first purpose of this study is to confirm and quantify the variation between genders. The second is to investigate the relationship between sex and some of the genetic markers associated with sheep resistance to nematode infection.

The specific objectives of this thesis are:

1- Investigate the association between faecal worm egg counts and gender of the host.
2- Study the association between a genetic marker (MHC) and faecal egg counts.
3- Interactions between interferon gamma as a genetic marker with faecal egg counts in relation to sex of the host.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2-1 EXPERIMENTAL ANIMALS

2-1-1 Breed, Location, Age and Sex

All the animals used in these experiments were Scottish Blackface sheep (Figure 2-1). Both castrated males and females were used in this study. Due to the variety of animal ages, locations and sampling time which have been used in this study, the details of the populations studied will be described in each chapter.

Figure 2-1 Scottish Blackface sheep

2-1-2 Blood collection

Blood was collected by jugular venepuncture into evacuated glass tubes containing the anticoagulant disodium EDTA (Becton Dickinson UK Ltd, Oxford, UK). The tubes were centrifuged at 1000g for 30 minutes at 4°C. Afterward the plasma layer was collected in 20ml plastic tube and then the leucocytes (buffy coat) was collected in a 1.5ml Eppiendorf tube and stored at -20°C till further use.

2-2 PARASITOLOGY

2-2-1 Faecal Worm Egg Counts

Faeces samples were taken directly from the rectum of the lambs and stored at 4°C until processed.

A modified McMaster salt flotation technique (Miller and Nawa 1979; Wells 1963) was performed to estimate the concentration of nematode eggs in the faeces. Three grams of faeces were added to 42 ml of tap water, the mixture was then homogenised mechanically and poured through a 250-micron aperture sieve and the filtrate was transferred to a 15 ml glass test tube and spun for two minutes at 560g. The supernatant was discarded and the faecal pellet was broken up by vortexing (Stuart Scientific Co. Ltd- UK), and the tube was filled to its former level with saturated sodium chloride solution. The tube was gently inverted six times, until the suspension was mixed. A sufficient amount of this suspension was withdrawn to fill both chambers of a McMaster egg counting slide (Gelman Hawksley Ltd., Northampton, England) using a plastic Pasteur pipette. The preparation was then examined using the x25 objective of a Stereomicroscope. The number of eggs present in both chambers was counted and the result was multiplied by 50 to give the number of eggs per gram. To improve the accuracy of the technique, four slides were counted per animal.

2-2-2 Herbage Analysis

A w-shaped route across the paddock (diagonally four times) was used to collect herbage samples (Taylor 1939). The samples were collected by hand, at fifty evenly spaced stops along each route. At each stop, four plucks of grass were taken to make a total of 200 plucks per plot. Herbage samples were collected in a plastic bag, which then was weighed and put into a small hand operated washing machine (Easy Pressure Washer, Classic Supplies Ltd., Leeds). Eight litres of lukewarm water were added and the bag was tied and the machine turned through two hundred revolutions. The herbage was filtered through a 2mm mesh sieve into a bucket. The washed grass was then spread on a tray and dried in an incubator at 70°C. When the grass was completely dried, the herbage was weighed again. The dry weight was then used in the final calculation of numbers of larvae per kilogram-dried herbage (L3/kdh).

A 38-micron sieve was used to filter the washings contained in the bucket where the material was retained. The larval suspension was drawn through a coarse filter paper (Whatmans Grade 113, 18.5cm) using a Buchner funnel and vacuum pump. A single milk filter paper (Maxa Milk filters, A. McCaskie, Stirling) was put on top of the retained material, the combination inverted and placed on a Baermann filter funnel filled with lukewarm water. After a minimum of six hours, 10 ml of fluid was withdrawn and the larvae were differentiated and counted.

2-3 MOLECULAR BIOLOGY

2-3-1 DNA Extraction

DNA was extracted from blood leucocytes. The following solutions were used.

2-3-1-1 Solutions and Media

1M Tris-HCl pH7.5

A 121g Trizma base (Tris [hydroxymethyl] aminomethane; Sigma-Aldrich Company Ltd, Poole, England), was weighed and dissolved in 800 ml distilled water (dH_2O) and the pH was adjusted to 7.5 using Microprocessor pH meter (HANNA Instrument). The volume was adjusted to 1 litre and the solution stored at 4°C.

1M $MgCl_2$

A 203.3g $MgCl_2.6H_2O$ (BDH Chemicals Ltd, Poole, England), was weighed and dissolved in 800 ml dH_2O. The volume was adjusted to 1L and autoclaved. The solution was stored at 4°C.

5M $NaCl_2$

292.2g NaCl (Sigma- Aldrich) was weighed and dissolved in 800ml dH_2O. The volume made up to 1L, autoclaved and stored at 4°C.

0.5M EDTA pH8.0

A 93.05g EDTA (Ethylene Diamine Tetra Acetic Acid- Sigma-Aldrich) was weighed and made up to 500 ml in dH_2O and the pH was adjusted to 8.0, autoclaved and afterward stored at 4°C.

10% SDS pH7.2

A 100g SDS (Sodium Dodecyl Sulfate, Sigma Aldrich) was weighed and mixed with 800 ml dH_2O. The solution was heated to dissolve the SDS, and then the pH adjusted to 7.2. The volume was made up to 1 litre and stored at room temperature.

Proteinase K

100mg proteinase K (Sigma-Aldrich) was dissolved in 25 ml dH_2O to make a 4 mg proteinase K/ml. 500 µl of this solution was mixed with 500 µl 10% SDS

3M Sodium Acetate pH5.2

A 204.1g $NaAc.3H_2O$ (BDH-Limited) was mixed with 350 ml dH_2O and the pH was adjusted to 5.2 with Glacial Acetic Acid. The volume was adjusted to 500 ml dH_2O, autoclaved and stored at 4°C.

TE Buffer

A 2ml 0.5M EDTA and 10 ml 1.0M Tris-HCl was mixed with dH_2O and made up to 1L. The solution was autoclaved and stored at 4°C.

Phenol: Chloroform: Isoamylalcohol (25:24:1)

250 ml equilibrated phenol (Sigma-Aldrich), 240 ml chloroform (Sigma-Aldrich) and 10 ml isoamylalcohol (Sigma-Aldrich), were mixed together and stored in a dark bottle at 4°C.

Chloroform: Isoamyalcohol (24:1)

240 ml chloroform (Sigma-Aldrich) was mixed with 10 ml isoamyalcohol (Sigma-Aldrich) and stored at 4°C.

Lysis Buffer

A 109.536g Sucrose (Sigma-Aldrich) was dissolved in 10 ml 1M Tris-HCl pH7.5 and 5 ml 1M $MgCl_2$ to make up 990ml with dH_2O. The solution was then autoclaved and 10ml of 10% Triton × 100 (Sigma-Aldrich) was added and the buffer was stored at 4°C.

Digestion Buffer

7.5ml 5M $NaCl_2$ and 25 ml 0.5M EDTA (pH8.0) was mixed with dH_2O to make up 500 ml. The solution was autoclaved and stored at 4°C.

2-3-1-2 DNA Extraction Procedure

50 ml cold lysis buffer was added to each frozen and thawed buffy coat. The content was mixed thoroughly several times by inverting the tubes. Tubes were then stood in ice for 10 minutes and afterward centrifuged at 800g for 10 minutes at 4°C. The supernatant was discarded and the tubes were blotted on paper towel, the pellet was resuspended and mixed in 5 ml Digestion buffer. Afterwards the tubes were centrifuged at 800g for 10 minutes at 4°C. The supernatant was discarded and the tubes were blotted on paper towel. The pellet was resuspended and mixed with 4 ml Digestion buffer. 1ml Proteinase K solution was added to every sample and incubated in a 56°C water bath for 24 hours. Then the samples were removed from the water bath and 5 ml equilibrated phenol (Sigma-Aldrich) was added and mixed gently to form an emulsion. The samples were left for 10 minutes, and then centrifuged for 10 minutes at 800g. The phenol layer was discarded and the clear aqueous layer was transferred to 50 ml tubes, where it was remixed gently with phenol:chloroform:isoamylalcohol (25:24:1) and samples after that were centrifuged for 10 minutes at 800g. The clear aqueous layer was collected and transferred to a 50 ml tube, where the previous step was applied once more. The new aqueous layer was transferred to 15 ml plastic tube where, 5ml chloroform:isoamylalcohol (24:1) was added and mixed. Tubes were centrifuged at 800g for 10 minutes. The clear aqueous layer was removed to fresh 30ml plastic tube containing 1 ml 3M sodium acetate, where the solution was mixed and 12.5 ml cold ethanol was added. The tube was inverted several times until the DNA precipitated. DNA was collected using a heated sealed pipette in 5 ml eppiendorf tube and rinsed in 500 µl 70% ethanol, allowed to air dry, then 200 µl TE buffer was added. The tubes were then

heated at 56°C in water bath for 1 hour. Lastly the tubes were gently shaken for 2-7 days at 4°C in a balanced shaker (IKA-Labortechnik- England) to resuspend the DNA.

2-3-2 Interferon gamma (IFN-γ) genotyping

2-3-2-1 Solutions and Media

IFG1 primer

20 µl of the IFG1 primer at 100 pmol/µl (MWG-Biotech AG) were diluted in 80 µl sterile water (SH_2O) to make up a working solution (20pmol/ul) of IFG1 primer solution.

Primer sequence 5'- TTG TGA CTG TTA GCT AGA TGT GTT- 3'.

IFG2 primer

20 µl of the IFG2 primer at 100 pmol/µl (MWG-Biotech AG) were diluted in 80 µl SH_2O to make up a working solution (20 pmol /ul) of IFG2 primer solution.

Primer sequence 5'-ATA CAC ATA TTA TGC CCA TCT TTT-3'

dNTPs

5 µl of 100-mmol stock solution of dATP, dGTP, dTTP and dCTP (Pharmacia Biotech) was diluted in 180 µl dH_2O to make up 10 mmol dNTPs.

3% Metaphor agarose gel

4.5g of metaphor agarose (Bio Whittaker Molecular Applieations – BMA) were weighed out and added to 150 ml 1xTBE buffer to a 500 ml conical flask. The flask was then placed on a magnetic stirrer (Stuart Scientific Co.Ltd- UK). When the agarose and the TBE had mixed, the flask was covered with Saran wrap and placed in microwave. It was boiled for 2 minutes at full power. Then the agarose was dissolved by turning the microwave to full power and heating for 10 seconds at a time. When the agarose had fully dissolved, the flask was placed

on a stirrer at low setting and 20µl Ethidium bromide (10 mg/ml) was added to the agarose and the solution topped with dH2O to replace losses due to evaporation. After the solution had cooled to approximately 55°C, the gel was poured into a levelled casting tray plate and any bubbles which appeared were removed by a needle, and the comb was put in the gel. The gel was allowed to set at room temperature for 30 minutes and then for a further 30 minutes at 4°C.

2-3-2-1 Procedure

Polymerase chain reaction (PCR) amplification of lamb DNA with a master mix (MM) solution was prepared using Taq polymerase to perform this experiment (Schmidt et al. 1996). PCR master mix was prepared in PCR hood as shown in Table 2-1.

Table 2-1 Master Mix solutions for IFN-G genotyping

REAGENT	CONCENTRATION	AMOUNT
IFN1 primer	**20pMoles**	**0.8µl x n+1**
IFN2 primer	**20pMoles**	**0.8µl x n+1**
dNTPs	**10mlmol**	**1.6µl x n+1**
10x BUFFER	**N/A**	**1.6µl x n+1**
$MgCl_2$		**2.0µl x n+1**
H_2O	**N/A**	**12.0µl x n+1**
The MM solution was irradiated for five minutes in an ultra violet (UV) box.		
Taq		**0.2µl x n+1**
Sample DNA		**1.0µl per sample**
Negative (H_2O)		**1.0µl for negative control**

19 µl of MM was mixed with 1µl DNA and 19 µl MM with 1µl H_2O for the negative control. Samples were placed in a thermocycler (Gene Amp- PCR system2700 Version2.0-Biosystems A&B), and the PCR conditions were as follows:

	94.0°C for 9mins
30 cycles	94.0°C for 1min
	53.0°C for 1min
	72.0°C for 1min
	72.0°C for 7mins
	4°C for ∞

The PCR products were then analysed on 3% metaphor agarose gel. The comb was removed from the gel, and the gel was placed in a tank, then 1xTBE buffer was added until the gel was covered by approximately 3mm.

For each sample, 7µl PCR product was added and mixed with 3µl gel loading buffer, which was loaded to the well along with 10b.p DNA ladder and negative control. The gel was run at 5v/cm (150v), until the dye front had reached the end. The gel then was removed and photographed.

2-3-3 Cloning and sequencing of MHC class II DRB1 alleles

2-3-3-1 Solutions and Media

ERB3

20µl of ERB3 at 100 pmol/µl (MWG-Biotech AG) was diluted in 80µl sterile distilled water (SH_2O) to make up a 20 pmol/µl ERB3 primer solution.

ERB3 primer 5'- CTCTCTCTGCAGCACATTTCCT

SRB3

20µl of SRB3 at 100 pmol/µl (MWG-Biotech AG) was diluted in 80µl sterile distilled water (SH_2O) to make up a 20 pmol/µl SRB3 primer solution.

SRB3 primer 5'-CGCTGCACAGTGAAACTC

1.5% Agarose gel

0.750g Seakem® LE Agarose (Cambrex Bioscience, Rockland, Inc. Rockland, ME USA) was mixed with 50 ml 1× TBE buffer in a 200 ml Pyrex beaker on a magnetic stirrer which was set at a low level. The beaker was then placed in a microwave and heated for 2 minutes at high setting until the TBE and agarose had fully dissolved. The beaker was placed again on magnetic stirrer at low level setting to cool down and 5µl ethidium bromide was added to the agaroseand losses due to evaporation replaced. When the agarose temperature reached approximately 55°C, it was poured in a levelled gel-casting tray with the coomb removed and any air bubbles that appeared were removed by a needle. At this point the comb was put in the gel-casting tray and the gel allowed to set for 30 minutes in the cold room (4°C) before it was used.

X-Gal

100mg X-Gal (5-bromo-4-chloro-3-indolyl-β-D- galactoside\ LIFE TECHNOLOGIES™, Paisley, UK) was mixed with 2 ml dimethylformamide (Sigma-Aldrich) and stored at -20°C.

LB agar

5g LB broth (Lennox L broth, Sigma-Aldrich) and 3.750g Agarose (Seakem® LE Agarose-Cambrex Bioscience Rockland, Inc. Rockland, ME USA) was mixed with 250 ml dH_2O. The solution was autoclaved and allowed to cool in a water bath at 42°C, and a 125µl of 50µg/ml ampicillin (Sigma) was added and mixed properly with the solution. 25 ml of this mixture was poured into a sterile Petri dishes in sterile conditions (the quantity of this solution is sufficient for 10 plates).

LB broth

5g LB broth (Lennox L broth, Sigma-Aldrich) was mixed with 250ml dH_2O and was autoclaved. This solution was left to cool until it reached 55°C when 125µl ampicillin was added and mixed with the solution. The solution was kept at 4°C until required.

2-3-3-2 Procedure

Preparation of PCR reaction

PCR products (DRB1 EXON2 allele) were performed using lamb genomic DNA, Taq polymerase (Qiagen) and Master Mix (MM) solution (J. Buitkamp 2001- Personal communication). PCR master mix was prepared in a PCR hood as shown in Table 2-2.

Table 2-2 Master Mix solutions for MHC Class II DRB1 EXON2 genotyping

REAGENT	CONCENTRATION	AMOUNT
ERB3	20pmol	1.0µl x n+1
SRB3	20pmol	1.0µl x n+1
DNTPs	10mlmol	1.0µl x n+1
MgCL	N/A	2.0µl x n+1
10x BUFFER	N/A	5.0µl x n+1
H_2O	N/A	39.8µl x n+1
The MM solution was irradiated for five minutes in an ultra violet (UV) box.		
Taq polymerase		0.2µl x n+1
DNA sample		1.0µl per sample
Negative (H_2O)		1.0µl for negative control

49µl of MM were mixed with 1µl DNA and 49µl MM with 1µl H_2O for the negative control using 0.2ml ependorf tubes. Samples were placed in a thermocycler (Gene Amp- PCR system2700 Version2.0- Bio systems A&B). The PCR conditions were as follows:

1 cycle pre-PCR	94°C for 6min
	61°C for 2min
	72°C for 2min
35 cycles	94°C for 1min
	61°C for 2min
	72°C for 2min
1 Hold	72°C for 15min
1 Hold	4°C for ∞

The reaction products were analysed by 1.5% agarose gel electrophoresis. The comb was removed from the gel, and the gel was placed in a tank, then 1xTBE buffer was added until the gel was covered by approximately 3mm. For each sample, 7µl PCR product was added and mixed with 3µl gel loading buffer, which then loaded into the well along with 5µL of 100bp DNA ladder and negative control. The gel was then run at 100v, until the dye font reached the end. The gel was then removed and photographed in order to confirm the presence of the PCR products (DRB1 EXON II).

TOPO® Cloning Reaction

The PCR products were TOPO® cloned into the pCR®2.1-TOPO® vector (Table 2-3) and chemically competent *E. coli* cells were transformed with the recombinant vector according to the manufacturers instruction.

Table (2-3) TOPO® Cloning Reaction

REAGENT	REACTION VOLUME
Fresh PCR product	**2.0µl**
Salt Solution	**1.0µl**
Sterile Water	**2.0µl**
TOPO® Vector	**1.0µl**
Final Volume	**6.0µl**

The reaction was mixed gently in a 0.5ml microtube, and incubated at room temperature for 5 mins. The reaction mixture was placed on ice. 2µl of the cloning reaction was mixed gently with TOPO 10 competent cells and incubated on ice for 5 mins. The cells were then heat shocked for 30 seconds at 42°C in a water bath. Immediately afterwards the tube was placed on ice. 250µl of room temperature SOC medium was added to the tube which was tightly capped and rotated horizontally at 200 rpm at 37°C for 1 hour. 20µl of transformation mixture was mixed with 30µl of SOC medium and spread on a prewarmed LB-agar plates containing X-Gal and 50µg/ml ampicillin. Plates were incubated overnight at 37°C. Blue and white colonies were observed the following day. 10 white or light blue colonies were isolated and every single colony was cultured overnight in 3 ml LB broth containing 50µg/ml ampicillin in universal tubes at 37°C in an orbital shaker (225 rpm). Plasmid DNA was checked for presence of the correct insertion by PCR amplification.

Purification of plasmid DNA

PCR products were purified following the QIAprep spin miniprep kit protocol (QIAGEN). Briefly, the DNA adhered to the filter within the column, separating it from all other

components of the PCR reaction, which were washed away with various buffer solutions. The plasmid DNA was finally eluted in 50µl Elution buffer (EB).

Sample preparation for DNA sequence analysis

Cloned fragments were chemically labelled with fluorescent dyes. The dyes facilitate the detection and identification of the DNA. More specifically, PCR reactions were performed using plasmid DNA samples in a total volume of 20µl containing 3µl of primers, 4µl 10x buffer, 7µl sterile water, 2µl DNA and 4µl of Big Dye™ Terminator Cycle sequences ready reaction (ABI Prism). Samples were prepared in the thermocycler (Gene Amp- PCR system2700 Version2.0- Bio systems A&B), incorporating 25 cycles of amplification as follows:

25 Cycles	96°C for 10 seconds
	50°C for 5 second
	60°C for 4 minutes

DNA was then purified by precipitation methods using 16µl of deionised water and 64µl of non-denatured 95% ethanol and kept at room temperature for 15 minutes. Pelleted DNA (1300 rpm for 20 minutes) was washed in 250µl of 70% ethanol and re-pelleted (1300 rpm for 10 minutes) before all ethanol was removed and dried at 90°C for 1 minute. Template suppression reagent (25µl formamide) was then added, and left at room temperature for 2 minutes. The mixture was heated to 95°C for 2 minutes and chilled before 20µl of this mixture was transferred to genetic analyser sample tubes.

DNA sequencing

Samples were run on the ABI PRISM® 3100 Genetic Analyzer (PE Applied Bio systems, UK), under standard sequencing conditions. The ABI PRISM® 3100 Genetic Analyzer is a multi-colour fluorescence-based DNA analysis system using the proven technology of capillary electrophoresis with 16 capillaries operating in parallel. The Genetic Analyzer is fully automated from sample loading to data analysis.

Sequence evaluation

The output was in the form a chromatogram, which was examined with Chromas software and a sequence file which were analysed with Genetic Computer Group (GCG) software.

2-4 STATISTICAL ANALYSIS

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

CHAPTER THREE

DISTRIBUTION OF FAECAL NEMATODE EGG COUNTS AMONG LAMBS

3-1 INTRODUCTION

The McMaster technique was introduced by (Gordon and Whitlock 1939) as a diagnostic method to assess nematode egg production by infected animals. This technique has been widely used to examine infected sheep. Faecal egg counts (FECs) are a product of both the number and the fecundity of adult female worms (Stear et al. 1995b).

Faecal egg counts have been widely adopted as an indirect measure of host resistance (Eady et al. 1996), especially in the selection of sheep for nematode resistance (Watson et al. 1995; Bisset et al. 1992; Woolaston 1992; Woolaston and Windon 1991; Albers et al. 1987 and Windon and Dineen 1981).

Lambs naturally exposed to *T. circumcincta* by grazing contaminated pasture showed considerable variation in faecal worm egg count (Stear et al. 1995a) . Worm burden and FEC in grazing lambs are under genetic control (Bishop et al. 1996). Stear et al. 1997 have reported that genetically resistant hosts in a flock of grazing lambs reduce nematode egg production by decreasing worm fecundity.

The distribution of FEC and worm burdens among sheep follow a negative binomial distribution, in which a minority of the animals account for the majority of the worm burden or egg output (Stear et al. 1995a; Barger 1985; Roberts and Swan 1982; Donald 1968 and Hunter and Quenouille 1952). The negative binomial distribution is perhaps the most widely used distribution to describe parasite burden (Gregory and Woolhouse 1993).

There are number of limitations in using the McMaster technique. These limitations arise due to the variation between parasite species and their laying egg capacity, where *Teladorsagia*

species lay fewer eggs per worm per day compared to more prolific parasites such as *H. contortus* (Eysker and Ploeger 2000). Egg production is also density dependent, and the presence of high levels of infection can lead to low FEC. Stear and Bishop (1999) reported that egg production by female *T. circumcincta* declines as the number of these parasites increased. Faecal egg counts are also less reliable in older animals due to the ability of the host to regulate parasite egg output without necessarily reducing the worm number (Douch and Morum 1993). Other influences can include faecal throughput and consistency, diurnal patterns of egg production by adult worms, and the variability of egg distribution within the faecal sample (Douch et al. 1995).

Reducing FEC provides a means of decreasing pasture contamination with epidemiological benefits (Gray 1997; Woolaston and Piper 1996). Faecal egg counts can be cheaply and easily performed, and have therefore formed the basis for much of the research into the quantitative nature of parasite resistance.

Several factors influence faecal egg production and its variation among animals. These include date of birth, sire, dam, sex, year and month of sampling, history of exposure to infection and genetic differences (Stear et al. 1995a). Many studies have suggested that variation decreases as animals mature due to a decrease in parasite survival (Maizels et al. 1993). Understanding these factors and its role in faecal output is vital for development of control strategies.

Pasture larval counts have also been used to determine the intensity of challenge (Boag and Thomas 1971b; Stear et al. 1995a). The number of infective larvae ingested by sheep will depend upon the number of eggs deposited by infected sheep, the rate of development of eggs to larvae, the larval survival rate and the grazing pattern of sheep (Stear et al. 1995a). In

spring, infected ewes often exhibit a peri-parturient rise in output of parasite eggs and as spring temperatures raise the eggs develop to infective larvae. Infected lambs also contaminate the pasture. By late July and August, pasture contamination levels rises to become a significant threat to lambs (Gettinby et al. 1989). The herbage number of *T. circumcincta* L3 increases markedly from mid-summer onwards and this is when disease appears, as the eggs deposited in the first half of grazing season from April to June give rise larvae from July to October (Urquhart et al. 1996; Boag and Thomas 1971).

The objective of this study was to examine the distribution of faecal egg counts in Scottish Blackface lambs, and to determine the influence of sex.

3-2 MATERIAL METHODS

3-2-1 Experimental animals

The lambs came from a commercial upland farm in the Borders region in southeast Scotland. The lambs were all straightbred Scottish Black face. The lambs were born from mid-April to early May and weaned at 4 months of age. Lambs were sampled in August, September and October 2001 to 2003. Each of the 3 cohorts had approximately 250 lambs, in which, approximately equal numbers of castrated male and female lambs were used in this experiment.

3-2-2 Faecal samples collection

Faeces were taken directly from the rectum of the lambs. All faecal samples from 2001 to 2003 were collected monthly for three months from August to October. After each sampling, all lambs were treated with a broad-spectrum anthelmintic Ivermectin (Oramec, drench), or levamisole (Nilverm, Schering-Plough Animal Health), which were rotated between years.

3-2-3 Faecal worm egg counts

Faecal nematode egg counts were determined monthly from August to October in the three years using protocol 2-3-1.

3-2-4 Herbage analysis

Lambs were moved from one field to another, relying on the field condition. As the field exhausted by grazing lambs, sheep were moved to another herbage-rich field. To facilitate further analysis, we gave number to each field in each month that lambs were grazed, but it is not necessarily to be the same field number in the following months or years.

Pasture larval counts was determined using protocol 2-3-2.

3-2-5 Statistical analysis

Means and ranges were calculated with the means procedure of the SAS statistical package (SAS Institute, Cary, North Carolina, Version 8). Chi-square tests were used to examine the goodness of fit to the negative binomial distribution.

The negative binomial distribution has often been used empirically to describe the distribution of parasites among individuals (Anderson & May, 1992). The distribution is defined by its arithmetic mean and a shape parameter k, which is an inverse index of the extent of dispersion; as k decreases toward zero the distribution become more dispersed. The SAS program estimates the parameter k by the maximum likelihood method developed by (Bliss and Fisher 1953). The relationships among faecal egg counts obtained on different dates were estimated by the correlation coefficient using the correlation procedure on the SAS statistical package. Egg counts were transformed prior to statistical analysis by taking

the logarithm of the egg count plus 10, to avoid zero values and to produce approximately normally distributed data.

3-3 RESULTS

3-3-1 General distributions of faecal egg counts among lambs

Tables 3.1, 3.2 and 3.3 show the arithmetic mean, standard error of the mean and range of three successive years of values observed in faecal egg counts in August, September and October. Table 3.1 shows that the mean faecal egg count in 2001was 363.9 in August, rose to 408.2 in September, and fell to 278.2 in October.

Table 3-1 Eggs per gram of faeces in lambs sampled in 2001

Date of sampling	Number of animals	Mean	Std. Error of mean	Minimum	Maximum
August	240	363.9	26.5	0	2900
September	216	408.2	29.6	0	2388
October	229	278.2	19.1	0	1700

The pattern of faecal egg count in 2002 differed from 2001 (Table 3.2). In August, the mean was 260.4 then decreased to approximately 200 and fell slightly in October to 181.6.

Table 3-2 Eggs per gram of faeces in lambs sampled in 2002

Date of sampling	Number of animals	Mean	Std. Error of mean	Minimum	Maximum
August	248	260.4	26.7	0	3275
September	256	201.1	16.9	0	2550
October	261	181.6	13.4	0	1200

Table 3.3 shows the mean FEC in August 2003 was 150.7, then increased in September to 271.3, and decreased slightly to 252.7 in October.

Table 3-3 Eggs per gram of faeces in lambs sampled in 2003

Date of sampling	Number of animals	Mean	Std. Error of mean	Minimum	Maximum
August	**254**	**150.7**	**26.9**	**0**	**5325**
September	**257**	**271.3**	**18.3**	**0**	**1875**
October	**253**	**252.7**	**14.5**	**0**	**1238**

From the above, the mean faecal egg counts in August 2003 were slightly less than the two previous years. The range reveals that high counts can occur even in quite young animals.

Tables 3.4, 3.5 and 3.6 present the correlation among transformed faecal egg counts between months in three successive years. Most correlations were positive, but not all were significant. Table 3.4 demonstrate the correlation of faecal egg counts in 2001. There was a highly significant but weak correlation ($r = 0.18$, $p < 0.01$) between August and October, while there was a very highly significant correlation between August and September, and between September and October ($r = 0.35$, $P < 0.001$); ($r = 0.34$, $P < 0.001$) respectively.

Table 3-4 Correlations among transformed faecal egg counts in 2001

	August	September	October
August	-		
September	**0.35*****	-	
October	**0.18****	**0.34*****	-

** $P < 0.01$. *** $P < 0.001$.

Table 3.5 shows the correlations of faecal egg counts in 2002. There was a highly significant but very weak correlation between August and September (r = 0.12, P <0.01). Counts in September and October showed a very highly significant correlation (r = 0.25, P <0.001), while there was no correlation between August and October.

Table 3-5 Correlations among transformed faecal egg counts in 2002

	August	September	October
August	-		
September	**0.12****	-	
October	**0.00**	**0.25*****	-

** $P < 0.01$. *** $P < 0.001$

Table 3.6 shows the correlation of faecal egg counts in 2003. There was a very weak non-significant correlation between August and September (r = 0.07, P = 0.24). A weak but significant correlation (r = 0.11, P <0.05) was found among transformed faecal egg counts between September and October. The counts in August and October were weakly but highly significantly correlated (r = 0.17, P <0.01).

Table 3-6 Correlations among transformed faecal egg counts in 2003

	August	September	October
August	-		
September	**0.07**	-	
October	**0.17****	**0.11***	-

* $P < 0.05$. ** $P < 0.01$.

Tables 3.7, 3.8 and 3.9 provide the correlation coefficients that were calculated from log-transformed faecal egg counts within each month for August, September and October 2001.

All correlations were positive and statistically very highly significant ($P< 0.001$), indicating the accuracy of the technique used in this experiment.

Table 3-7 Correlations among transformed FECs in August 2001

	Log epg1	Log epg2	Log epg3
Log epg1	-		
Log epg2	0.91	-	
Log epg3	0.93	0.92	-
Log epg4	0.94	0.91	0.93

$P < 0.001$

Table 3-8 Correlations among transformed FECs in September 2001

	Log epg1	Log epg2	Log epg3
Log epg1	-		
Log epg2	0.90	-	
Log epg3	0.91	0.90	-
Log epg4	0.96	0.95	0.97

$P < 0.001$

Table 3-9 Correlations among transformed FECs in October 2001

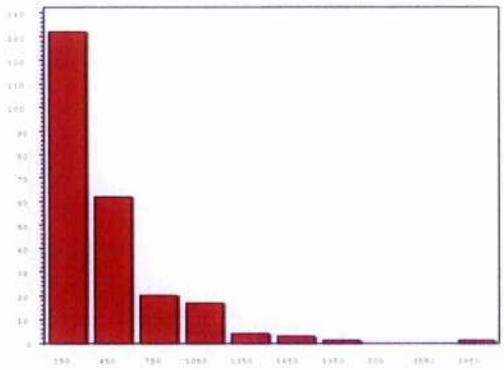
	Log epg1	Log epg2	Log epg3
Log epg1	-		
Log epg2	0.90	-	
Log epg3	0.87	0.90	-
Log epg4	0.93	0.95	0.90

$P < 0.001$

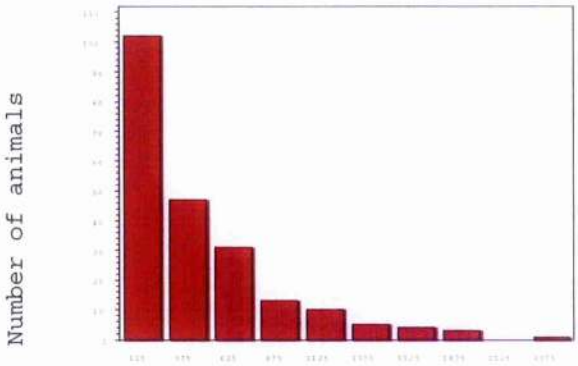
The distributions of faecal egg counts in each month in each year were positively skewed; most of the lambs had relatively few egg counts but a small number had very high counts.

Figure 3.1 (A, B and C) illustrates the skewed distribution of faecal egg counts of *T. circumcincta* among lambs in August, September and October 2001.

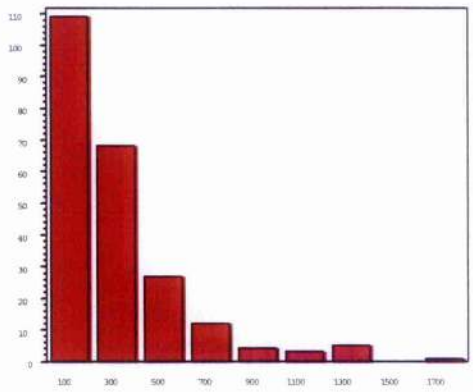
A



B



C



Faecal egg counts (epg)

Figure 3-1 Distribution of faecal egg counts in (A) August, (B) September and (C) October 2001

Tables 3.10, 3.11 and 3.12 present k the inverse index of dispersion for the negative binomial distribution and its standard error. The k value increased from August to October through September in all three years. The negative binomial distribution was fitted to the number of faecal egg count. Faecal egg counts sampled in September 2001 gave a good fit to the negative binomial, while the August and October samples did not (Table 3.10).

Table 3.10 Index of dispersion when negative binomial distribution were fitted to faecal egg counts of sheep sampled in 2001

Date of Sampling	Index of dispersion	SE	Goodness-of-fit probability
August	0.615	0.146	0.07
September	0.668	0.141	0.21
October	0.762	0.126	0.01

The distribution in September 2002 was not compatible with negative binomial, but the other two sets of samples in 2002 gave acceptable fits (Table 3.11).

Table 3.11 Index of dispersion when negative binomial distribution were fitted to faecal egg counts of sheep sampled in 2002

Date of Sampling	Index of dispersion	SE	Goodness-of-fit Probability
August	0.561	0.154	0.75
September	0.767	0.116	0.08
October	0.772	0.118	0.28

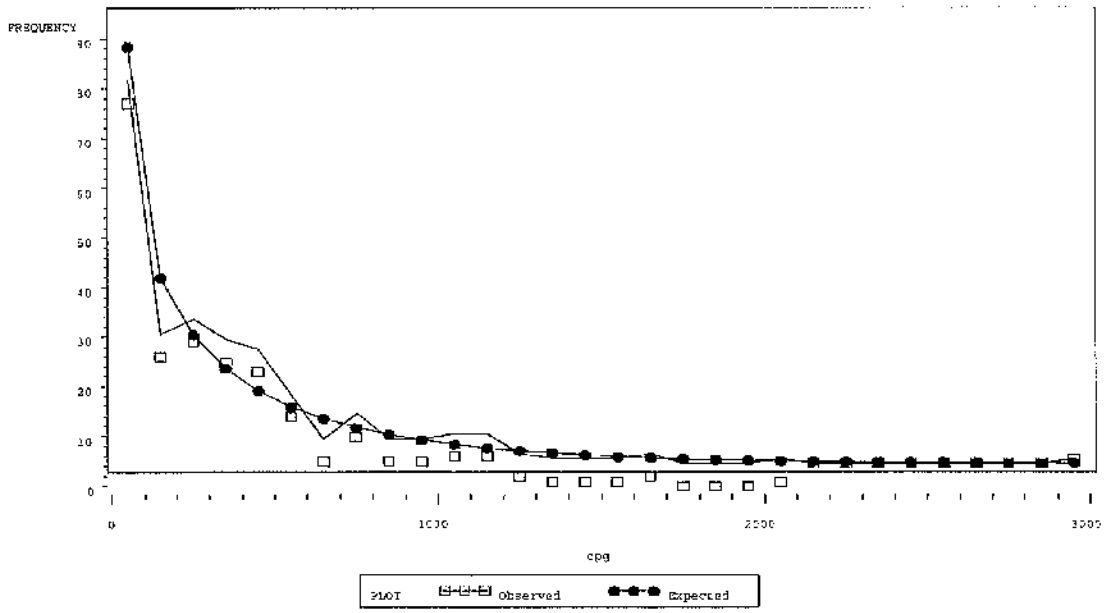
Faecal egg samples in September and October 2003 gave a good fit to negative binomial, but the August sample did not, also, k value was very low (0.199) in August 2003 and this was probably due to the high range of faecal egg counts (0-5325) (Table 3.12).

Table 3.12 Index of dispersion when negative binomial distribution were fitted to faecal egg counts of sheep sampled in 2003

Date of Sampling	Index of dispersion	SE	Goodness-of-fit probability
August	0.199	0.518	0.00
September	0.767	0.117	0.22
October	1.091	0.085	0.16

Fig 3-2 A- 3-2 J illustrates the negative binomial distribution in all three years.

A



B

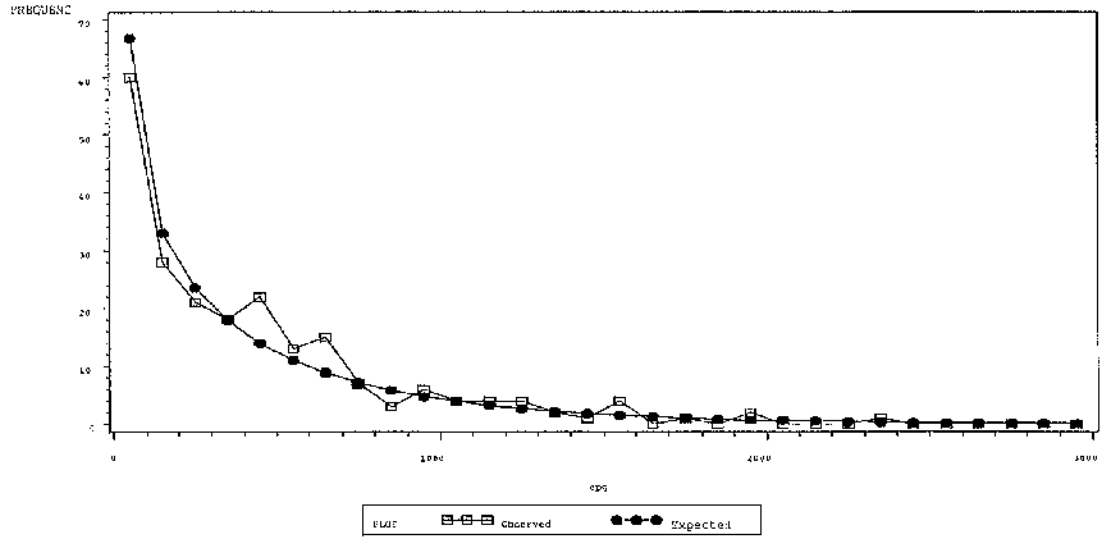
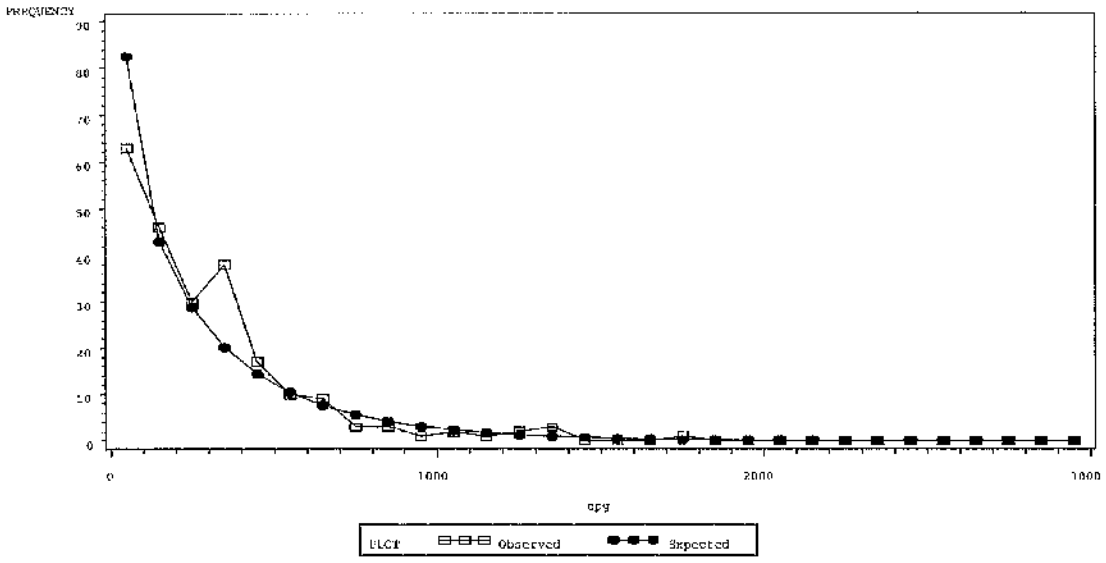


Fig 3-2 Negative binomial distribution in (A) August 2001 and (B) September 2001

C



D

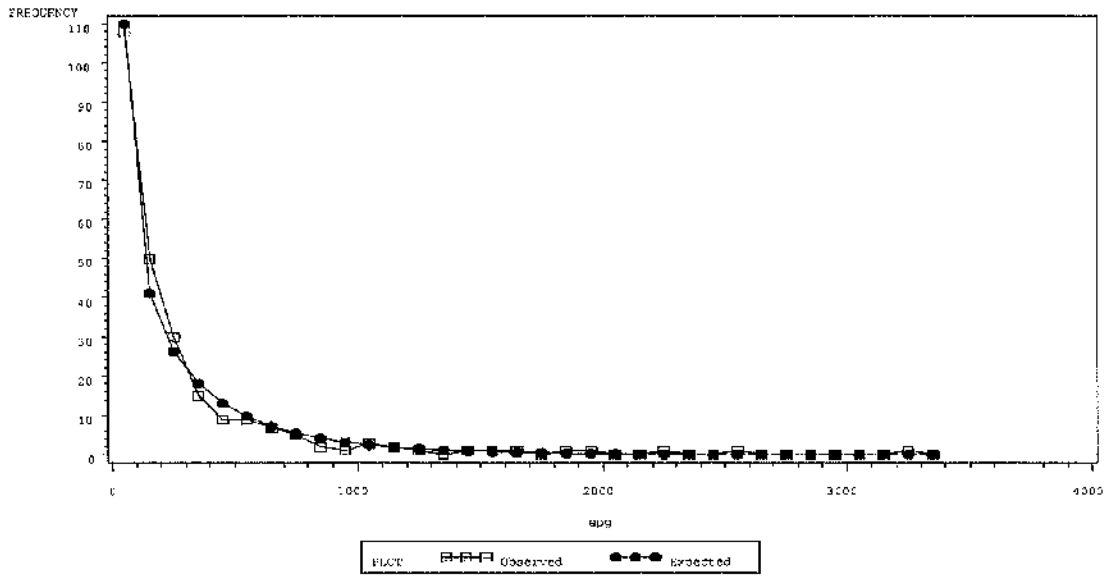
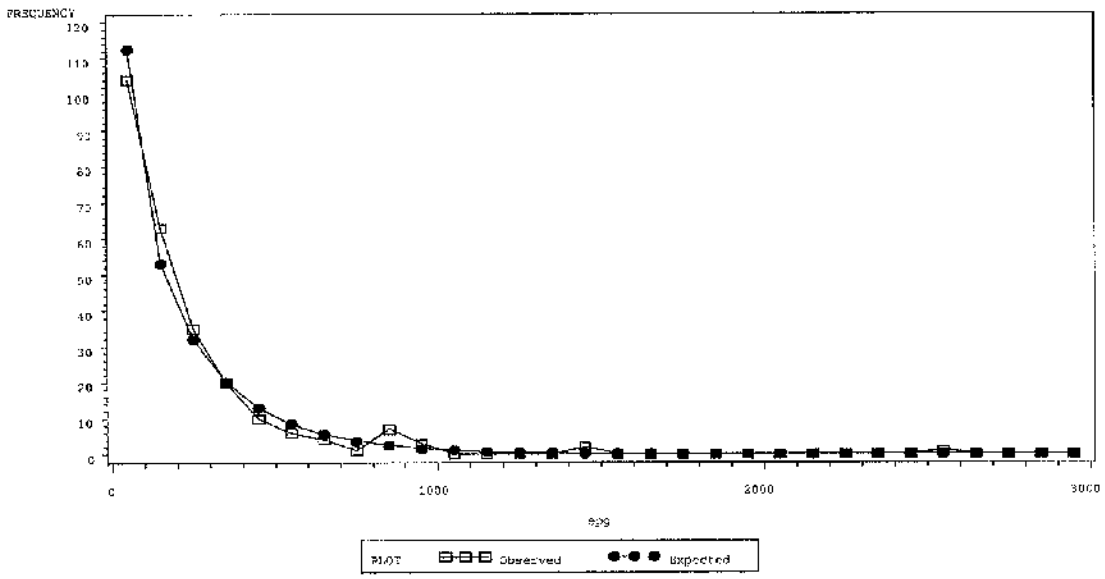


Fig 3-2 Negative binomial distribution in (C) October 2001 and (D) August 2002

E



F

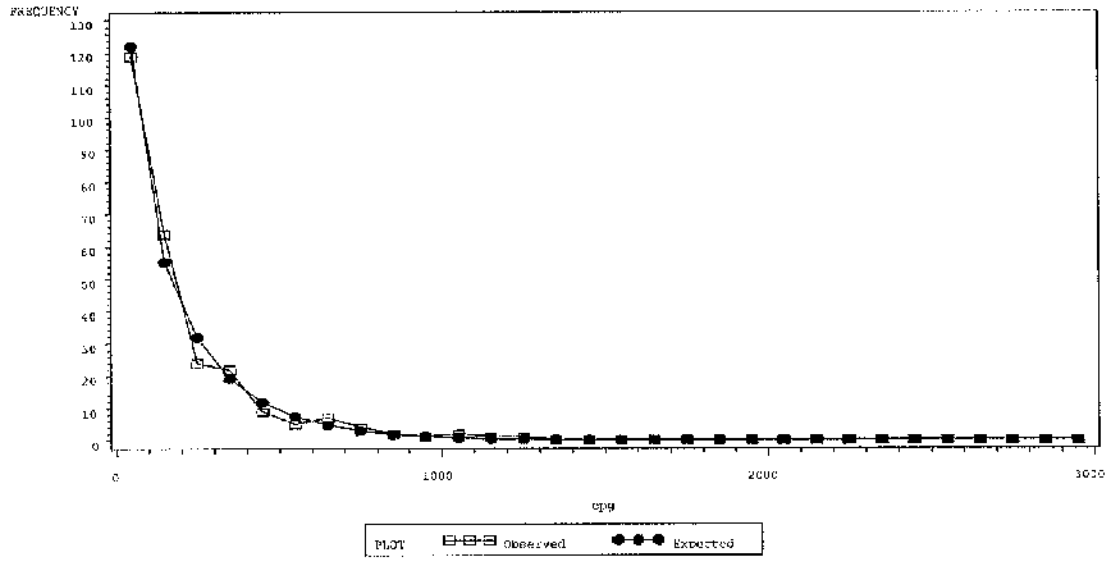
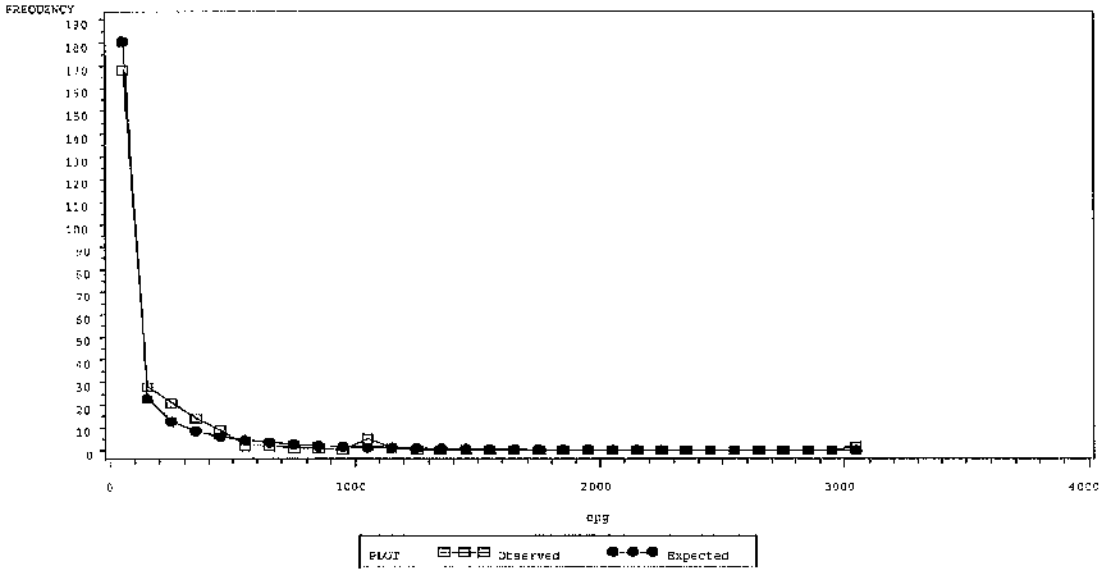


Fig 3-2 Negative binomial distribution in (E) September 2002 and (F) October 2002

G



H

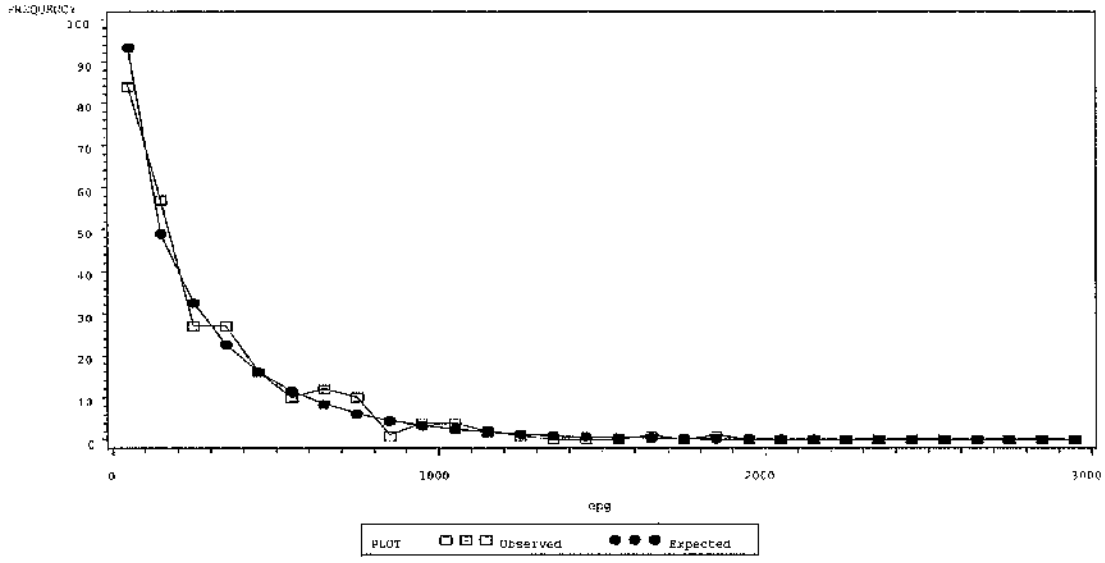


Fig 3-2 Negative binomial distribution in (G) August 2003 and (H) September 2003

J

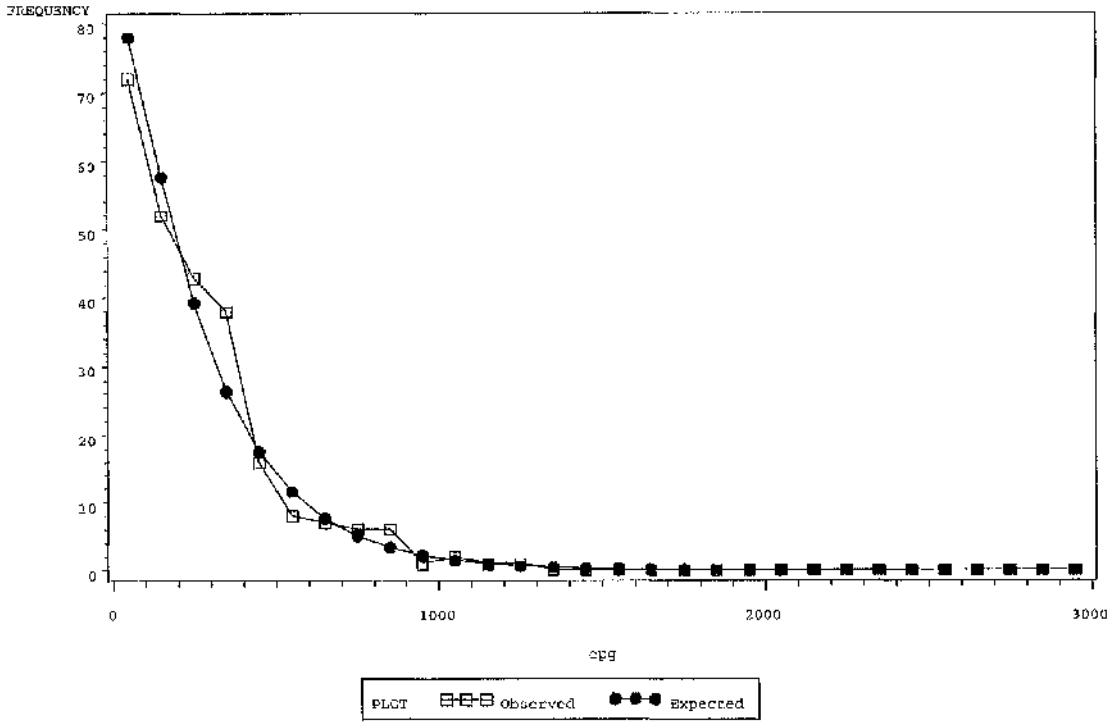


Fig 3-2 Negative binomial distribution in (J) October 2003

The pasture larval cultures showed that generally the overwhelming majority of larvae were *Teladorsagia*, but other larvae such as *Nematodirus*, *Ostertagia* and *Cooperia* were also observed with relatively low counts compared with *Teladorsagia* (Table 3-13, 3-14 and 3-15)

Table 3-13 Total pastural larval counts (13/kdh) in 2001

		Teladorsagia	*Ostertagia*	*Nematodirus*	*Cooperia*
August	Field 1	12	0	6	0
	Field 2	70	0	14	0
	Field 3	72	0	18	0
September	Field 1	100	130	11	0
	Field 2	56	72	40	0
October	Field 1	143	55	33	0
	Field 2	182	26	52	0

Table 3-14 Total pastural larval counts (13/kdh) in 2002

		Teladorsagia	*Ostertagia*	*Nematodirus*	*Cooperia*
August	Field 1	81	40	40	20
September	Field 1	35	0	53	0
October	Field 1	21	0	21	0

Table 315 Total pastural larval counts (13/kdh) in 2003

		Teladorsagia	*Ostertagia*	*Nematodirus*	*Cooperia*
August	**Field 1**	**55**	**0**	**147**	**36**
September	**Field 1**	**164**	**0**	**185**	**65**
	Field 2	**142**	**33**	**44**	**0**
October	**Field 1**	**59**	**39**	**78**	**39**

3-3-2- Sex differences in transformative faecal egg counts

Differences between the sexes were estimated by least square means from models which fitted year and sex. Table 3-16, 3-18 and 3-20 give the mean, standard error and range of values observed in transformed faecal egg counts between male and female lambs in August, September and October over three years (2001,2002,2003). There was no significant differences between the two genders in August when the data were pooled across years (Table 3.16), but there was a very highly significant year effect *(P>0.001)* on this month (Table 3.17), indicating that the egg counts differed between years.

Table 3-16 Sex differences in transformed faecal egg counts in August 2001/02/03

Effect	Nmber of animals	MEAN	SE	Minimum	Maximum
Male	337	1.97	0.039	1.89	2.04
Female	341	1.98	0.038	1.90	2.05

Table 3-17 Influence of year and sex on transformed faecal egg counts in August 2001/02/03

Effect	DF	F Value	Pr>F
Year	392	51.65	0.0001
Sex	392	0.08	0.775

For transformed faecal egg counts in September, there were again no significant differences between male and female lambs (Table 3.18), with a very highly year effect *(P>0.001)* (Table 3.19).

Table 3-18 Sex differences in transformed faecal egg counts in September 2001/02/03

Effect	Nmber of animals	MEAN	SE	Minimum	Maximum
Male	339	2.22	0.045	2.13	2.31
Female	336	2.15	0.045	2.06	2.24

Table 3-19 the influence of year and sex on transformed faecal egg counts in September 2001/02/03

Effect	DF	F Value	Pr>F
Year	381	10.34	0.0001
Sex	381	2.11	0.146

October revealed a significant sex difference *(P>0.05)* (Table 3.20), with no significant year effect (Table 3.21).

Table 3-20 Sex differences in transformed faecal egg counts in October 2001/02/03

Effect	Nmber of animals	MEAN	SE	Minimum	Maximum
Male	354	2.16	0.055	2.05	2.27
Female	348	2.08	0.055	1.97	2.19

Table 3-21 the influence of year and sex on transformed faecal egg counts in October 2001/02/03

Effect	DF	F Value	PR>F
Year	398	2.88	0.057
Sex	398	3.95	0.047

The distribution of transformed faecal egg counts in male and female lambs of each month over three years revealed that the majority of the lambs had relatively few egg counts and a small number had very high egg counts (Fig 3-3- 3-11) and the variations in both genders varies among years.

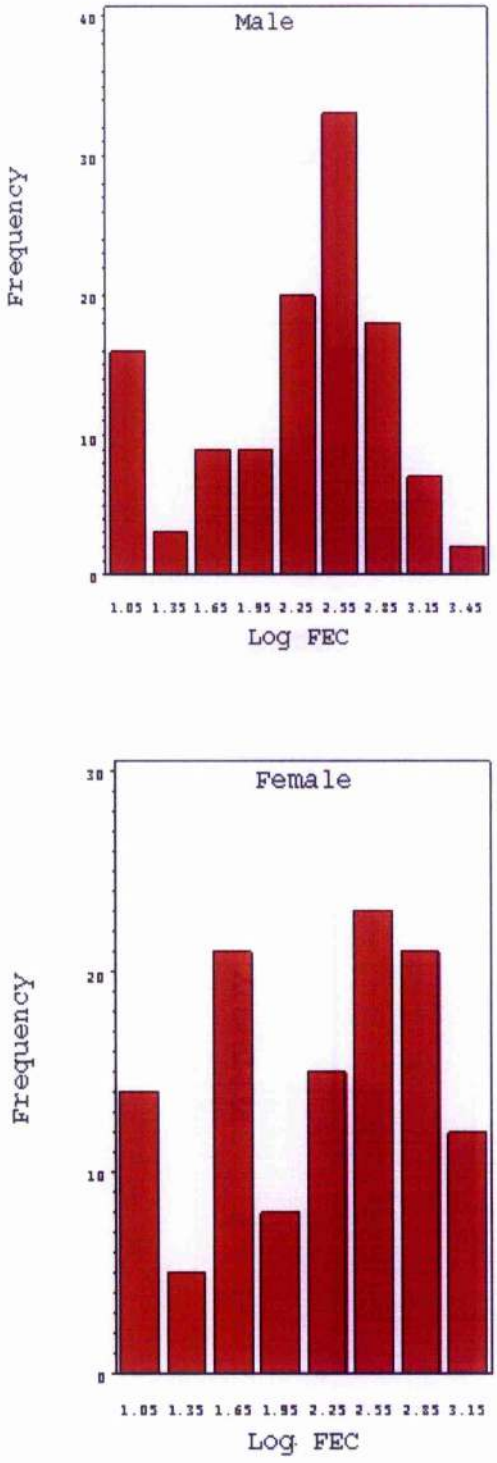


Fig.3-3 Distribution of Log egg counts in male and female lambs - August 2001

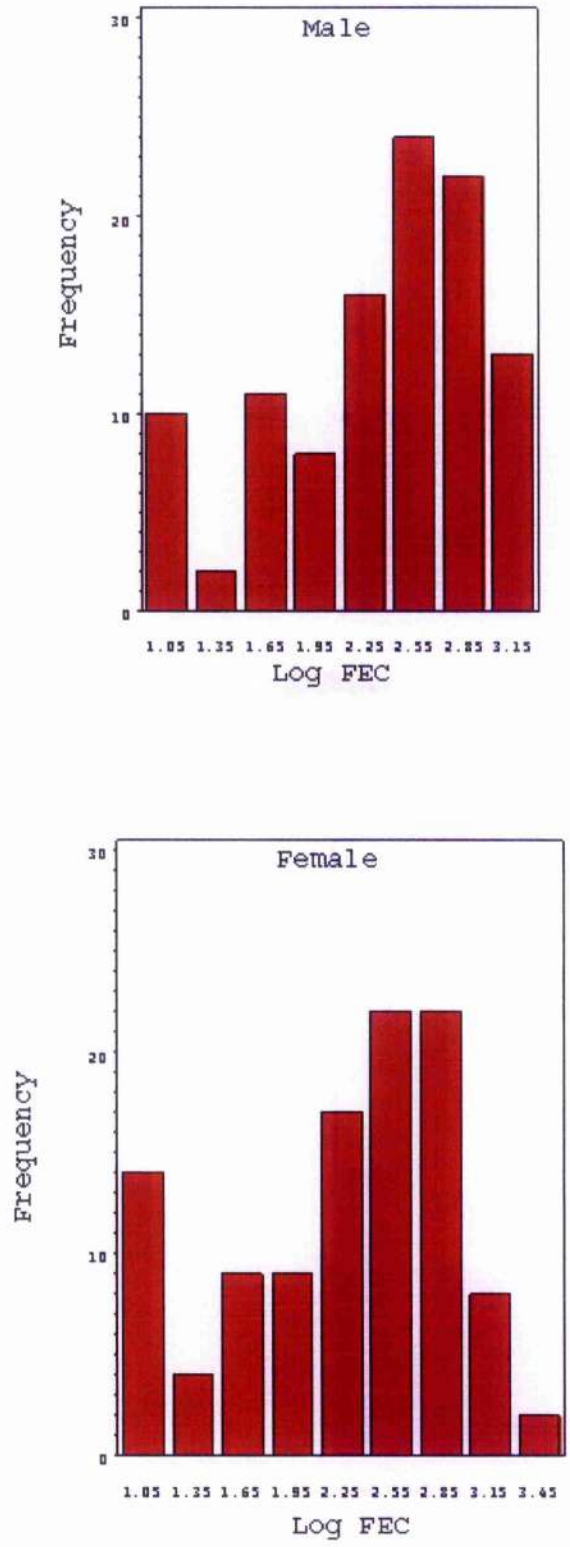


Fig. 3-4 Distribution of Log egg counts in male and female lambs – September 2001

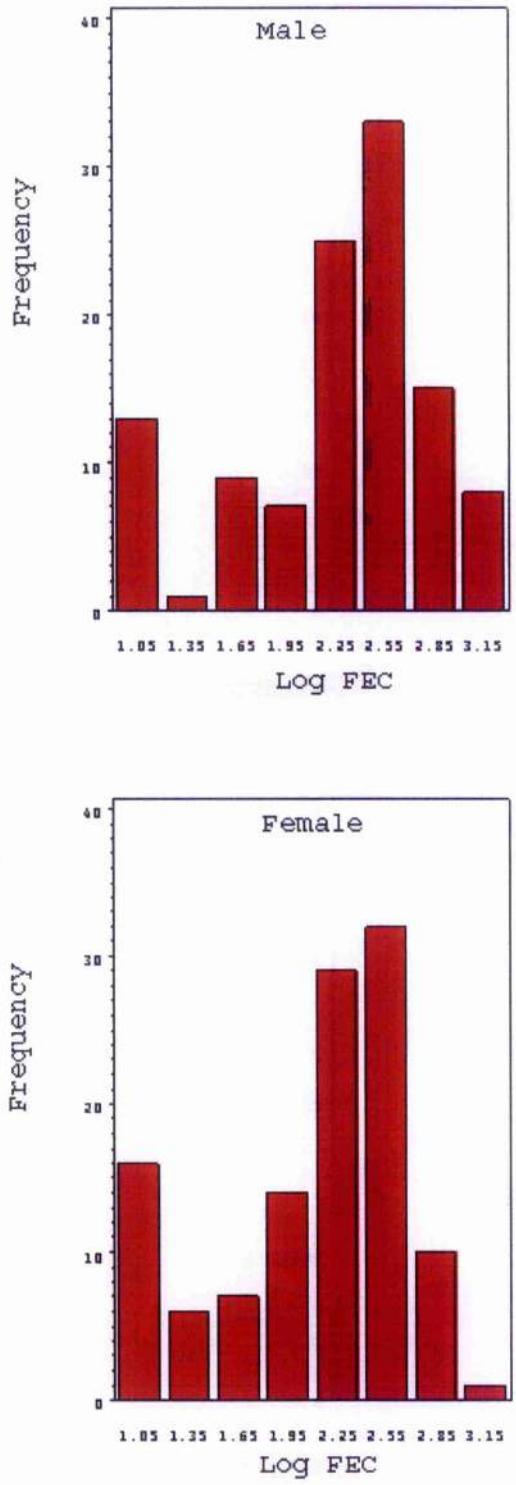


Fig.3-5 Distribution of Log egg counts in male and female lambs – October 2001

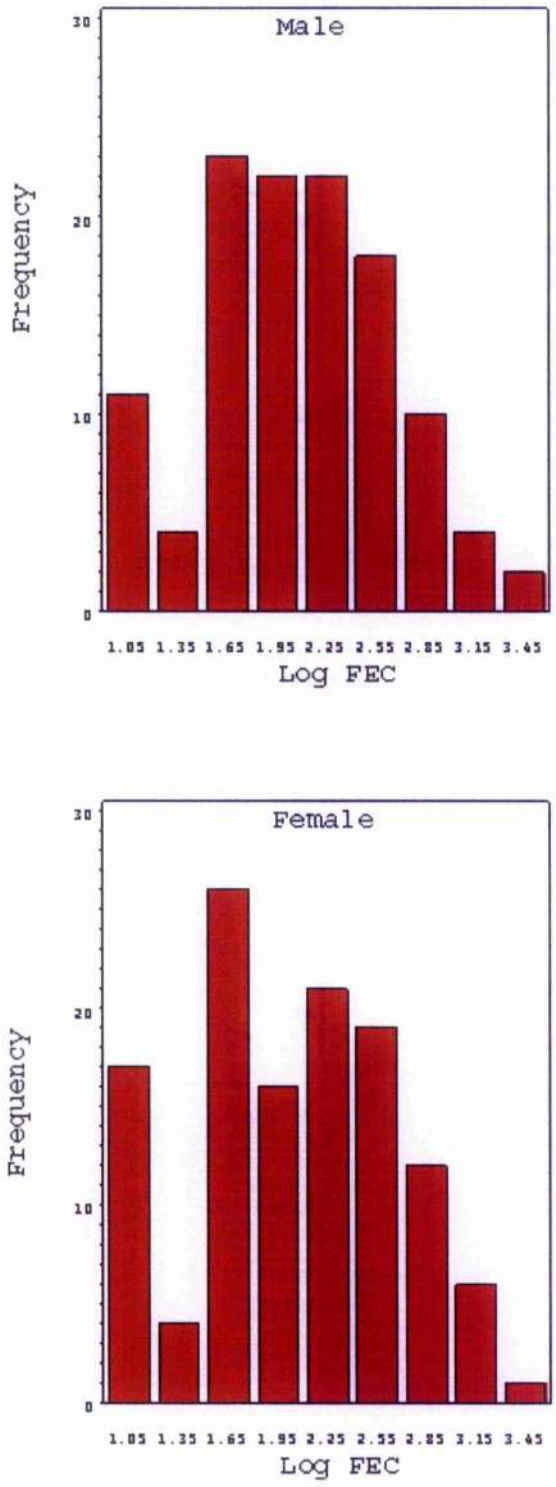


Fig.3-6 Distribution of Log egg counts in male and female lambs – August 2002

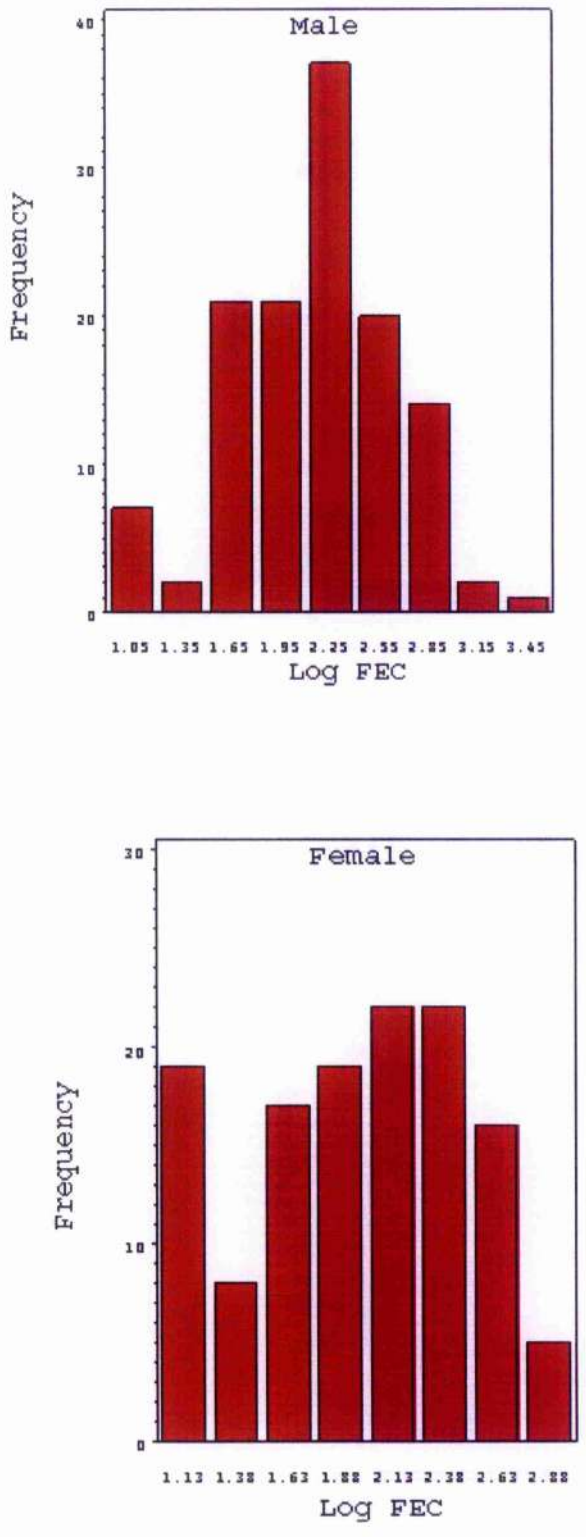


Fig.3-7 Distribution of Log egg counts in male and female lambs – September 2002

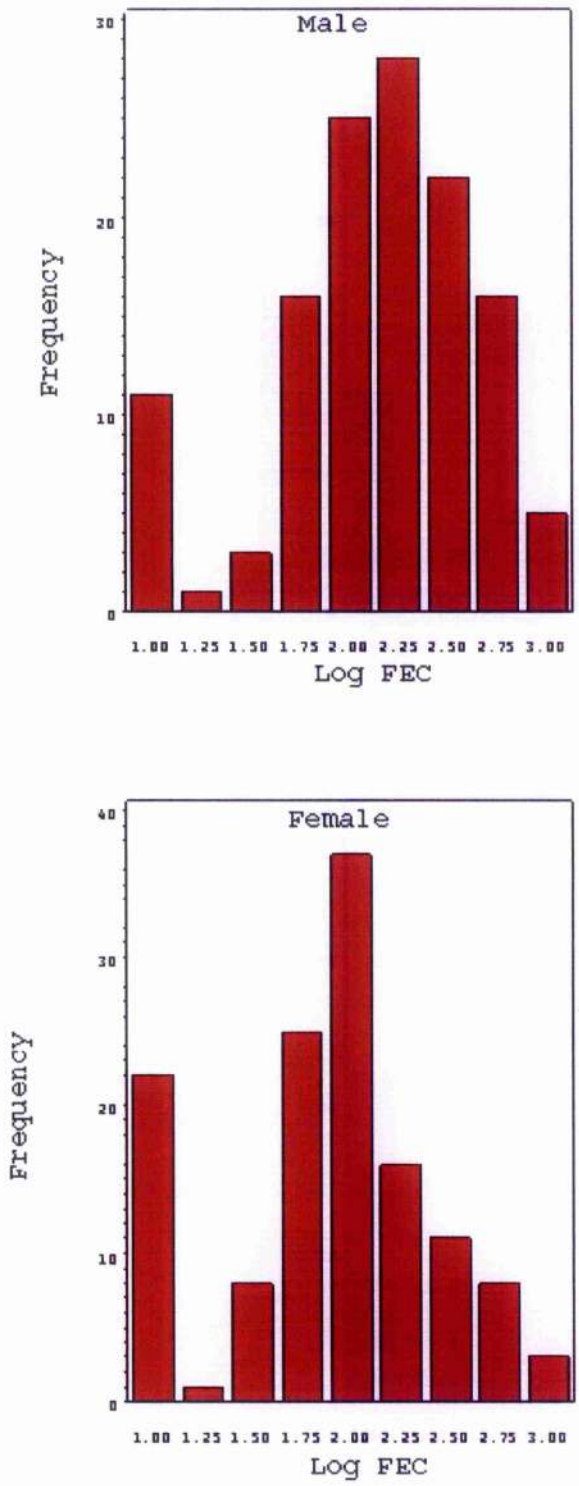


Fig.3-8 Distribution of Log egg counts in male and female lambs – October 2002

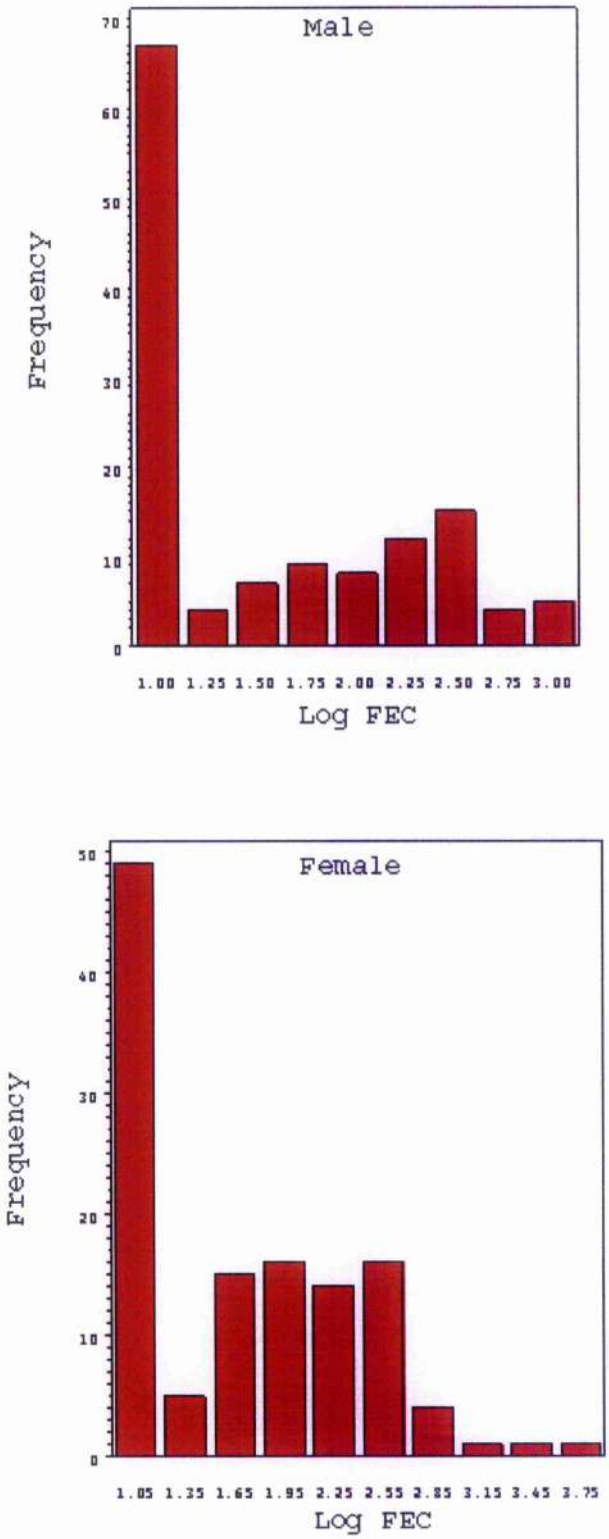


Fig.3-9 Distribution of Log egg counts in male and female lambs – August 2003

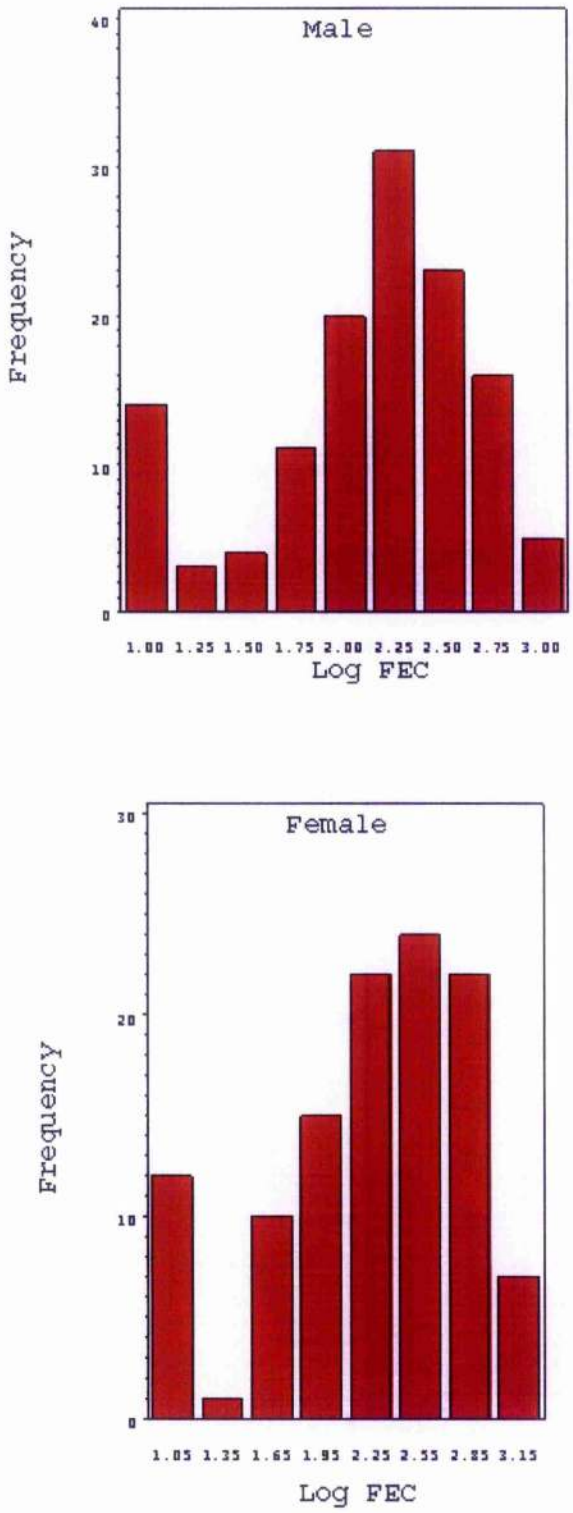
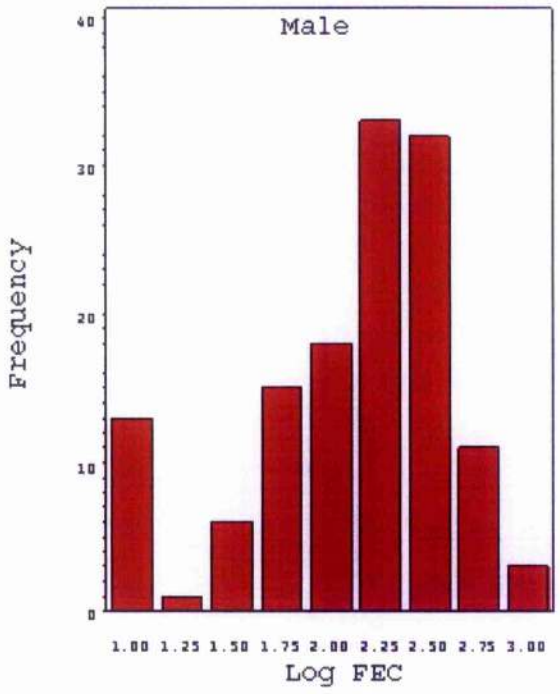


Fig.3-10 Distribution of Log egg counts in male and female lambs – September 2003



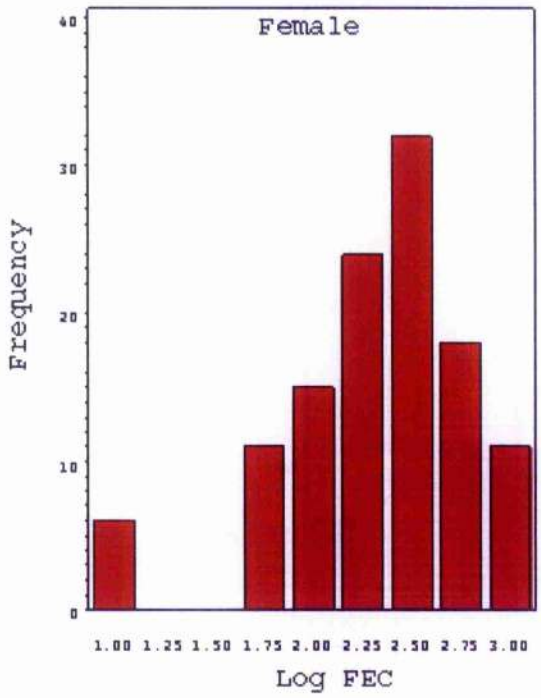


Fig.3-11 Distribution of Log egg counts in male and female lambs – October 2003

3-4 Discussion

The mean and distribution of faecal egg counts in lambs of Scottish Blackface sheep following natural infection with gastrointestinal nematodes changes over times. A longitudinal study of faecal egg counts was made in naturally infected Scottish Blackface sheep over three years at monthly intervals between August to October. This study has shown that there was no discernible pattern to egg counts within each season; each year out of the three had its own pattern. The results of these experiments are not strictly comparable with other studies because lambs of these experiments were given monthly anthelmintic treatment after each collection of faecal samples. However, the infection pattern is similar to that described by Thomas and Boag (1972) and by Boag and Thomas (1973).

The factors that influence these changes in faecal egg counts from month to month and from year to year are possibly grazing management, anthelmintic treatment and differences in the weather especially temperature and humidity which influences the development of infective larvae ingested by lambs (Stear et al. 1995a).

The mean egg count in October is lower than that in September in each year which may reflect the development of host resistance (Smith et al. 1985) and possibly differences in the number of infective larvae ingested. The correlations among FECs over the three years were significant but they were relatively small. The correlations were significant between August and September during 2001 and 2002, similar results were obtained by Stear et al. (1995a) who reported that the correlations were only significant and positive between August and September and not in preceding months. However, in our data there was a non-significant correlation in 2003. The correlations were significant between September and October for all three years. This result means that lambs with high egg counts in September tend to have higher egg counts in October. A comparison of the negative binomial parameter k increased

from August to October in each year, indicating that the distribution became less dispersed as lambs matured; this result agreed with that of Maizels et al. (1993) who stated the amount of over dispersion is less in the older populations, but disagreed with earlier results by Stear et al, (1995a), who reported that the amount of variation increased as the lambs matured.

However, in August 2003 the *k*, the inverse index of dispersion, was very low. This low value of *k* was observed because two lambs had a very high faecal egg count (3125 and 5325 epg) which gave a very high range in August. These results suggest that an exceptionally small proportion of animals were producing most of the total pasture contamination in this month.

Egg counts in males were consistently higher than females but the differences were only significant in October ($P>0.05$), which means that as animal mature, differences start to appear; this result is similar to a study by Stear et al, (1995a), who reported that female lambs egg counts were lower than in males but the differences were only significant from August. There was a very highly significant year effect in August and September ($P>0.0001$).

These differences between females and intact castrated males could be due to the influence of female endocrine hormones on the immune system (Barger 1993). Numerous interactions between the endocrine system and the immune system have been described (Khosraviani and Davis 1996). Growth hormone, insulin-like growth factors and prolactin play an important role in the growth, maintenance, repair and function of the immune system (Clark et al. 1997). Prolactin has a variety of physiological effects on growth and reproduction in seasonal breeding species, and also in the timing of puberty in female vertebrates (Nicoll 1974; Noakes et al. 2001). Prolactin can induce interleukin-2 receptor expression in

splenocytes and can potentiate the proliferation of ovine peripheral blood mononuclear cells (Khosraviani and Davis 1996). Thus females express better immune responses than males, which may be due to the role of the prolactin in immune response. Alternatinely it may be the differences in grazing behaviour. The influence of sex on ruminant susceptibility to other gastrointestinal nematodes has been reviewed by Barger (1993).

In conclusion there was considerable variation between male and female lambs to faecal egg counts. Female lambs appeared to be more resistant to *T. circumcincta* than male lambs at 6-months of age.

CHAPTER FOUR

VARIATION IN FAECAL EGG COUNTS AMONG LAMB POPULATIONS

4-1 INTRODUCTION

Faecal egg counts are widely used to estimate the relative susceptibility of infected sheep to nematode infection (Bisset et al. 2001;Woolaston and Windon 2001). The dominant nematode species in cool, temperate climates such as the UK, is *T. circumcincta* (Stear et al. 1998), and faecal egg counts following natural infection are used to guide selection decisions when breeding sheep for nematode resistance. Faecal egg counts following natural infection are very variable both within and between populations but several issues remain unresolved. Faecal egg counts in some sheep populations show a good fit to the negative binomial distribution while others do not (Stear et al. 1995a). However, the reasons for this are unclear. The lack of consistency hinders the application of general linear mixed models for data analysis. The variation among populations has not been quantified yet and an assessment of variation would assist the design of selection schemes that use several different farms. Furthermore some populations show much higher levels of variation among animals than others. Understanding the reasons for this variation would lead to better characterisation of resistant animals and could help to identify the mechanisms underlying resistance.

The aim of this study was to investigate the poor fit of the negative binomial to egg counts in lambs, to quantify the variation in faecal egg counts among populations and to examine the sources of this variation.

4-2 MATERIAL AND METHODS

4-2-1 Experimental animals

42 cohorts from two upland farms in Scotland; one in west central Scotland and one in the Borders region in southeast Scotland were used in this study. The sheep were all straightbred Scottish Blackface. The lambs were born in late spring (April and May) and

weaned at 3 or 4 months of age. The management regime on the first farm has been previously described (Stear et al. 1995a). On this farm, the lambs were sampled every 28 days from 8 to 24 weeks of age from 1990 to 1996. Additionally lambs born in 1992 to 1995 were also sampled at 4 weeks of age. Over half the animals were necropsied at 30 or 31 weeks after the last sample date. All lambs were given a broad spectrum anthelmintic (albendazole sulphoxide) according to the manufacturer's recommendations every 4 weeks from 4 to 20 weeks of age. The second farm population has been described in chapter 3. Each of the seven cohorts from the first farm consisted of 200 lambs while each of the three cohorts from the second farm had approximately 250 lambs. Only 70-95% of lambs were sampled on most occasions due to deaths, missing records, lost tags and insufficient faeces in the sample. As the four populations sampled at necropsy contained fewer lambs than the other populations they were not included in the initial assessment of means and variances.

4-2-2 Parasitological methods

The parasitological methods are described in chapter two. In addition replicate aliquots from the same faecal preparation were counted in September 1993, October 1993, October 1994, and all samples from 1995 onwards.

Standard parasitological procedures were used at necropsy to identify and count all nematodes present in the abomasum and small intestine (Armour et al. 1966; Stear et al. 1998) in lambs from the first farm. The large intestine was not examined as the frequency of anthelmintic treatment would prevent any large intestinal parasites surviving to the egg laying stage (Stear et al. 1998).

4-2-3 Pasture larval counts

The procedures used to obtain pasture larval counts methods have been described in Chapter two.

4-2-4 Statistical analysis

The SAS suite of statistical programs version 8.2 was used for all analyses (SAS Institute, Cary, N. Carolina). The univariate procedure was used to estimate means, variances, standard deviations and ranges for each population sampled on each occasion. When replicate aliquots were counted, only the first aliquot was used to estimate means, variances and the mean-variance relationship. There was an outlying population with a relatively low mean egg count of 201 epg but a high variance of 831,550 eggs per gram^2. This sample was taken in May 1994 and comprised only 88 lambs. All other samples had over 140 animals. This outlying population was discarded from further analyses. The distributions of means and variances were both skewed to the right. Gamma distributions were fitted to the data with the Capability procedure in the SAS/QC suite of programs. The gamma and lognormal distributions have three parameters: threshold, scale and shape. The threshold parameter was set to zero while maximum likelihood estimates of the scale and shape parameters were calculated iteratively by the Newton-Raphson approximation. Goodness of fit was tested by the Anderson-Darling statistic; this test belongs to the quadratic class of empirical distribution function statistics (D'Agostino and Stephens 1986).

The relationship between the mean and variance was estimated by fitting a regression line between log transformed variance and log-transformed mean: log(variance) = $\alpha + \beta$ *log(mean) (Perry 1981;Taylor 1961). This regression was fitted with the GLM procedure in SAS. When back-transformed, this gives a power relationship of the form: variance = a * mean **b.

Among nematode eggs, only Nematodirus spp. are counted separately. Eggs from the other species cannot be distinguished from each other and their eggs are counted together. Multiple regressions with the SAS GLM program was used to examine the relationship between the non-Nematodirus egg count and the number of nematodes present from the five common taxa: *T. circumcincta*, Cooperia spp., *T. vitrinus*, *T. axei* and *H. contortus*.

There is a nonlinear relationship between the number of adult *T. circumcincta* in the abomasum and the number of eggs produced by this species (Bishop and Stear 2000; Stear and Bishop 1999). The egg output depends upon the number of adult nematodes and their mean egg output. The mean egg output is strongly associated with the mean length of the adult female worms (Stear and Bishop 1999). The mean egg output per worm was estimated as worm length to the power 0.4 multiplied by 1.12; one was subtracted from the sum. The predicted egg output was then calculated by multiplying the total number of adult worms by the mean egg output per worm. The predicted egg count for *T. circumcincta* was subtracted from the observed egg count to create a residual egg count. Multiple regressions were then used to examine the relationship between the residual egg count and the number of nematodes from the four taxa: Cooperia spp., *T. vitrinus*, *T. axei* and *H. contortus*. Due to the presence of negative numbers 1000 was added to all residual counts prior to log transformation.

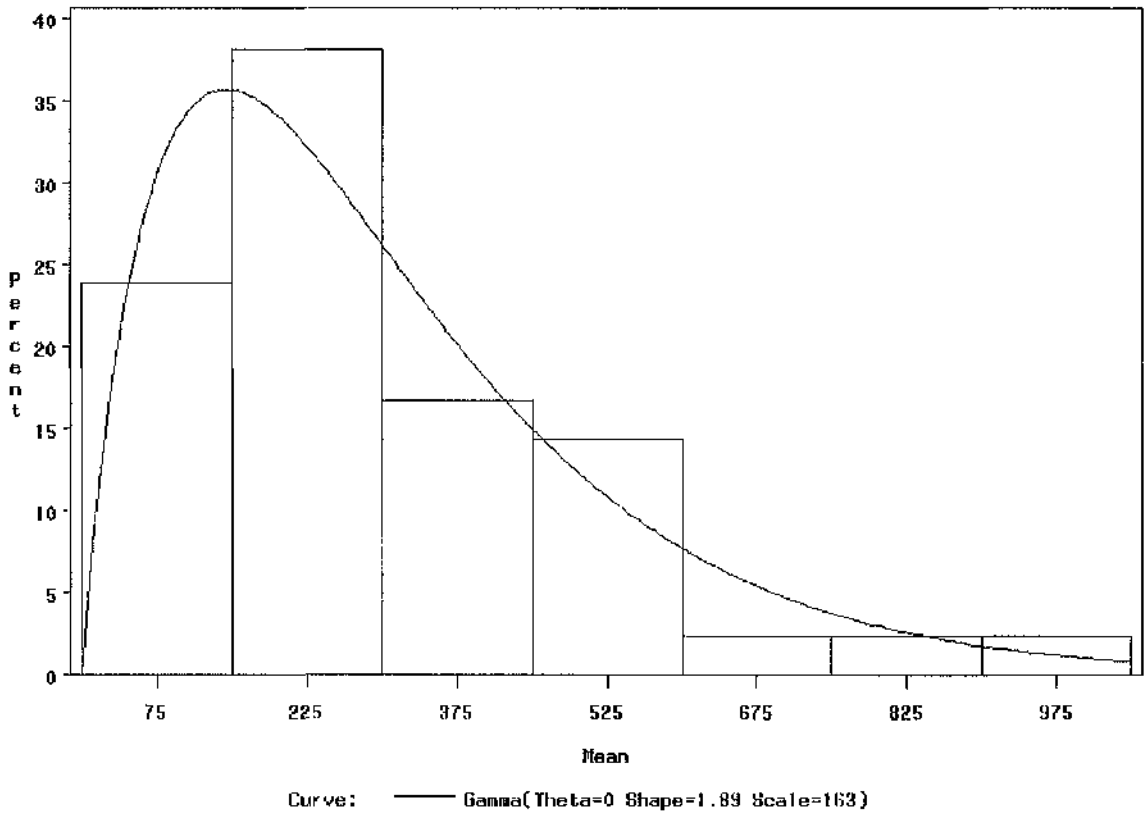
Generalised linear modelling was carried out with the GLIMMIX macro in SAS (Littell et al. 1996).

4.3 RESULTS

Fig 4.1 demonstrates the distribution of mean egg count among the populations sampled. Each of the 42 populations sampled represents the mean egg count for a particular cohort on

one farm at a single date. The distribution was right skewed with a mean of 305 and a median of 255 eggs per gram. The data appeared to follow a gamma distribution (Anderson-Darling statistic $p > 0.50$). Maximum likelihood estimates for the shape and scale parameters were 1.89 and 163 respectively. The gamma distribution with these parameters has been superimposed on the histogram (Fig 4.1).

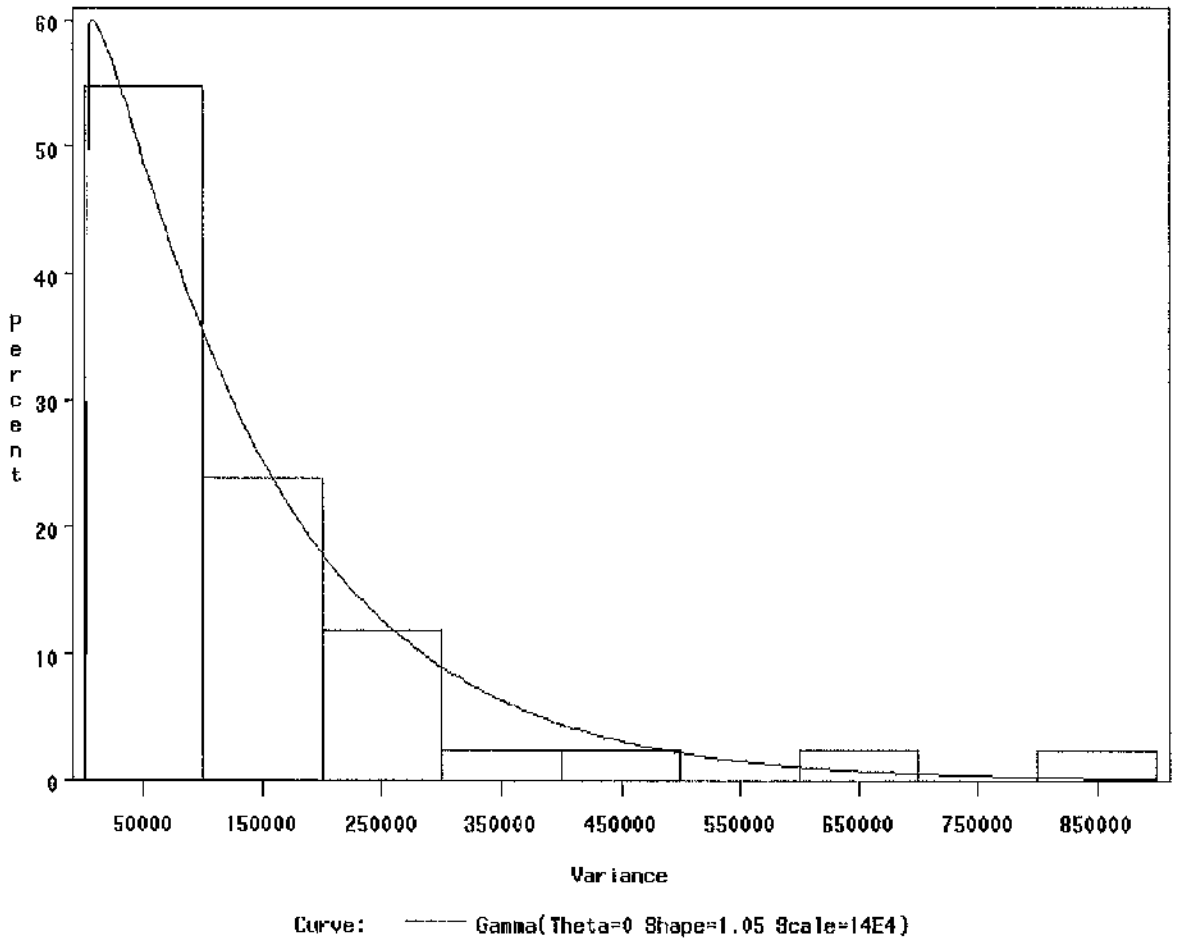
Fig 4-1 The distribution of mean egg counts



The distribution of the variances of the egg counts is presented in Fig 4.2. This distribution was also right skewed. Most populations had relatively small variances but a small number had quite high variances. The median variance was 89890 and the mean variance was higher at 162743 eggs per gram^2. As with the distribution of means, the distribution of variances among the sampled populations appeared to follow a gamma distribution (Anderson-Darling

statistic p = 0.169). Maximum likelihood estimates for the scale and shape parameters were 139462 and 1.05. The gamma distribution with these parameters has been superimposed on the histogram (Fig 4.2).

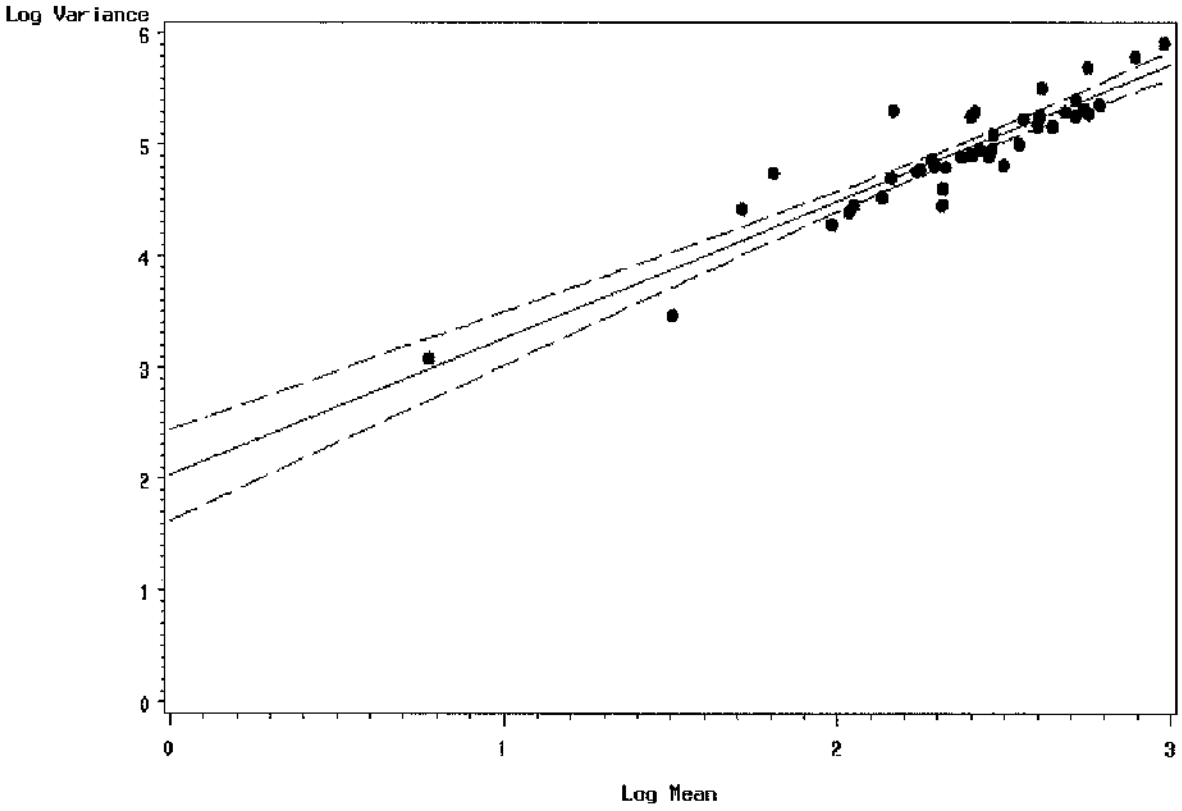
Fig 4-2 The distribution of egg count variances



Regression analysis (Fig 4.3) demonstrated that the variance = 120 * (mean epg) $^{1.23 \pm 0.08}$. The 95% confidence limits on the scalar term were 39 and 372. Fig 4.3 illustrates this relationship between the mean and the variance. The R-square value was 0.84, indicating

that variation among populations in their variances largely reflected variation among populations in their mean egg counts.

Fig 4-3 The relationship between transformed egg count mean and variance. Means and variances were transformed by taking logarithms to the base 10. The solid line represents the regression and the dotted lines represent 95% confidence limits.



An additional four populations from farm one were examined at necropsy. Table 4.1 presents the mean egg count and variance for these four populations as well as the number of species present. As the numbers of fourth and fifth-stage larvae do not influence the egg count they have not been included in table 4.1. Larvae were found for only *T. circumcincta*, *Cooperia* spp. and *T. vitrinus*. Most fourth-stage larvae will be inhibited but a small number

may arise from recent infection. In 1992, the mean numbers of fourth-stage and fifth-stage larvae in each lamb were respectively 5738 and 574 for *T. circumcincta,* 2 and 7 for Cooperia spp. and 4 and 5 for *T. vitrinus.* In 1993, mean numbers of fourth-stage and fifth-stage larvae were respectively 528 and 100 for *T. circumcincta,* 18 and 19 for Cooperia spp. and 1 and 1 for *T. vitrinus.* In 1994 and 1995 all recovered larvae were *T. circumcincta.* There were 705 and 31 fourth and fifth-stage larvae in 1994 and 3221 and 82 respectively in 1995.

Table 4.1 demonstrates that the variance increased as the egg count increased in line with the previous analysis. The interesting feature of table 1 is that high egg counts and high variances are not due to high numbers of adult nematodes *per se* but to high numbers of species other than *T. circumcincta.* For example the lowest egg counts (87 epg) occurred in 1992. This year had the second highest mean number of nematodes (6860) but over 95% of these were *T. circumcincta.* In contrast, the mean egg counts were much higher in 1993 (317 epg) and 1994 (494 epg) but the number of adult nematodes was much lower at 3309 and 2336 respectively. However, the proportion of *T. circumcincta* was lower at 84% in 1993 and 66% in 1994. Together, these results suggest that high means and variances in faecal egg counts in October at the end of the grazing season are not due to high intensities of infection but to the presence of species other than *T. circumcincta.*

Table 4-1 The mean and variance of faecal egg count and the number of adult nematodes present at necropsy.

Year	Number of Lambs	Mean Epg	Variance Epg^2	*T. circumcincta*	*T. axei*	*H. contortus*	*Cooperia spp.*	*T. vitrinus*	*B. trigonocephalum*
1992	110	87	22,359	6538	0	0	74	246	0.9
1993	100	317	65,306	2778	65	0	350	114	0
1994	169	494	218,000	1554	1.2	0	523	246	0
1995	151	1767	2,710,068	3000	101	17	4382	1020	0

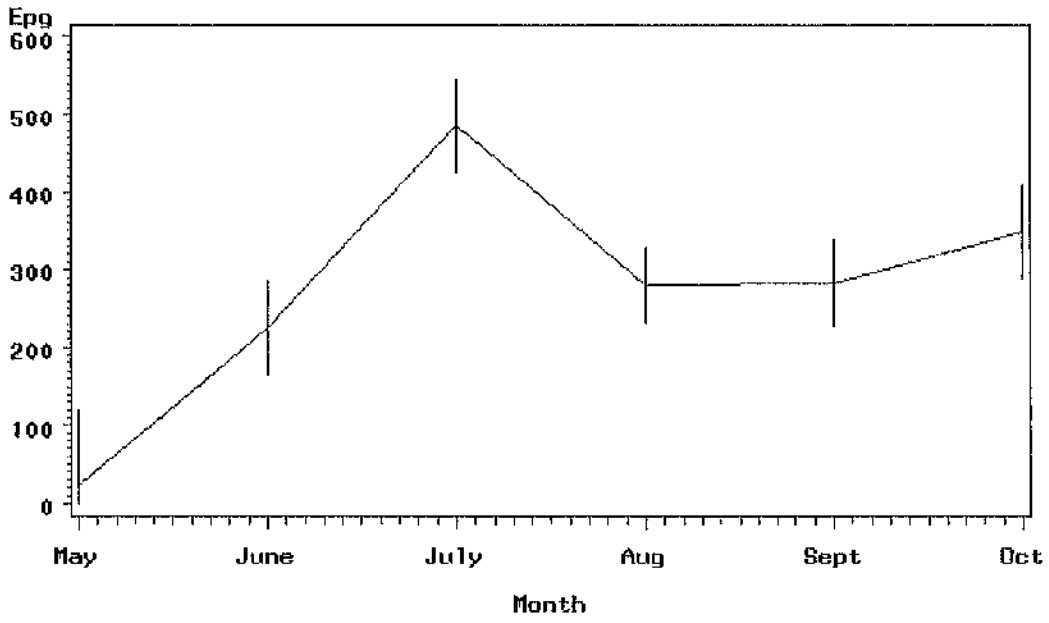
Multiple regressions were used to examine the relationship in these lambs in October between the total faecal egg count and the number of adult parasites of the five taxa (*T. circumcincta*, Cooperia spp., *T. vitrinus*, *T. axei* and *H. contortus).* Both faecal egg count and adult parasite numbers for each species were transformed by log10(x+1). The initial analysis showed a negative relationship (-0.23 $\pm$ 0.09; $p < 0.05$) between faecal egg count and transformed number of *T. circumcincta* and positive relationships between transformed egg count and transformed number of Cooperia spp. (+0.33 $\pm$ 0.03; $p < 0.001$) and the transformed number of *T. axei* (+0.15 $\pm$ 0.07; $p < 0.05$). The relationships between faecal egg count and the transformed numbers of *T. vitrinus* and *H. contortus* were not significant (p=0.21 and p=0.50) respectively.

As the relationship between egg count and number of adult *T. circumcincta* is non-linear, the *T. circumcincta* egg count was predicted from the number of adult *T. circumcincta.* This predicted egg count was then subtracted from the actual egg count and the residual egg count transformed by log 10(residual+1000). Multiple regression analysis demonstrated highly significant effects between transformed residual egg count and the transformed numbers of Cooperia spp. (+0.07 $\pm$ 0.01; $p < 0.001$), *T. axei* (+0.04 $\pm$ 0.01; $p < 0.05$) and *T. vitrinus* (+0.03 $\pm$ 0.01; $p < 0.001$). The relationship between faecal egg count and the transformed number of *H. contortus* was not significant (p=0.64), possibly because only 8 of 483 lambs examined were infected with this parasite.

Generalized linear modelling with a gamma distribution and a reciprocal link function was used to test the relationship between mean egg count and farm, year and month. Three separate univariate analyses were carried out and each variable was fitted

separately as a fixed effect. These analyses showed that there were no significant differences in mean egg count between the two farms (p=0.455), an inconclusive result for year (p=0.053) and significant differences among months (p=0.024). Egg counts were low in May, rose in June, peaked in July, fell in August, remained stable in September but rose again in October (Fig 4.4). The highest July mean egg counts occurred in 1993 (572 epg).

Fig 4-4 Egg count means plus standard errors by month of sampling. Lambs were born in a 3-week interval then sampled every 28 days. All lambs were treated with anthelmintic at each sample date.



4.4 DISCUSSION

There was considerable variation among the populations sampled in faecal egg count means and variances. The distribution of means and variances were both skewed. Most populations had relatively low means and variances but a small proportion had high means and variances. The variance was related to the mean to the power 1.23; this exponent was significantly greater than 1.0 and significantly less than 2.0. Analysis of necropsy data suggested that high means and variances were not simply due to high intensities of infection but to the presence of species other than *T. circumcincta,* particularly *Cooperia* spp. and *T. vitrinus.*

The contribution of other nematode species to high egg counts is consistent with previously published reports on the density-dependent regulation of fecundity in *T. circumcincta* (Bishop and Stear 2000). As the intensity of infection with *T. circumcincta* increases, an increasing number of larvae arrest development while those that do develop into adults produce fewer eggs per day. Previously published results has been used to predict egg output from the number of adult parasites (Bishop and Stear 2000; Stear and Bishop 1999). After subtracting this predicted output from the observed egg count, multiple regression analysis on the transformed residual egg counts gave highly significant positive relationships with the numbers of Cooperia spp., *T. axei* and *T. vitrinus.* Care is needed in interpreting these results because the residual egg count is an imprecise estimate of the egg count due to species other than *T. circumcincta.* Nonetheless the conclusion that the egg count is influenced by all nematodes present is plausible. Although *T. circumcincta* is the predominant species, the egg count does not necessarily reflect this. Indeed lambs

with many adult *T. circumcincta* produce fewer nematode eggs than lambs with moderate infections (Bishop and Stear 2000).

There was no significant difference in mean egg counts between the two farms sampled. However, the mean egg counts varied with the month of sampling. Egg counts rose to a peak in July then declined before rising again in October. A similar bimodal pattern with slightly earlier timings was reported previously for untreated lambs (Thomas and Boag 1972). This study was carried out under commercial conditions with regular anthelmintic usage; therefore the values observed each month represent independent infections and are not influenced by pre-existing infections derived from previous months.

The decline from the first peak has been explained by the onset of immunity in lambs (Stear et al. 1999b). However part of the peak could be contributed by time-dependent variation in other nematodes such as *Cooperia* spp. Necropsies of large numbers of infected lambs at regular intervals during the grazing season are needed to clarify the contribution made by different species of nematodes.

Faecal egg counts in sheep are not particularly well-described by the negative binomial distribution (Stear et al. 1995a) this lack of fit is surprising because the negative binomial distribution is a flexible distribution that is widely used to describe parasite distributions among hosts (Bliss and Fisher 1953; Hunter and Quenouille 1952). The poor fit of the negative binomial distribution may be explained by the observation that several species contribute to the egg count. The dominant nematode is *T. circumcincta* but other species can contribute to the egg count. If each species

egg counts follow a negative binomial distribution, the combined distribution would not conform to a negative binomial (Grafen and Woolhouse 1993). In addition males have higher egg counts than females and this too could lead to departures from the negative binomial distribution (Stear et al. 1995a).

There was a strong relationship between the mean and the variance for egg counts. This relationship followed Taylor's power law (Taylor 1961). Taylor's power law has been used previously in a subset of these data (Stear et al. 1998). Then the regression line was drawn through the origin but visual examination (Fig 3) of the larger data set analysed here suggests that an intercept was more appropriate. Estimating the slope of the regression line is subject to error (Boag et al. 1992; Perry 1981) because both the mean and variances are estimates of the true values. However, there is no agreement on the best way to avoid this problem (Sokal and Rohlf 1995). A coefficient of 1 is consistent with a Poisson distribution and implies a square root transformation while a coefficient of 2 implies a logarithmic transformation is most appropriate. Here the estimate lies between 1 and 2, implying that neither transformation is ideal.

Taylor's Power law has been widely used to describe the relationship between variability in population size and mean abundance of a species over space and time (Anderson et al. 1982; Keeling 2000). Taylor (1961) considered the scalar to be of less importance than the exponent which generally lies between 1 and 2. Mathematical modelling suggested that the value of the exponent was determined by relative magnitude of birth, death, immigration and emigration rates (Anderson et al. 1982) while Kilpatrick and Ives (2003) argued that negative interactions among species interactions could produce exponents between one and two.

In conclusion, faecal egg counts vary in naturally infected sheep and mean egg counts vary among different populations and among the same population sampled at different times. The variance was largely determined by the mean and high means are not necessarily due to high intensities of infection but probably reflect the contribution of species other than *T. circumcincta*.

CHAPTER FIVE

INTERFERON GAMMA GENOTYPING

5-1 INTRODUCTION

Control strategies for gastrointestinal nematode infection include anthelmintic chemotherapy and/or grazing management; however, drug treatment is costly and drug resistance has evolved in all major parasite species (see chapter one). One of the additional methods for controlling nematode infection in domestic sheep is deliberate selection for parasite resistance, which has been undertaken in many countries (Beh and Maddox 1996; Eady et al. 1998; Bisset et al. 2001) .

The identification of Quantitative Trait Loci (QTL) that have a significant association with resistance to internal nematodes, would provide more through understanding of the genetic mechanisms underlying nematode resistance. A number of genes have been associated with nematode resistance in sheep. These include genes within the Major Histocompatibility Complex genes (Schwaiger et al. 1995; Buitkamp et al. 1996; Buitkamp et al. 1999), the 5' end of the IgE gene (Clarke et al. 2001), microsatellites markers for sheep chromosome 5 located near the interleukin (IL-3, IL-4, IL-5) genes (Benavides et al. 2002), and near the interferon γ (IFN-γ) gene (Coltman et al. 2001; Crawford and McEewan 1999; Sayers et al. 2005).

QTL influencing nematode resistance has been reported by several groups (reviewed by Coltman *et al.*, 2001). One likely candidate gene is interferon gamma, which has been mapped to sheep chromosome 3q23 by fluorescence in situ hybridization (Goldammer et al. 1996). A more detailed scan of the q arm of chromosome 3 using markers spaced at approximately 4 cM intervals has also been performed (Crawford and McEewan 1999). Four markers on ovine chromosome 3 within 2 cM of the IFN gene were located, which included one microsatellite marker located in the first intron (Schmidt et al. 1996). This showed the strongest association with resistance. In addition, significant differences between the

frequencies of the resistant and susceptible alleles at the IFN-γ microsatellite locus have been seen between the original selections lines (Crawford and McEewan 1999).

While it is not yet clear if a functional polymorphism in a coding region of the IFN-γ gene or in a regulatory sequence is the actual source of the QTL effect (Coltman et al. 2001), IFN-γ gene is a member of the interferon family that codes for cytokines. Furthermore IFN-γ plays an important role in the regulation of the immune response to parasitic infection (Wakelin 1996; Else et al. 1994) and has the potential to alter resistance to a wide variety of infectious disease and vaccines (Radford et al. 1991).

IFN-γ is a glycoprotein, derived from T cells and NK cells, which activates macrophages and up-regulates Th1 cell responses, while it down-regulates Th2 cell responses (Wakelin 1996; Tizard I 1992). The Th1 response is relatively beneficial to the survival of the nematode parasite, while the Th2 cells are associated with resistance to infection from extracelluar parasites such as gastrointestinal nematodes (Else and Finkelman 1998; Else et al. 1994; Grencis 1997).

Relatively little is known about the effector mechanisms that operate against intestinal nematode infection. Depletion of IFN-γ in normally susceptible mice results in expulsion of the parasites. This is evidence for an IFN-γ role in the establishment of chronic helminth infection (Else et al. 1994). In another study, the effect of the IFN-γ locus on FEC may be mediated by IgA in four months old lamb (Coltman et al. 2001). Stear et al. (1999a) and Stear et al. (1999b) have found that genetic variation in faecal egg output among naturally infected 6-7 months old lambs is largely caused by variation in worm length, which in turn is controlled by the strength and specificity of the local IgA response to fourth-stage larvae. Resistance to gastrointestinal parasites in older sheep may be mediated by other mechanisms

(Stear et al. 1999b). Thus, polymorphism leading to differential expression of IFN-γ could influence resistance to gastro-intestinal nematodes (Coltman et al. 2001).

The purpose of this chapter is to determine if microsatellite polymorphism near the IFN-γ locus is associated with resistance in Scottish-Blackface sheep. In addition, we examined differences between sexes in the effect of the IFN-γ microsatellite locus.

5-2 MATERIAL AND METHODS

5-2-1 Experimental Animals

A flock of 800 straight bred Scottish Blackface lambs was sampled over a four-year period (1992-1995). All lambs were from a commercial farm in west central Scotland. Only samples collected in August, September and October were used in this experiment. Castrated male and female lambs were used in this experiment.

5-2-2 Blood samples

See protocol 2-1-2

5-2-3 Faecal worm egg count

See protocol 2-3-1.

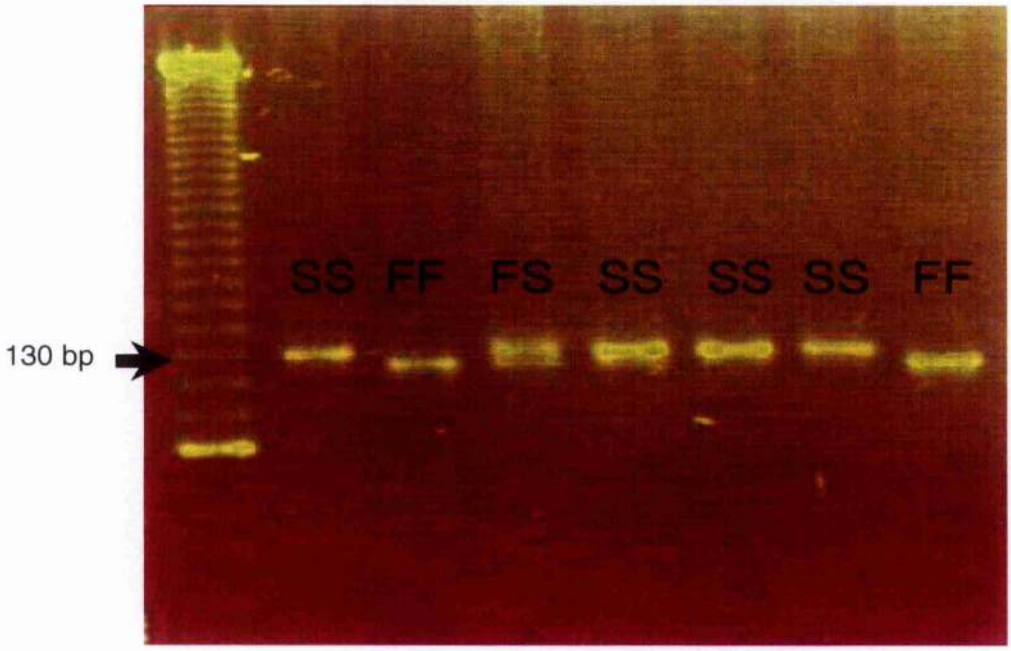
5-2-4-DNA Extraction

DNA was extracted using the protocol 2-4-1.

5-2-5 Interferon gamma (IFN-γ) genotyping

IFN-γ genotyping was performed using protocol 2-4-2. Two different microsatellite alleles were distinguished (figure 5-1), nominated the slow allele (S) which was 130 base pairs (bp) in length, and the fast allele (F), which was approximately 4 bp shorter.

Figure 5-1 IFN-γ genotyping



5-2-6 Statistical analysis

All analyses were carried out with the SAS suite of statistical programs version 8.2 (SAS Institute, Cary, N. Carolina). The test for the Hardy- Weinberg Law, was calculated with the allele procedure of the SAS Genetics statistical package. The mixed model procedure on the SAS system was used to analyse the IFN-γ effect on faecal egg counts and the interaction

with sex. All traits were analysed with a model that included, year, sex, IFN- γ as fixed effects and log FEC August, log FEC September and log FEC October as dependent variables. In addition average logepg was also used as a dependent variable for the analyses of the sex effect on IFN- γ genotyping. Sire, dam and residual were the random effects in this model. All egg counts were transformed prior to this analysis by taking the logarithm to the base 10 of the count plus one. The frequency procedure on the SAS system was used to calculate the IFN- γ genotype frequency among lambs.

5-3 RESULTS

Tables 5-1A, B and C illustrate the effect of IFN-γ, year and sex on transformed faecal egg counts over four years (1992-1995). There was no significant effect of the IFN- γ locus on transformed egg counts in August, but there was a very highly significant year effect (P>0.0001), and a significant sex effect (P>0.05) on the August counts (Table 5.1A).

Table 5-1B shows, that there was a very highly significant year effect (P>0.0001) and highly significant sex effect (P>0.001) on transformed egg counts in September. There was a considerable IFN- γ locus effect on transformed egg counts in September and transformed egg counts in October (Table 5-1C), which also showed very highly significant year and sex effects (P>0.0001) on log FEC October.

Table 5-1 Analysis of transformed egg counts over four years in A-log FEC August, B-log FEC September and C-log FEC October.

A

EFFECT	ESTIMATE	S.E	DF	T VALUE	PR> | T |
Intercept	2.319	0.095	29	24.17	>.0001
Year:1992	-0.837	0.121	195	-6.88	>.0001
Year:1993	-0.283	0.127	195	-2.22	0.027
Year:1994	-1.059	0.112	195	-9.43	>.0001
Year:1995	0	.	.	.	
IFN-γ	-0.128	0.102	195	-1.26	0.210
Sex	0.196	0.073	195	2.66	0.008

B

EFFECT	ESTIMATE	S.E	DF	T VALUE	PR> | T |
Intercept	2.80	0.095	29	23.84	>.0001
Year:1992	-0.748	0.122	211	-6.13	>.0001
Year:1993	-0.521	0.126	211	-4.12	>.0001
Year:1994	-1.183	0.111	211	-10.66	>.0001
Year:1995	0	.	.	.	.
IFN-γ	-0.169	0.0987	211	-1.71	0.088
Sex	0.251	0.0718	211	3.50	0.0006

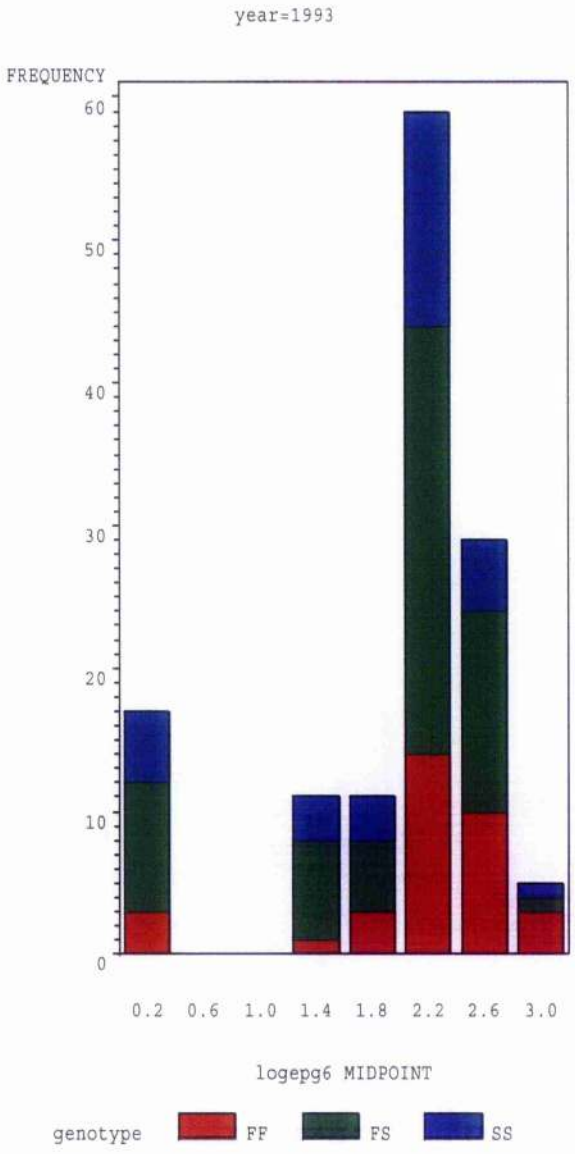
C

EFFECT	ESTIMATE	S.E	DF	T VALUE	PR> | T |
Intercept	2.509	0.079	25	31.45	>.0001
Year:1993	-0.703	0.103	115	-6.83	>.0001
Year:1994	-0.695	0.090	115	-7.72	>.0001
Year:1995	0	.	.	.	.
IFN-γ	-0.150	0.087	115	-1.72	0.088
Sex	0.294	0.066	115	4.45	>.0001

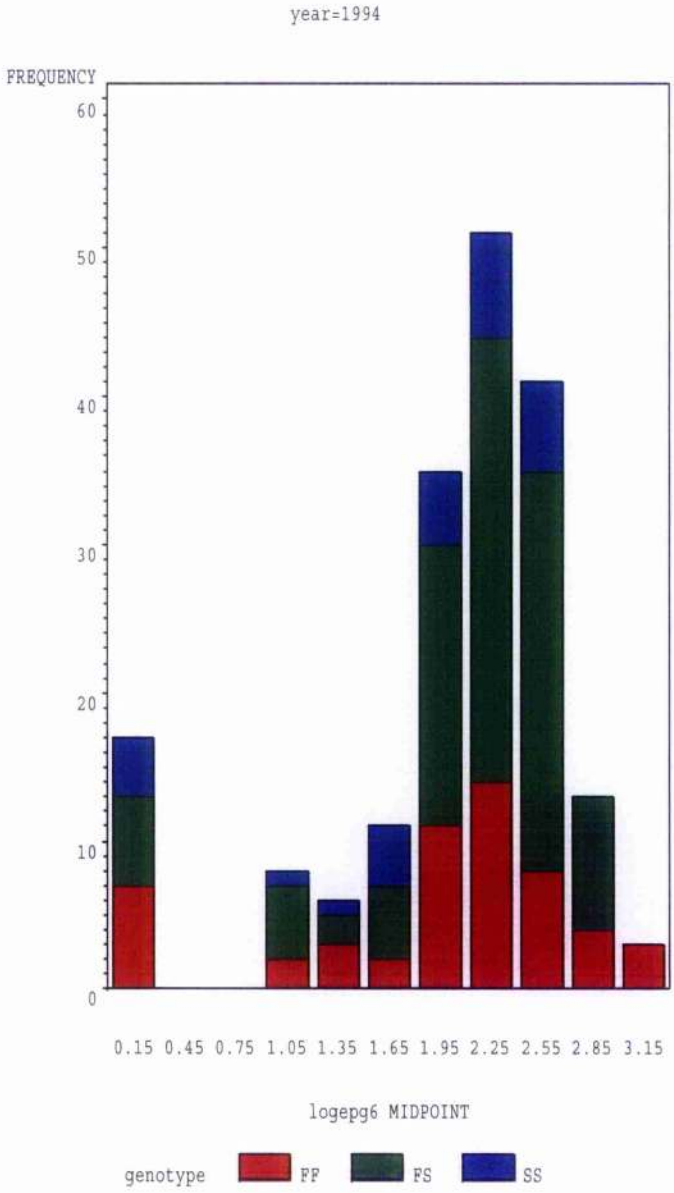
Figure 5.1A, B and C show the association between the IFN- γ locus genotypes and transformed egg counts in October over three years (1993-1995). In 1993, FF genotypes were most common in lambs with high faecal egg counts (Fig 5-1A). In 1994 and 1995, high faecal egg output was associated with the FF and FS genotypes at the IFN- γ locus (Fig 5-1B and C).

Fig.5-2 Association between IFN-γ microsatellite alleles and log FEC October in A-1993, B-1994 and C-1995.

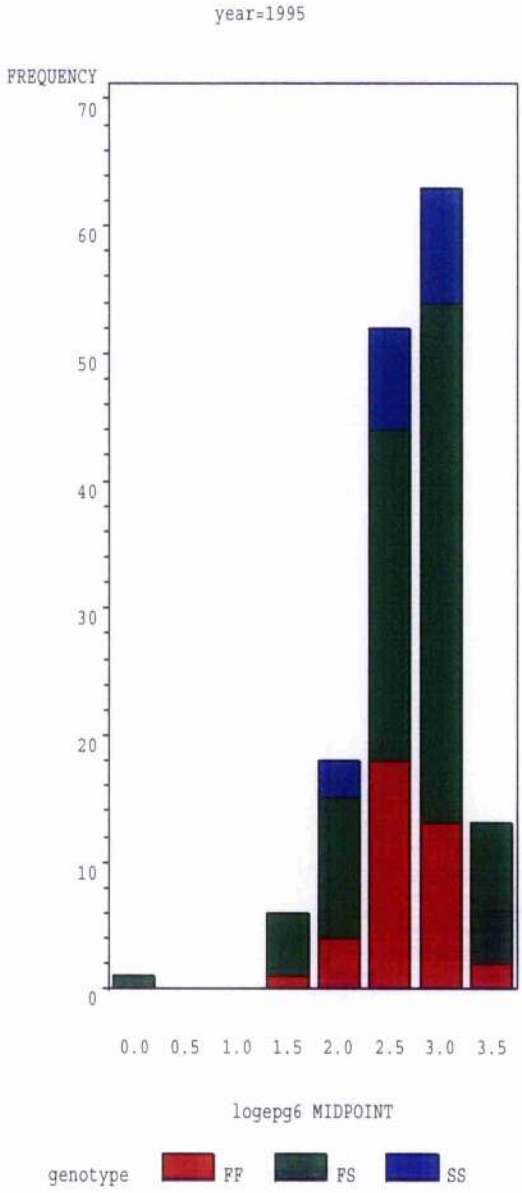
A



B


year=1994
FREQUENCY
60
50
40
30
20
10
0
0.15 0.45 0.75 1.05 1.35 1.65 1.95 2.25 2.55 2.85 3.15
logepg6 MIDPOINT
genotype
FF
FS
SS

C


year=1995
FREQUENCY
70
60
50
40
30
20
10
0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
logepg6 MIDPOINT
genotype
FF
FS
SS

Tables 5-2A, B, C and D, present the genotype frequency, at the IFN- γ gene among lambs over four years (1992-1995). Table 5-2A shows that 56.02% of the lambs were genotyped FS, 28.80% were FF and 15.18% were SS in 1992. In 1993, 50% of the lambs were genotyped FS, 26.06% FF and 23.94% were SS (Table 5-2B). In 1994 and 1995 FS allele was accounted for 54.92% and 64.32%, FF allele was 30.05% and 24.32%, and SS allele was 15.03% and 11.35% respectively (Table 5-2C and D).

Table 5-2 Genotype frequencies at the IFN-γ microsatellite locus in A-1992, B-1993, C-1994, D-1995

A

GENOTYPE	FREQUENCY	PERCENT	CUMULATIVE FREQUENCY	CUMULATIVE PERCENT
FF	55	28.80	55	28.80
FS	107	56.02	162	84.82
SS	29	15.18	191	100.00

B

GENOTYPE	FREQUENCY	PERCENT	CUMULATIVE FREQUENCY	CUMULATIVE PERCENT
FF	37	26.06	37	26.06
FS	71	50.00	108	76.06
SS	34	23.94	142	100.00

C

GENOTYPE	FREQUENCY	PERCENT	CUMULATIVE FREQUENCY	CUMULATIVE PERCENT
FF	58	30.05	58	30.05
FS	106	54.92	164	84.97
SS	29	15.03	193	100.00

D

GENOTYPE	FREQUENCY	PERCENT	CUMULATIVE FREQUENCY	CUMULATIVE PERCENT
FF	45	24.32	45	24.32
FS	119	64.32	164	88.65
SS	21	11.35	185	100.00

Tables 5-3A, B and C demonstrate the effects of the IFN- γ microsatellite locus on transformed egg counts in male and female lambs over four years. Each sex was analysed separately. The estimate represents the difference in log FEC between the resistant SS genotype and the susceptible FF genotypes. There were no significant effects for both genders in August (Table 5-3A). On the other hand, there was a possible effect of the IFN-γ locus in September in male lambs (P = 0.088), but not in female lambs (Table5-3B). Male lambs showed a significant effect of the IFN- γ locus in transformed egg counts in October (P<0.05), while female lambs did not (Table 5-3C).

Table 5-3 Effect of the IFN-γ microsatellite locus on A-log FEC August, B-log FEC September and C- log FEC October, in the different sexes

A

EFFECT	NMBER OF ANIMALS	ESTIMATE	S.E	T VALUE	PR> | T |
Male IFN-γ	429	-0.2154	0.126	-1.71	0.0932
Female IFN-γ	280	0.0332	0.731	0.19	0.8484

B

EFFECT	NMBER OF ANIMALS	ESTIMATE	S.E	T VALUE	PR> | T |
Male IFN-γ	448	-0.2101	0.1215	-1.73	0.0887
Female IFN-γ	294	0.1528	0.1689	-0.90	0.3696

C

EFFECT	NMBER OF ANIMALS	ESTIMATE	S.E	T VALUE	PR> | T |
Male IFN-γ	342	-0.2206	0.097	-2.27	0.0304
Female IFN-γ	213	-0.1364	0.1746	-0.78	0.4410

5-4 DISCUSSION

In only one year, did the genotype frequencies conform to Hardy- Weinberg equilibrium. The observed deviation from Hardy-Weinberg equilibrium could be due to differences in frequency between male and female parents. As the number of male parents was small, differences could arise by chance alone (Falconer and Mackay 1996).

The results showed that faecal egg count was associated with the IFN-γ microsatellite locus, suggesting there may be a QTL associated with reduced FEC segregating at or near the IFN-γ gene in Scottish Blackface sheep. This result confirmed previous results in Merino and Romney sheep (Crawford and McEewan 1999), feral Soay sheep (Coltman et al. 2001) and in Texel sheep (Sayers et al. 2005). In addition, this result indicated that the effect was becoming stronger as the animal matured, from 4 to 6 month of age. Furthermore, two genotypes (FF and FS) at the IFN-γ microsatellite locus are associated with relatively high faecal egg output, while the SS genotype is associated with low faecal egg output. This implies that allele F has a dominant effect on susceptibility.

This study has also showed a significant association between the IFN-γ microsatellite locus and six month old male lambs, while there was no association with female lambs at any age. This could be because mature male lambs were putting more resources into gaining weight, while female lambs were responding better immunologically (see section 3-4 in chapter 3).

The observed effect of IFN-γ may be caused by another uncharacterized gene in the adjacency of IFN-γ gene. The aim of future studies should be to identify the locus responsible for increased nematode resistance.

In conclusion, there was an association between faecal egg output and IFN-γ gene, and this effect appears more clearly in older lambs. In addition this study suggested that two genotypes at the IFN-γ microsatellite gene appear to be responsible for moderate and high faecal egg output.

CHAPTER SIX

QUANTITATIVE TRAIT LOCI ASSOCIATED WITH NEMATODE INFECTION

6-1 INTRODUCTION

Animals infected with nematode parasites are traditionally treated by anthelmintic compounds, however, treatment is costly. Cases of multiple anthelmintic resistance in gastrointestinal parasites of small ruminants have been reported (Bartley et al. 2004). Reports of complete multi-drug resistance to anthelmintics are now emerging (Chandrawathani et al. 2004b) and there is a growing demand from consumers for products free of any kind of chemical treatment (Sangster 1999).

Due to the absence of commercially available vaccines or new anthelmintic drugs, one of the most promising methods of control is the selection and breeding of animals genetically resistant to nematodes (Albers and Gray 1987; Windon 1990; Baker 1999; Bishop and Stear 1999). Nematode-resistant sheep would reduce the cost of anthelmintic treatment and minimise the effect of nematodes on animal production. Selective breeding is based on selection for faecal egg counts (FEC). This trait can be misleading due to the variation in faecal egg production following changes in the weather or anthelmintic treatment.

A number of studies on field and experimental sheep flocks clearly demonstrated the presence of considerable within and between-breed variation in resistance to parasites infection (see section 1-5-2 in chapter 1). The desire to see whether the genetic variation revealed in these studies could be used to enhance nematode parasite resistance in sheep led to the establishment of experimental flocks, selectively bred for resistance to parasite infection. These require the utilization of phenotypic traits associated with expression of parasite resistance, which should be heritable and correlated closely with host worm burden and /or production losses (Beh and Maddox 1996). These phenotypic traits are continuously variable and are known as quantitative traits (Beh and Maddox 1996; Nicholas 2003). Genetic variation in quantitative traits is due to segregation at many loci. These loci are

known as quantitative trait loci (QTL), and with the developments in molecular and genomic biology, it is now feasible to identify QTL (Nicholas 2003). The abundance of genetic markers has made it possible to identify QTL- the regions of a chromosome or, individual sequence variants that are responsible for trait variation. This technique depends on the proximity of the marker/s on the chromosome to the gene of interest (Kahn and Watson, 2003). If resistance is under the control of many genes, all with small effects, it is unlikely that a genetic marker will be useful in identifying resistant animals. However, if resistance is due to the effect of a single gene or the actions of only a few genes of moderate or large effect the probability of identifying a suitable marker is more likely (Kahn and Watson, 2003). Identification of a QTL or closely linked markers that have significant association with nematode resistance would assist a selection scheme. Selection would be substantially simplified if animals could be selected on genotype (Diez-Tascon et al. 2005)

Genetic markers have been sought for genes known to influence immune responses, especially genes of the Major Histocompatability Complex (MHC). The ovine MHC is a polymorphic multi-gene complex located on chromosome 20 (20q15-q23) (Mahdy et al. 1989; Nicholas 2003), and their high polymorphism make them suitable candidates for investigating disease associations. Associations between the MHC and disease resistance have been found in sheep (Schwaiger et al. 1995), humans (Hohler et al. 1997), and cattle (Acosta-Rodriguez et al. 2005).

The MHC is divided into three regions, MHC class I, II and III. At a molecular level variation in the immune response is thought to be a result of genetic variation in the amino acid sequence of the antigen-presenting site in both class I and class II MHC molecules (Hohenhaus and Outteridge, 1995). The MHC class I and II genes encode structural glycoproteins that bind and present peptides to T cells of the immune system resulting in T

cell stimulation (Tizard and Schubot 2004) and as such these genes play a key role in the initiation of an immune response.

The MHC class I region contains several genes, which control type of antigens on the surface of most nucleated cells. The class II region contains genes that encode two different types of polypeptide (α and β), and are expressed on the surface of antigen presenting cells such as macrophages, dendritic cells and B-cells where they present processed antigen to CD4 expressed T helper (T_H) cells (Nicholas 2003; Tizard and Schubot 2004). The class III region contains a mixture of genes having a wide range of functions, only some of which are involved in the immune response (Nicholas 2003).

The literature published on QTL in nematode resistance in sheep has been based on diverse analytical approaches involving a variety of sheep breeds and nematode species (Diez-Tascon et al. 2005; Beh et al. 2002; Coltman et al. 2001; Buitkamp et al. 1996; Buitkamp et al. 1999; Schwaiger et al. 1995).

The purpose of this chapter is to firstly identify QTL associated with nematode resistance which segregate in Scottish Blackface lambs using the FEC and immunoglobulin A (IgA) activity as indicator traits for host resistance and response to infection. Secondly it is to demonstrate the relationship between MHC class II DRB1 microsatellite polymorphism and allele sequences.

6-2 MATERIALS AND METHODS

6-2-1 Animals

The study population comprised of 816 straight bred Scottish Blackface lambs, from which 789 animals, comprising 9 half-sib families ranging from 23-141 individuals, were studied. The animals were bred over a 3-year period (2001 – 2003) and all were of known parentage. The complete pedigree contained 4847 animals with records dating back to 1986.

The lambs were born outside and were continually exposed to natural mixed nematode infection by grazing. Lambs were kept in two groups each year with the group being representative of the field grazed. Husbandry procedures followed standard commercial practice.

6-2-2 Phenotypic Measurements

6-2-2-1 Parasitological findings

Faecal samples were collected from the rectum of the lambs at 16, 20 and 24 weeks of age. Faecal egg counts were done using the protocol 2-3-1. Eggs were classified according to whether they were *Nematodirus* spp or other species.

6-2-2-2 Plasma IgA activity

Blood samples were collected at 24 weeks of age. The activity of plasma Immunoglobulin A (IgA) against a somatic extract of 3rd-stage larvae from *T. circumcincta* was measured by indirect ELISA (Strain et al. 2002). Relative IgA activity was measured as: (observed – standard) / (high control – standard), where the observed value is the sample mean from 3 replicates for the animal, the standard is the mean of 3 replicates from a pooled sample of helminth-naive lambs and the high control is the mean of 3 replicates from a pool of high-responder lambs (Sinski et al. 1995). The pool of high responder lambs was created by

combining equal quantities of plasma from 6 lambs that gave strong IgA responses following natural infection. The value for each animal was therefore expressed as a proportion of a positive control.

6-2-3 Genotyping and map construction

All animals were genotyped using microsatellite markers. This typing was carried out by a commercial company Agresearch Ltd in New Zealand using DNA supplied by us. Eight chromosomes were examined; they were chromosomes 1, 2, 3, 5, 14, 18, 20 and 21. These regions were chosen because previous studies had suggested QTL for nematode resistance (chromosomes 3 and 20) or lamb performance traits such as growth rate or meat quality. Each chromosome contained between 9 and 34 markers.All sires were genotyped for markers across each region. The markers used for specific chromosomes have been described in the figures 6-1, 6-2 and 6-3. Offspring were subsequently genotyped for markers that were heterozygous in their sire. In total 139 markers were genotyped. Relative marker locations were established by creating a linkage map (Davies et al. 2005a) for each chromosome using Cri-map (Green et al. 1990).

6-2-4 Data analysis

Data analysis began with an assessment of the distribution of the traits. All traits were transformed prior to further analysis; all FEC traits (both mixed species and Nematodirus) were subjected to a log transformation ln (trait + x) where x is a constant used to avoid zero values. Typically x = half the measurement increment, however some results are sensitive to the value of x, and the impact of other values was also investigated.

IgA data was transformed using a cubed root transformation. These transformations successfully reduced the skewness of these traits resulting in approximately normally distributed data.

For the QTL analysis the traits analysed were IgA, FEC at weeks 16, 20 and 24 for both mixed species and Nematodirus and an average animal effect that is described below. A restricted maximum likelihood program, ASREML (Gilmour et al. 1996) fitting an animal repeatability model (i.e. ignoring genetic effects), was used to create an average effect for each animal for both strongyles and nematodirus FECs. This effect was an average effect across time as FEC was measured at 3 time points. This animal model also fitted fixed effects: year, management group, sex, type (twin or single) and day of birth (fitted as continuous effect). The average effect was calculated from the transformed FEC data.

Heritability estimates were calculated using ASREML (Gilmour et al. 1996). An animal model, including all known pedigree relationships (4847 animals), was fitted. This included fitting the following fixed effects: year, management group, sex, type (twin or single) and day of birth (fitted as continuous effect. This analysis was repeated fitting a litter effect (c^2); the significance of the litter effect was tested using a likelihood ratio test.

6-2-5 Estimation of QTL position

QTL analysis was performed using regression techniques implemented by QTL express (Seaton et al. 2002). The probability of inheriting a particular sire chromosome at a particular position was calculated for each offspring at 1cM intervals (Knott et al. 1996). Phenotypes were then regressed upon the conditional probability that a particular haplotype is inherited from the sire, along each chromosome, fitting fixed effects of year, sex, litter size, management group and day of birth (fitted as a covariate).

For each regression an F-ratio of the full model including the inheritance probability versus the same model without the inheritance probability was calculated across families, the location of the QTL was indicated by the largest F-value.

6-2-6 Significance threshold

The 5% chromosome-wide threshold was determined for each chromosome by permutation testing (1000 permutations) (Churchill and Doerge 1994). A 5% genome–wide threshold was then obtained by applying the Bonferroni correction (Knott et al. 1996):

$$P_{genome\text{-}wide} = 1 - (1 - p_{chromosome\text{-}wide})^{n}$$

Where n is the number of chromosomes. The genome-wide threshold is based on the assumption that by chance you would expect 0.05 significant results per genome analysis.

6-2-7 Confidence intervals

For each QTL estimate that was significant at the 5% chromosome-wide level confidence intervals were calculated using the bootstrap method (Visscher et al. 1996). 1000 samples with replacement were used to estimate 95% confidence intervals.

6-2-8 QTL effects

The proportion of phenotypic variance explained by the QTL was calculated using:

Phenotypic proportion = 4 (1- MS_{full} / $MS_{reduced}$) where MS is the residual mean square from the regression analysis (Knott *et al.* 1996). Dividing this phenotypic value by the heritability estimates the proportion of genetic variance explained by the QTL. As these results came from a half-sib analysis it was necessary to adjust the genetic proportion (GP) value to account for the proportional reduction in phenotypic variance expressed within sire families:

Adjusted GP = GP (1 - h^2 / 4). The resulting value is an estimate of the size of the effect of the QTL, i.e. the proportion of total additive genetic variance that is explained by the QTL.

6-2-9 Cloning and sequencing of MHC class II DRB1 alleles

See protocol 2-3.

6-3 RESULTS

6-3-1 QTL associated with nematode resistance

Table 6-1 shows, the summary statistics for the traits (FEC and IgA activity), age (week), number of observations, mean, maximum, transformed mean and transformed standard deviation over three years. This data shows that mixed species FEC were much higher than Nematodirus FEC. mixed species FEC ranged from 0 to 5325, while Nematodirus FEC ranged from 0 to 1888. Mixed species FEC and Nematodirus FEC were significantly higher in August than September or October. IgA activity ranged from 0 to 1.24 with a standard deviation of 0.21.

Table 6-1 Summary Statistics over three years (2001-2003)

Trait	Age (weeks)	No.of obs.	Mean	Max.[A]	Transformed mean	Transformed standard deviation
IgA Activity	24	757	0.13	1.24	0.42	0.21
Nematodirus FEC August	16	740	39.0	1888	3.62	0.74
Nematodirus FEC September	20	722	22.2	675	3.61	0.6
Nematodirus FEC October	24	741	30.3	600	3.67	0.71
Mixed species FEC August	16	740	256	5325	4.85	1.28
Mixed species FEC September	20	721	288	2550	5.19	1.12
Mixed species FEC October	24	741	236	1700	5.12	1.02

[a] The minimum value for each trait was zero

Heritability estimates for all traits are shown in Table 6-2. For both Strongyle and Nematodirus FECs, heritability estimates were somewhat varied. This study found that heritability estimates calculated with a restricted maximum likelihood algorithm were sensitive to data transformations. IgA activity appears to have a relatively low heritability in this study however there is a significant maternal effect.

Table 6-2 Heritabilities estimate

Trait	h^2	s.e.	c^2	s.e.
Nematodirus FEC August	**0.30**	**0.11**		
Nematodirus FEC September	**0.21**	**0.09**		
Nematodirus FEC October	**0.19**	**0.09**		
Nematodirus Average Animal Effect	**0.24**	**0.09**		
Mixed species FEC August	**0.50**	**0.12**		
Mixed species FEC September	**0.11**	**0.07**		
Mixed species FEC October	**0.21**	**0.09**		
Mixed species Average Animal Effect	**0.23**	**0.09**		
IgA Activity	**0.18**	**0.09**	**0.13**	**0.06**

Table 6-3 and 6-4 demonstrate significant QTL and their size effect associated with Nematodirus FEC traits. QTL analysis suggested putative QTL on chromosomes 2, 3 and 14. A QTL at chromosome 2 was associated with Nematodirus FEC in September, at an estimated position of 134 cM and this QTL was significant at 5% genome wide significance threshold (Table 6-3). The size of the QTL effect was 0.52, suggesting that 52% of the genetic variation was attributable to this single QTL (Table 6-4). Another QTL associated with Nematodirus FEC in August was on chromosome 3 at an estimated position of 174 cM, with significance at both 5% chromosome-wide threshold and 5% genome wide threshold (Table 6-3). This QTL accounted for 26% of the genetic variation (Table 6-4). A third QTL was associated with the following Nematodirus traits; average animal effect, FEC in August and FEC in October at estimated positions of 103cM, 100 cM and 104 cM respectively on chromosome 14, with significance at 5% genome-wide level (Table 6-3). The QTL accounted for 79% of the genetic variation for average animal effect, 40% of the genetic variation for FEC in August and 71% of the genetic variation for FEC in October (Table 6-4).

Table 6-3 QTL significant at 5% chromosome- wide significance level associated with Nematodirus FEC traits.

TRAIT	CHROMOSOME	POSITION (CM)	MARKER REGION	F RATIO	5% CHROMOSOME-WIDE THRESHOLD	5% GENOME-WIDE THRESHOLD	95% CONFIDENCE INTERVAL
Nematodirus FEC September	2	134	BM81124-CP79	3.06	2.88	2.96	44 – 203
Nematodirus FEC August	3	174	BM6433-BMS772	3.43	3.41	2.96	0 – 202.5
Nematodirus Average Animal Effect	14	103	ILSTS002-LSCV30	5.26	2.42	2.96	65 – 123
Nematodirus FEC August	14	100	BMS833-ILSTS002	3.54	3.17	2.96	0 – 151
Nematodirus FEC October	14	104	ILSTS002-LSCV30	3.74	2.61	2.96	32 – 146.5

Table 6-4 Proportions of variation attributable to QTL effect associated with Nematodirus FEC traits.

TRAIT	CHROMOSOME	HERITABILITY	PHENOTYPIC PROPORTION	GENETIC PROPORTION
Nematodirus Average Animal Effect	14	0.24	0.20	0.79
Nematodirus FEC August	14	0.30	0.13	0.40
Nematodirus FEC October	14	0.19	0.14	0.71
Nematodirus FEC September	2	0.21	0.12	0.52
Nematodirus FEC August	3	0.30	0.08	0.26

Evidence for QTL associated with mixed species FEC traits and IgA activity was observed in chromosomes 3 and 20. Chromosome 3 indicated a QTL associated with Strongyle average animal effect at 150 cM, with significance at the 5% genome wide threshold (Table 6-5). This QTL accounted for 37% of the genetic variation (Table 6-6). On chromosome 20 a QTL was observed for Strongyle FEC October at 10cM and was significant at 5% genome wide threshold (Table 6-5). The estimated QTL effect was 31% of the genetic variation (Table 6-6).

IgA activity was associated with a QTL in chromosome 3 at estimated position of 118 cM, with a significant 5% genome wide threshold (Table 6-5). The QTL accounted for 41% of genetic variation (Table 6-6). On chromosome 20 indications of a QTL were observed for

IgA activity at 40cM. This QTL was significant at the 5% chromosome-wide threshold (Table 6-5) and the QTL accounted for 51% of genetic variation (Table 6-6).

Table 6-5 QTL significant at 5% chromosome-wide significance level associated with Strongyle FEC traits and IgA activity.

Trait	Chromosome	Position (cM)	Marker Region	F	5% Chromosome-wide Threshold	5% Genome-wide Threshold	95% Confidence Interval
IgA Activity	3	118	KD103-LYZ	2.48	2.48	2.96	36 – 189.5
Strongyles Average Animal Effect	3	150	CSRD111-TEXAN15	2.59	2.44	2.96	0 - 205
Strongyles FEC October	20	10	DYA-MCMA36	2.64	2.44	2.96	0 – 59
IgA Activity	20	40	BM1815-DRB1	2.90	2.45	2.96	1 – 65

Table 6-6 Proportions of variation attributable to QTL effect associated with Strongyle FEC traits and IgA acitivity.

Trait	Chromosome	Heritability	Phenotypic proportion	Genetic proportion
Strongyles FEC October	**3**	**0.21**	**0.08**	**0.37**
Strongyles Average Animal Effect	**20**	**0.23**	**0.08**	**0.31**
IgA Activity	**20**	**0.18**	**0.10**	**0.51**
IgA Activity	**3**	**0.18**	**0.08**	**0.41**

Figures 6-1, 6-2 and 6-3 illustrate the effect of significant FEC traits and IgA activity on chromosome 3 (Fig. 6-1), chromosome 14 (Fig. 6-2) and chromosome 20 (Fig. 6-3).

Figure 6-1 QTL contour plot of chromosome 3 for FEC, Nematodirus and IgA activity

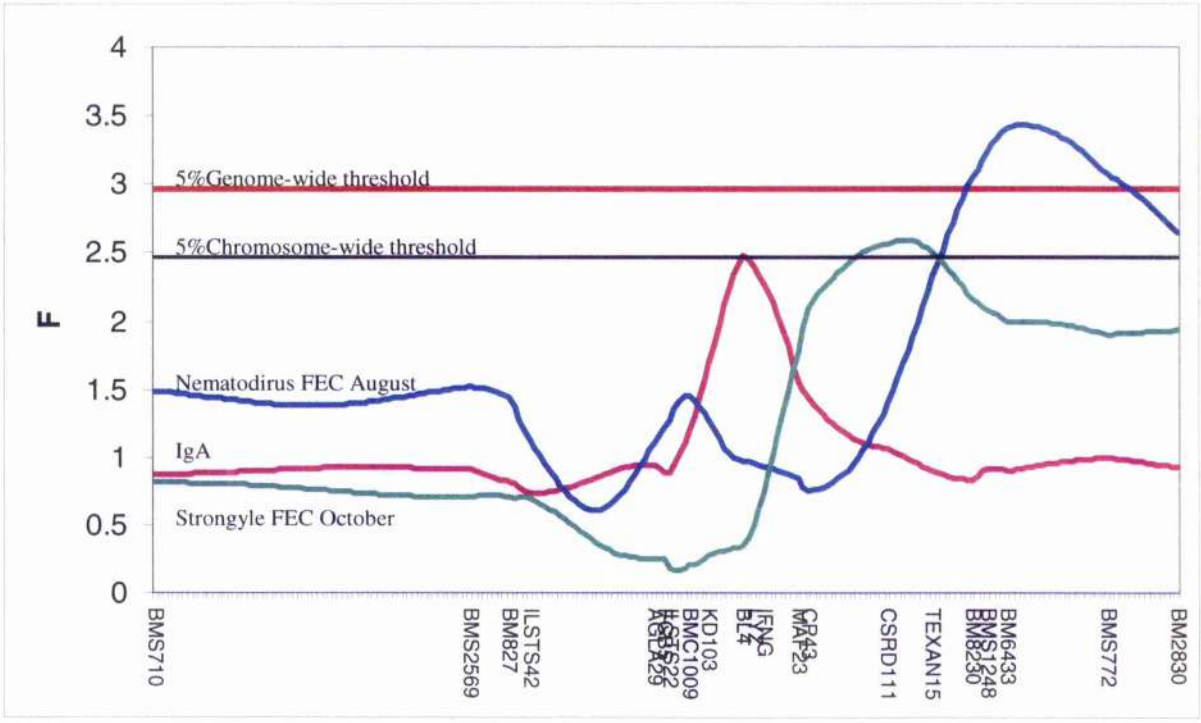


Figure 6-2 QTL contour plot of chromosome 14 for Nematodirus FEC traits

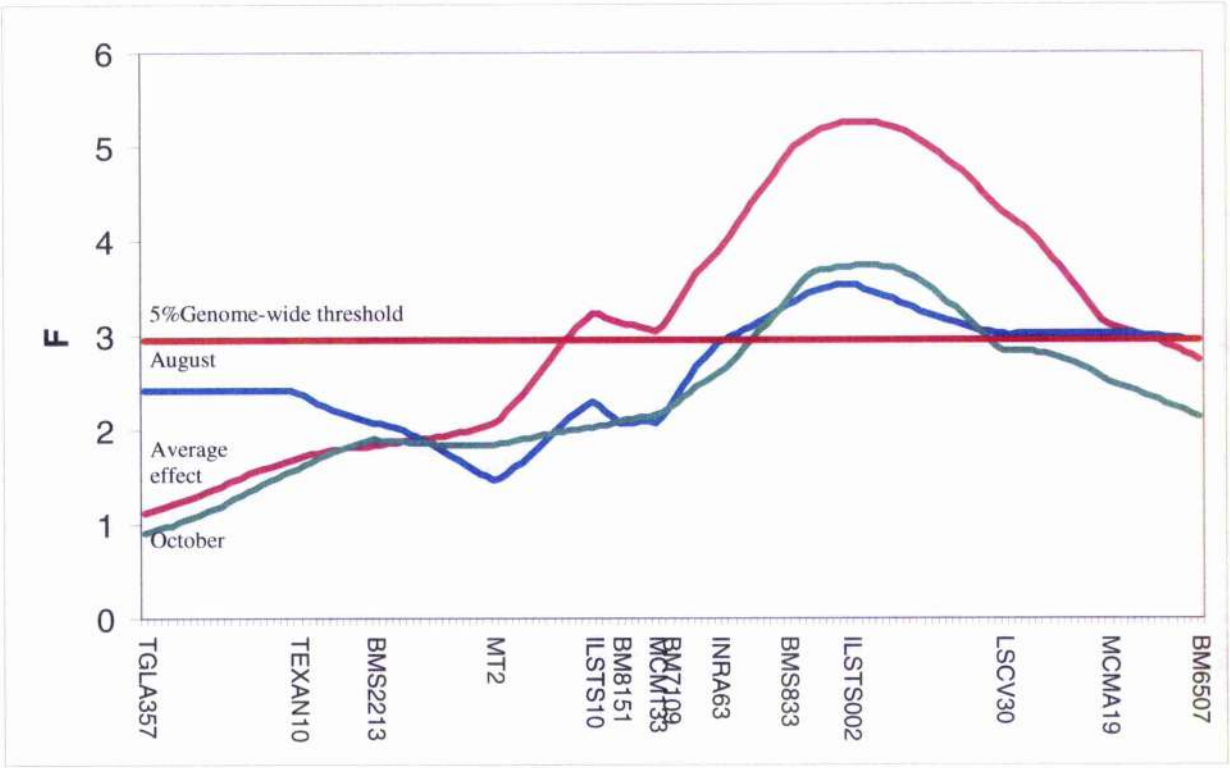
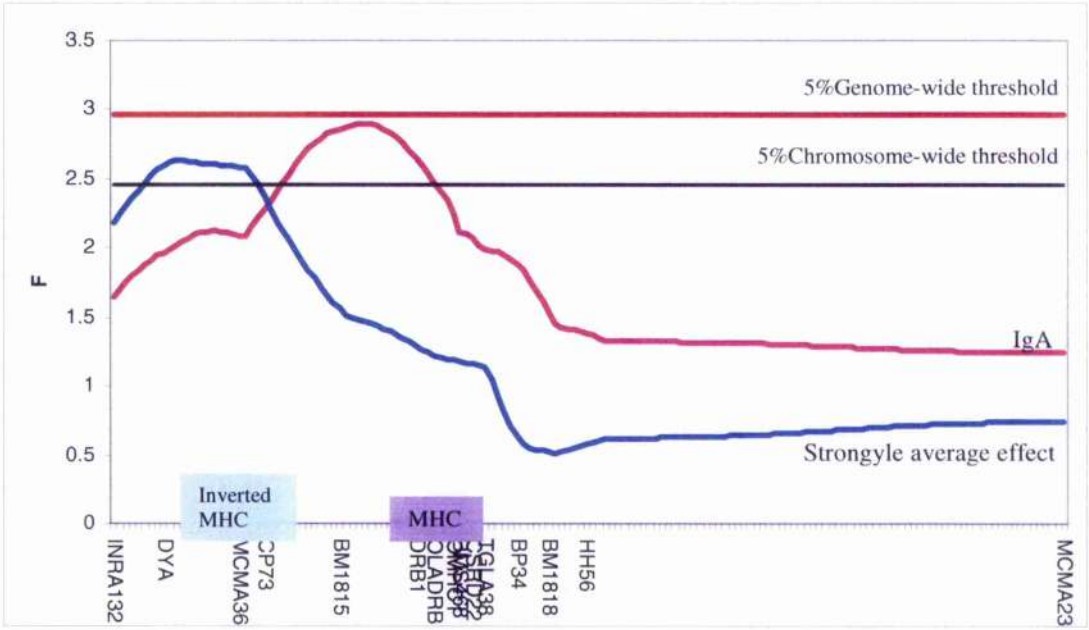


Figure 6-3 QTL contour plot of chromosome 20



6-3-2 Cloning and sequencing of alleles at the exon 2 of the MHC- class II DRB1

The DRB1 locus lies within a QTL region on chromosome 20 which is a putative candidate because of its role in immunity (Zinkernagel and Doherty 1979; Lechler 1994). Investigations of the DRB1 locus have focused on the second exon which forms part of the MHC class II antigen-binding cleft. Amino acids in this region determine the size, form and charge of the cleft and more importantly, the side chains of foreign antigen that may be accommodate in the cleft (Thorsby 1999; Stern et al. 1994). The highly polymorphic nature of the DRB1 gene, where there are over than 90 alleles identified, means the most accurate method of genotyping is sequence-base typing. We therefore sequenced alleles at this locus, which were previously genotyped based on their sizes.

Table 6-5 demonstrates the microsatellite polymorphism of the second exon of the MHC-class II DRB1, their length and sequences. A total of 19 different alleles were identified and 17 alleles could be distinguished on the basis of their length (Table 6-5). Sequence analysis of the DNA samples comfirmed new sequences of the exon 2 of the DRB1 gene associated with microsatellite lengths of 498bp, 500bp, 512bp, 568bp and 572bp. However, some of the microsatellite alleles differ in their length, but appear to have the same sequences in the coding region (E&F), (I&K), (N&O) and (T, U and V). While a number of alleles have the same length but has different sequences (G1&G2) and (H1&H2).

Table 6-5: Sequences and size of microsatellite alleles at *DRB1*.

Microsatellite allele	Size (bp)	Sequence(Gene bank accession number)
A	416	ab017231
B	476	ab017228
C	480	ab017214
D	484	u00216
E	498	y035-1 *
F	500	y035-1 *
G1	512	y082-5 *
G2	512	ab017206
H1	522	af036562
H2	522	u00206
I	526	y10248
K	534	y10248
L	546	ab17230
M	556	ab17205
N	568	y072-2 *
O	572	y072-2 *
T	>800	ab01720
U	>800	ab01720
V	>800	ab01720

* Newly identified sequences

6-3 DISCUSSION

This chapter has identified three unpublished new sequences at the MHC class II DRB1 gene. The new sequences are Y035-1, Y082-5 and Y072-2, which were associated with microsatellite lengths of 498bp-500bp, 512bp and 568bp-572bp respectively.

This study has provided evidence for QTL on 4 chromosomes associated with FEC and IgA activity. The QTL identified on two of these chromosomes are close to regions linked to immune function.

The QTL on chromosome 3 which was associated with Nematodirus FEC, mixed species FEC and IgA activity is very close to Interferon gamma (IFNG). This could be a possible candidate gene as it is linked to immune function. Previous evidence for QTL associated with parasitic infection on chromosome 3 in the region of IFNG has been reported in several studies. Paterson et al. (1999) suggested a QTL in the interval IFNG - BMS1617 for a multispecies parasite challenge in Romney divergent selection lines. Evidence for a QTL associated with *T. circumcincta* was reported in Soay sheep again close to IFNG (Coltman et al. 2001) and a QTL for *Trichostrongylus colubriformis* was observed in Merino divergent selection lines in the IFNG region (Beh et al. 2002). These QTL are very close in position to the QTL identified in this study and they come from diverse breeds that were challenged with different species of nematodes.

In sheep the Major Histocompatibility Complex (MHC) is found in 2 regions of chromosome 20. The QTL found on chromosome 20 in this study are both very close to the MHC regions. These regions could contain possible candidate genes as the MHC consists of a group of closely linked genes involved in antigen presentation to the vertebrate immune system. The ovine MHC is a polymorphic multi-gene complex located on chromosome 20 (20q15-q23).

The MHC class I and II genes encode structural glycoproteins that bind and present peptides to T cells of the immune system resulting in T cell and as such these genes play a key role in the initiation of an immune response.

The MHC region may contain a QTL (Schwaiger et al. 1995; Stear et al. 1996; Buitkamp et al. 1996). Significant association between microsatellite polymorphism in DRB1 gene and faecal nematode egg count on Polish Heather headed sheep was also observed (Charon 2002). Furthermore, Outteridge et al. (1996) identified an association between the MHC DRB1 exon 2 and nematode resistance in the Merino breed. 3 QTL were reported in a Roehnschaf flock for haematocrit level (CP73), IgA level (DYMS1) and FEC (BM1815) after an artificial challenge with *Haemonchus contortus* (Janssen et al. 2002). This again is evidence within a similar chromosomal region.

The sizes of the QTL effects calculated in this study were very large for some of the traits. This may be due to the fact that the heritability estimates are quite low and also are very sensitive to the data transformation used. As it was necessary to investigate the effects of the transformations on the heritability estimates, the effect on QTL locations was also considered. However although the transformation was found to affect the heritability estimate, the regression techniques used in QTL mapping were found to be robust and not sensitive to the transformation. In particular, the position of the QTL was essentially identical irrespective of the transformation used, and the F ratio was only slightly affected. Nematodirus is not the predominant parasite in the flock used for this study; hence the data is skewed by an abundance of 0 values. Even when transformed the Nematodirus data is not normally distributed and the variance component analysis method used by ASREML is significantly affected by this deviation from normality.

In conclusion this chapter has provided strong evidence for QTL linked to parasitic infection and immune response on 4 chromosomes. Two chromosomes have potential candidate genes/regions which have been previously shown to be linked to immune function. The results of this study suggest that some aspects of parasite resistance are under strong genetic control and this information could be used to select sheep for increased resistance to parasitic infection in a marker assisted selection scheme.

CHAPTER SEVEN

GENERAL DISCUSSION

Essentially all grazing animals are infected with nematodes. These infections threaten the health and welfare of livestock and compromise the efficiency of livestock production. Nematodes are possibly the major disease challenge facing ruminants (Perry and Randolph 1999). They are ubiquitous and have a major impact on all production areas (Urquhart et al. 1996).

Many different species of nematodes are pathogenic and they differ in their natural history, epidemiology and pathology (Urquhart et al. 1996). The mixture of species differs among different climates. In the UK, the dominant nematode is *T. circumcincta* but most animals are infected with a mixture of species, including Nematodirus spp., Cooperia spp. and Trichostrongylus spp.

Nematodes cause disease but perhaps their major economic impact is the reduction in growth of young lambs (Coop et al. 1977). The severity of disease and the loss of production depend upon the intensity of infection, immunity of the host and its relative nutritional status (Coop and Kyriazakis 2000; Stear et al. 2003). The intensity of infection is influenced by the weather and management factors such as stocking rate, frequency of anthelmintic treatment and the number of times animals are moved to less contaminated pastures. Host immunity is strongly influenced by genetic factors but also depends upon age, nutrition and history of exposure. Relative nutritional status depends not only on past and current diet, especially protein intake (Coop and Kyriazakis 2000) but also upon the intensity of infection. Clearly, there is a complex interplay which makes the study of nematode infection fascinating and scientifically challenging as well as strategically important.

Efficient and welfare-friendly livestock production demands the control of nematode infection. Current control measures rely upon anthelmintic treatment but are threatened by

the widespread evolution of drug-resistance in parasite populations (Bartley et al. 2004). A variety of potential control methods have been advocated including vaccination (Knox et al. 2003), supplementary feeding (Coop and Kyriazakis 2000), and biological methods (Waller and Faedo 1996). Selective breeding of sheep for resistance to nematode production is an attractive, sustainable method of nematode control (Bisset et al. 2001; Eady et al. 2002; Stear et al. 2001a).

The exploitation of host genetic variation, using faecal egg count (FEC) in commercial sheep breeding programmes, is a well-established breeding practice in New Zealand ('WormFEC') and Australia ('Nemesis'). In the periparturient ewe, FEC are moderately heritable (Bishop and Stear, 2001, Morris et al., 1998; Watson et al., 1995; Woolaston, 1992) and genetically correlated with resistance in the lamb (Morris et al., 1998). Therefore selection of lambs should also lead to ewes that produce fewer nematode eggs. The reduced pasture contamination and decreased larval challenge should have tangible benefits on health and performance (Bishop and Stear, 2003).

In Australia and probably New Zealand the different mix of nematodes means that FEC are a better marker of nematode resistance than in the UK. Egg counts show a linear relationship with the number of adult nematodes in Australian conditions (Roberts and Swan 1981). The relationship is more complex in Scottish lambs (Bishop and Stear 2000). Lambs infected with high numbers of adult nematodes can have lower egg counts than more lightly infected contemporaries (Stear et al. 1998). One consequence of this complex relationship in Scottish sheep is that a selection scheme based solely on egg counts will make slower progress than comparable schemes elsewhere. Even so, alternatives to FEC are routinely used in New Zealand (parasite specific antibody responses) and in Australia (haematocrit values).

Two themes run through this thesis: the validation of genetic and phenotypic markers and the difference between the sexes. Faecal egg counts are the most widely used marker for nematode resistance and they were investigated in chapters three and four. The results are consistent with the idea that faecal egg counts are a useful marker for nematode resistance but have their limitations with natural mixed, predominantly *T. circumcincta* infection. Replicate counts on the same sample were shown to be repeatable but the correlation between samples from the same animals taken at monthly intervals was quite low. The negative binomial distribution provided only a good fit to the data on some but not all occasions. This was quite surprising as the negative binomial distribution is a flexible distribution that has been widely used to describe parasite counts.

Chapter 4 pursued the examination of faecal egg counts and necropsy data suggested that the failure of the negative binomial distribution to describe the distribution of faecal egg counts may be due to the mixed species infection. Each nematode species could produce eggs that followed the negative binomial distribution but the aggregate distribution need not conform to the negative binomial. More research is necessary to determine the joint distribution of several negative binomial variables. The most powerful statistical method for analysing faecal nematode egg counts will depend upon their distribution. Currently several methods are used including generalised linear modelling with negative binomial errors or general linear models on data transformed by taking the logarithmic, square root or cube root. None of these methods is entirely satisfactory and relatively trivial decisions such as the increment to add prior to transformation can give different results; a topic addressed in chapter 6.

However, the main problem with the use of faecal egg counts as a marker is their complex relationship with worm numbers. This was illustrated in chapter 4 where populations with relatively low egg counts had substantially more nematodes than populations with higher

mean egg counts. The explanation is the relatively low fecundity of *T. circumcincta* and especially the density-dependent relationship between egg output per worm and worm number for this parasite (Bishop and Stear 2000).

The relatively complex relationship between egg count and resistance to nematode infection has prompted the search for other markers. There are several markers for nematode resistance in naturally infected Scottish sheep that may offer greater responses to selection. They may be cheaper and more convenient for farmers than egg counts. These markers are both phenotypic and genetic. The phenotypic markers include plasma IgA activity against larval stages of *T. Circumcincta* (Strain et al. 2002), pepsinogenaemia (Stear et al. 1999a), fructosamine concentrations in the plasma (Stear et al. 2001b) and eosinophilia (Stear et al. 2002). Phenotypic indicators have been investigated on a number of occasions and consistently show moderate to strong heritabilities in commercial conditions. For example, IgG specific to *Trichostrongylus colubriformis* (Douch et al. 1995); plasma IgA activity (Strain et al. 2002), pepsinogenaemia (Stear et al. 1999a), fructosamine concentrations in the plasma (Stear et al. 2001b) and eosinophilia (Stear et al. 2002). Davies et al. (2005b) reported that these indicator traits were genetically correlated with worm size and fecundity.

Quantitative trait loci (QTL) provide genetic markers and QTL for nematode resistance have been detected in New Zealand, Australia, Kenya, US and Europe, including UK, France, Italy and Spain. QTL for FEC occur on chromosome 1 (for *T. colubriformis* (Beh et al., 2002; Diez Tascon et al, 2002) and chromosome 3 for *T. colubriformis* (Beh et al., 2002), and mixed natural infection in New Zealand (Paterson et al, 2000), and the UK (Coltman et al. 2001; Davies et al., 2005a), chromosome 6 for *T. colubriformis* (Beh et al., 2002) and chromosome 14 for Nematodirus (Davies et al. 2005a) and chromosome 20 for mixed natural infection in the UK (Buitkamp et al. 1996; Schwaiger et al. 1995; Stear et al. 1996; Stear et

al. 2005). The most useful markers are those that have been identified in several studies. All analyses involve a trade-off between false positives and false negatives. Many methods of QTL analysis accept the risk of false positives in order to minimise the risk of false negatives. Replication of positive findings provides confidence in the results and also suggests that the QTL are likely to be segregating in several populations.

The genetic markers used in this study were the interferon gamma region (Coltman et al. 2001) and the major histocompatibility complex (Schwaiger et al. 1995; Stear et al. 2005). These two QTL on chromosomes 3 and 20 have been confirmed in several independent studies.

Chapter 5 looked at the relationship between the interferon gamma region and faecal egg count. There was a relationship but only in older animals. Interestingly, the relationship was more pronounced in some years and in males. The stronger effect in males suggests that a study involving only male lambs would be more powerful than a study involving both sexes.

The final chapter used linkage analysis to demonstrate a relationship between the interferon gamma region and faecal egg counts. Linkage analysis also demonstrated a relationship between IgA activity and a region on chromosome 20 including the major histocompatibility complex. There was also a relationship between faecal egg count and a region on chromosome 20 that contained the other inverted part of the major histocompatibility complex. More research is needed to determine whether these regions contain the same or two distinct QTL. Further research should concentrate on fine-mapping these gene(s).

Ideally, genetic markers will show strong and consistent effects at all sample dates. This is potentially possible for genes that influence the efficiency of nutrient absorption and growth

rate. However, it is unlikely that all genetic markers for disease resistance will behave this way. For example, lambs develop an acquired immune response to nematode infection (Miller 1984) and markers that operate through the immune response will not show effects until the immune response develops. In addition to the systematic effects of age and sex there may also be pseudo-random variation. If a genetic marker has differential effects on different species or if it has a linear association with worm number then this marker is not expected to show a consistent relationship with faecal egg counts. This is not a problem with the marker but simply a consequence of the fact that faecal egg count is not a consistent marker for nematode resistance. Future studies should consider necropsy to avoid this problem. Of course, these problems with identifying nematode resistant animals emphasise the value of genetic markers.

Another theme running through this thesis is the examination of differences in nematode resistance between sexes. Biologists seek to explain relationships on two levels: proximate and ultimate. A proximate explanation emphasises the physiological mechanisms responsible for the relationship while an ultimate explanation attempts to explain why the physiological mechanisms have evolved to produce the observed effect. Females have lower egg counts than males and they also have stronger immune responses, especially parasite-specific IgA (Strain et al. 2002). In addition, the effect of the interferon gamma gene is less marked in females. As the male lambs were castrated, male hormones, such as testerone cannot explain the observed difference and female sex hormones have been implicated (Barger 1993). An alternative hypothesis is that males are growing more quickly and they allocate greater resources to growth. Consequently, immune responses are relatively weaker than in females and the influence of immune response genes is more apparent. The two explanations are not necessarily incompatible but more research is needed to unravel the influence of sex

hormones and nutrition on the differences between male and female lambs in parasite-resistance.

The ultimate explanation for the difference between males and females is that male lambs need to grow as quickly and as big as possible in order to capture and keep females for their harem. Consequently they are under selective pressure to devote the minimum resources necessary to fight disease. Sex differences are an area for further study and necropsy will be necessary to rigorously quantify differences in worm number and parasite resistance.

In conclusion, this thesis has examined markers for resistance to nematode infection and elucidated some of the strengths and weaknesses of faecal egg counts and the two most convincing genetic markers. Selective breeding is already taking place on a small number of commercial farms. The research reported here will help to advance that process by providing better understanding of the methods used to identify resistant and susceptible sheep.

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