GENETIC RESISTANCE OF SCOTTISH BLACKFACE SHEEP TO GASTRIC NEMATODES. ASSOCIATION WITH THE EOSINOPHIL

by

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To God be glory for the great things he has done!

DECLARATION

I hereby declare that the work reported in this thesis is original and has not been submitted for another degree. Except for the technical assistance from the staff of the departments of Veterinary Medicine and Clinical Biochemistry, Veterinary Parasitology and Veterinary Pathology, the rest of the work was done by me.

Signature ...

DEDICATION

To my dear wife, Rosemary Wambeti

and our two daughters,

Patience Wanjiru and Faith Nyawira

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ABBREVIATIONS

ABC	Avidin biotin complex
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BWT	Body weight
Cr	Chromium
DAB	Diaminobenzidine tetrachloride
E	Eosinophil(s)
ECP	Eosinophil cationic protein
EDTA	Ethylene diamene tetra acetic acid
EEC	European Economic Commission
EPG	Eggs per gram
FAO	Food and Agriculture Organisation of the United Nations
Fe	Iron
gm	Gram
GM CSF	Granulocyte-macrophage colony stimulating factor
h	Heritability
Hb	Haemoglobin
HCl	Hydrochloric acid
H/E	Haematoxylin-eosin
HR	High responder
HT	Haematocrit (calculated)
IEMVT	Institut d'elevage et de Medecine Veterinaires des pay Tropicaux
IgA	Immunoglobulin A

IgE	Immunoglobulin G
IL	Interleukin
ILCA	International Livestock Centre for Africa
KARI	Kenya Agricultural Research Institute
kd	Kilodalton
Kg	Kilogram
L	Litre
L1,L2,L3,L4	Larval stages 1,2,3,4
LR	Low responder
MBP	Major basic protein
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
mg	Milligram
MHC	Major histocompatibility complex
ml	Millilitre
mm	Millimetre
ug	Micrograms
ul	Microlitres
n	Number of observations
NaCl	Sodium chloride
NBF	Neutral buffered formal saline
NS	Not significant
C	Degrees celsius
ODA	Overseas Development Administration
OLA	Ovine lymphocyte antigens
p	Probability
PAF	Platelet activating factor
PAP	Peroxidase-anti-peroxidase

PBEOS	Peripheral blood eosinophilia
PCV	Packed red cell volume (%) - spun
PBS	Phosphate buffered saline
r	Spearmans rank correlation coefficient
RBC	Red blood cell
SAS	Statistical Analysis System
SBF	Scottish Blackface
SD	Standard deviation
SE	Standard error
TNF	Tumour necrosis factor
USA$	United States of America dollars
v/v	Volume per volume
WBC	White blood cells
WHO	World Health Organisation

SUMMARY

The gastric nematodes, *Haemonchus contortus* and *Ostertagia (Teladrosagia) circumcincta*, are the most pathogenic and economically important parasites of sheep in tropical and temperate areas, respectively. There are no vaccines available and the control methods rely entirely on grazing management and anthelmintic treatment. However, there are increasing constraints on the continued use of these conventional methods and alternative approaches for controlling gastric nematodes are under investigation. One of these, namely, genetic resistance to helminth infection is considered in this thesis.

Chapter 1 is a general introduction and review of gastric nematodes with respect to production losses, pathogenesis, limitations of the conventional methods of control, and evidence for genetic variation in resistance. The association between eosinophilia and parasite infection, including, the role of T-lymphocytes, chemotactic factors and possible effector mechanisms, are considered.

Chapter 2 is a general description of the materials and methods and includes, the type of sheep, their management, laboratory techniques and the methods of statistical analysis used to test for differences between groups or individual animals, including other interactive factors such as sex, time, drug treatment.

Chapter 3 reports the results of an investigation on the eosinophil response of lambs to natural mixed nematode infection, predominantly *O.circumcincta*. Faecal egg counts and peripheral blood eosinophil counts were found to be overdispersed, with wide variations among the lambs. The correlation coefficient between faecal egg count and peripheral blood eosinophilia was negative and significant suggesting that peripheral blood eosinophilia might be an indication of resistance to gastric nematode infection.

The repeatability of faecal egg counts taken three times at monthly intervals was not significant from zero (repeatability was 0.06), while that of peripheral blood eosinophil counts was 0.25 and was significant.

Chapter 4 describes the results of an investigation on the existence of diurnal variation of peripheral blood eosinophil counts in sheep infected with 50,000 L3 *O.circumcincta*. Diurnal variation was observed during low to moderate levels of peripheral eosinophilia with the highest counts around 0900 hrs and the lowest around midnight. However, at time of peak peripheral blood eosinophilia following infection, diurnal rhythm was not observed. Thus, the time of sampling peripheral blood eosinophilia is an important factor to consider during low to moderate levels of infection, but not at peak infection period. The factors that might influence diurnal variation, including cortisol levels in blood are considered.

Chapter 5 presents the results of an investigation on eosinophil responses of 20 Scottish Blackface lambs following three experimental infections with 10,000 L3 *H.contortus*. The greatest decrease in growth rate of infected lambs was observed during the first infection. Following the second infection, there was a slight increase in growth rates, while following the third infection, growth further increased.

There were marked differences in the degree of resistance to infection with *H.contortus*. Four lambs died before the end of the experiment and were classified as susceptible. The 16 lambs that survived, classified as resistant, showed marked differences in weight gains, in faecal egg counts, in the ability to maintain stable packed red cell volumes, in peripheral blood and abomasal tissue eosinophilia, and in worm burdens.

There was a significant negative correlation between faecal egg output and body weight gains during the second and third infections

and, between PCV and body weight gains, there was a significant positive correlation during the third infection. In all three infections, the correlation between faecal egg output and PCV was significant and positive.

With respect to peripheral blood eosinophilia, it was significantly and positively correlated with body weight gains during the first and second infections, significantly and negatively correlated with faecal egg counts during the second and third infections, and significantly and positively correlated with PCV values in the second and third infections. At the same time, peripheral blood eosinophilia and abomasal tissue eosinophilia were significantly and positively correlated; both were significantly and negatively correlated with worm burdens.

Thus, peripheral blood eosinophilia was significantly correlated to parameters that reflect resilience (body weight) and parameters that reflect resistance (worm burden, faecal egg output and PCV) and might have a potential as a marker for selection.

Chapter 6 is a general discussion with proposals for further investigations on eosinophils, including, heritability estimates of eosinophilia, association of eosinophilia with major histocompatibility complex, and eosinophil chemotaxis and adherence.

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The Food and Agriculture Organisation of the United Nations (FAO, 1990) has estimated that the total population of sheep in the world is slightly over 1,000 million. Table 1.1 indicates that the population of sheep has experienced a faster growth in the developing countries than in the developed countries in the last 25 years. Over the last decade, there has been an increase of 17.1% in Africa and by 6.5% in Asia, with even greater growth in some individual countries, such as Kenya, from 3.1 to 7.0 million; Sudan, from 13.9 to 19.0 million; Algeria, from 9.3 to 18.0 million; Syria, from 5.9 to 13.7 million (FAO, 1990). These increases are due to the ability of sheep to survive and produce in relatively poor environments on low-cost feeds; their particular adaptability to arid and semi-arid conditions; and their suitability for the small, capital-scarce family farms commonly found in developing countries.

Although the number of animals has grown, there has not been a corresponding increase in the performance of individual animals (FAO, 1990). The most important factors that limit the efficiency of animal production are poor nutrition and high morbidity and mortality rates from infectious diseases. Often, these factors are interactive and are aggravated by poor flock management.

The productive potential of the indigenous animals is also believed to be a limiting factor (FAO, 1990). The transfer of animals from developed to developing countries to improve the indigenous breeds has so far not been very successful (FAO, 1990). For example, exotic livestock often do not thrive well in environments where adapted indigenous breeds exist. At the same time, the full benefit of any improvement in genetic potential through selection and cross-breeding often requires improved nutrition and flock husbandry.

Table 1.1

Changes in the distribution of sheep in different parts of the world.

	1969/71	1974/76	1979/81	1984/86	1988/90
Africa	112.9	114.1	128.8	133.5	144.7
Sub-Saharan Africa	78.5	89.2	93.1	102.6	
Developing World	523.3	535.2	584.1	590.0	617.4
Developed World	557.5	519.3	512.7	535.7	558.2
World	1080.8	1054.5	1096.8	1126.7	1175.6

FAO (1990) in millions

Of the infectious diseases of sheep, gastric nematode infections are recognised as one of the most important constraints to productivity (Armour and Coop, 1991). This is because control can be difficult due to lack of vaccines (Smith and Munn, 1990) and the increasing incidence of drug resistance in nematode populations (Coles, 1988; Pritchard, 1990).

1.2 GASTRIC NEMATODE PARASITES OF SHEEP

Haemonchus contortus is the most pathogenic and economically important nematodes of sheep in the tropical, sub-tropical and warmer temperate regions (Allonby and Urquhart, 1975; Schillhorn van Veen, 1978; Fabiyi, 1986).

In the temperate countries, *Ostertagia (Teladorsagia) circumcincta* is the most pathogenic and economically important parasite of sheep (Urquhart *et al.*, 1987; Armour and Coop, 1991), but it is also prevalent in the subtropical regions where winter rainfall is experienced (Assoku, 1980; ILCA, 1991).

1.3 PRODUCTION LOSSES CAUSED BY GASTRIC NEMATODES

Surveys indicate that up to 95% of the small ruminant population in Sub-Saharan Africa is infected with parasites. Economic losses can be the direct result of neonatal mortality (5 to 25%) or reduced productivity, estimated in terms of weight loss at 0.6 to 1.2 kg/animal/ year (IEMVT, 1980) Infection also results in reduced reproductive efficiency; decreases milk production; reduces the quantity and quality of wool; increases the cost of production through purchase of anthelmintics and their administration; and incurs an opportunity cost through deferred grazing of contaminated pastures (Windon, 1990).

However, there are only a few reports which estimate actual loss of production caused by nematode parasites of sheep in monetary terms. Small ruminant losses in Kenya due to gastrointestinal nematode infection of small ruminants have been estimated at US$ 25 million a year (ILCA, 1991). In Nigeria, one report has estimated that 11% of the total flock value is lost per annum due to gastrointestinal parasites (ILCA, 1991). Similarly, in Chad, 11% of the total flock value is lost as a result of internal parasites (Schillhorn van Veen, 1978). In Australia, the economic loss including the cost of anthelmintics and their administration was estimated at A$ 362 million in 1985 (Windon, 1990).

1.4 PATHOGENESIS AND IMMUNITY TO GASTRIC NEMATODES OF SHEEP

The pathogenicity of gastric nematodes depends on the site of infection (Dunsmore, 1966; Dash, 1985), their mode of feeding, and the total number of worms present, including co-infection with other nematodes and other pathogens (Miller, 1984).

The life cycle of *H.contortus* and *O.circumcincta* is direct, and there is a free-living and a parasitic phase (Urquhart *et al.*, 1987; Roberts, 1991). The free-living phase, includes, the egg, first, second and third stage larva (L1-L3). L3 is the infective stage which develops into the parasitic phase in the host, including, L4, L5 and the mature adult.

Development and survival of the free-living stages are dependent on factors, such as soil surface moisture and temperature (Pandey *et al.*, 1989; Roberts, 1991). The population biology of the parasitic phase is dominated by the host's immune response to

infection and the accumulation of large numbers of immature larvae as a result of arrested development.

1.4.1. Arrested larval development

The ability of *H.contortus* and *O.circumcincta* to survive the host immune response is well documented (Gibbs, 1986; Urquhart *et al.*, 1987). In some circumstances, the fourth stage larvae are able to persist in the gastric mucosa in a metabolically dormant state. The accumulation of arrested larvae often coincides with the onset of cold autumn or winter conditions in the temperate countries, or very dry conditions in the subtropics or tropics (Urquhart *et al.*, 1987). The total faecal egg output continues to fall because the recruitment to the adult stage declines, even though the infective larvae may still be ingested. Maturation of larvae starts when environmental conditions become suitable for the development of the free-living stages of the parasite, but it is not clear what triggers the maturation.

Arrestment is an important feature of the population biology of gastric nematodes of sheep because, development ceases at a point in the parasite phase when, apparently, there is very little effective immunological response from the host (Snider *et al.*, 1981). Therefore, the death rate of the parasites is low, and perpetuation of the parasite population is guaranteed until survival of the free-living stages is assured. The point at which development ceases is also prior to the very damaging phase of emergence, so that the likelihood of host death diminishes as the rate of arrestment increases.

Arrested larval development may have several causes, including the effect of adverse climatic conditions (Armour and Duncan, 1987). Host immunity may also play a role in the induction of arrest (Gibbs, 1986) For example, Adams (1983) found that more

arrested *H.contortus* were present when challenge infections were superimposed on existing infections, suggesting that resident worms or a factor activated by their presence induced developmental arrest. In the case of *O.circumcincta* in sheep, Gibson and Everett (1978) have reported that lambs given daily doses of a few thousand larvae gradually became resistant to infection, and this was accompanied by the accumulation of a high proportion of arrested larvae.

1.4.2. Pathogenesis of ostertagiasis

The presence of sufficient numbers of *O.circumcincta* in the abomasum of sheep gives rise to extensive pathological and biochemical changes with severe clinical consequences. The main pathology occurs when parasites are emerging from the gastric glands (Urquhart *et al.*, 1987), as early as the third week after infection, but may be delayed for several months after infection by arrestment of larval development.

The developing parasites cause a reduction in the functional gastric gland mass, especially in parietal cells which produce hydrochloric acid. These cells are replaced by rapidly dividing, undifferentiated non-acid secreting cells. Initially, these cellular changes are confined to parasitised glands but as the parasites continue to grow, the changes spread to the surrounding non-parasitised glands resulting in a thickened hyperplastic nonfunctional gastric mucosa (Urquhart *et al.*, 1987). Macroscopically, the main lesion is a raised nodule with a central orifice, which in heavy infections coalesce to give a crazy paving-like appearance. The abomasal mucosa is often oedematous and hyperaemic and the regional abomasal lymph nodes become reactive and enlarged.

In heavy infections with more than of 40,000 adult worms, the main biochemical changes include, a reduction in the acidity of the abomasal fluid, the pH increasing from 2.0 to 7.0 (Jennings *et al.*, 1967). Therefore, pepsinogen is not converted to the proteolytic enzyme pepsin, and as a result there is failure to denature proteins and initiate digestion. The elevated pH means that the bacteriostatic effect is lost and there is a dramatic increase in abomasal bacteria (Urquhart *et al.*, 1987). The hyperplastic abomasal mucosa becomes highly permeable to macromolecules, such as pepsinogen and plasma proteins (Urquhart *et al.*, 1987). The end result is a leakage of pepsinogen into the circulation resulting in elevated plasma pepsinogen levels and a loss of plasma proteins into the gut lumen leading to hypoalbuminaemia. Clinically, the consequences are reflected as inappetence, weight loss, and diarrhoea. In lighter infections the main effect is a reduction in growth rate.

1.4.3. Pathogenesis of haemonchosis

The main effect of infection with *H.contortus* is an acute haemorrhagic anaemia due to the blood-sucking habits of the adult worms (Fourie, 1931). Anaemia may become apparent as early as 2 weeks after infection and is characterised by a dramatic fall in the packed red cell volume (PCV). Subsequently, the degree of anaemia may stabilise but at a lower level. Compensatory erythropoiesis occurs, but with continuous loss of iron and protein into the gut, and increasing inappetence, the bone marrow becomes exhausted and the PCV can fall further leading to death of the animal. Dargie and Allonby (1975) studied red cell loss and the ferrokinetics in single experimental infections in sheep using ^{51}Cr-labelled red cells and ^{59}Fe labelled transferrin. Their studies showed that there was a significant loss of

red blood cells into the abomasum and an increase in the rate of red blood cell production in infected animals.

At necropsy, adult worms are readily recognised on the surface of the abomasal mucosa, accompanied by numerous small haemorrhagic foci. The abomasal contents are fluid and dark brown due to presence of blood. The carcass can be pale and oedematous. Changes also occur in the bone marrow with an erythropoietic responsive marrow, expanding from the epiphysis into the medullary cavity (Urquhart *et al.*, 1987). In heavy infections, sheep may suddenly die from severe haemorrhagic gastritis; this is termed hyperacute haemonchosis.

Anorexia is one of the clinical signs observed in infections with gastric nematode infections (Symons, 1985). In both ostertagiasis and haemonchosis, it would appear that reduction in voluntary food intake is a major factor in production loss (Symons, 1985; Nansen, 1986). While Dargie and Allonby (1975) reported that one-year old Merino sheep infected with 10,000 *H.contortus* third stage larvae increased their intake of nitrogen during the first 5 weeks, Evans *et al.*, (1963) found that anorexia appeared after 9 to 10 weeks following oral infection with 3,500 larvae per week and was more severe with continuous daily infection of 500 larvae than with intermittent weekly infection.

A chronic form of haemonchosis can develop during a prolonged dry season when reinfection is negligible but pasture becomes deficient in nutrients. Thus, prolonged loss of blood from small persistent worm burdens is sufficient to produce clinical signs of weight loss, weakness and inappetence rather than a lethal degree of anaemia (Urquhart *et al.*, 1987).

1.4.4. Immunity of sheep to gastric nematodes

The presence of gastric nematodes is associated with increased numbers of B lymphocytes and T lymphocytes expressing CD4 and CD8 antigens in the parasitised abomasal tissue (Emery and Wagland, 1991; Gorrel *et al.*, 1988). Non-lymphoid cells such as mast cells, globule leukocytes, eosinophils and basophils, also accumulate in the submucosa (Miller, 1990; Rothwell, 1989).

There is strong evidence that acquired immune responses play a major role in resistance to gastric nematode parasites of sheep (Miller, 1984, 1987; Watson *et al.*, 1986; Barger, 1989; Adams, 1988; Douch, 1990; Gill, 1991; Windon, 1990, 1991; Gamble and Zajac, 1992). Douch (1990) reported that immune responses to gastric nematode parasites of sheep involve a cascade of events starting with the recognition of nematode antigen(s), followed by stimulation of the immune system and finally, the release of products that promote elimination of the parasite from the abomasum.

Smith *et al.*, (1982a,b) noted that, in immune sheep, worm expulsion is brought about by a combination of factors, involving T cells, specific antibody and nonspecific factors, including mucus and a wide range of inflammatory mediators, such as mast cell proteases, histamine, leukotrienes, prostaglandins (Kelly and Dineen, 1976), thromboxane and platelet-activating factors, that are released into the gut contents and mucus. Several of these mediators have been shown to immobilise L3 *in vitro* (Douch *et al.*, 1984; Jones *et al.*, 1990) and increased levels of these mediators in the gut contents after challenge coincide with periods of plasma leakage into the gut and worm rejection (Miller, 1984). This reaction might account for the rapid expulsion of *H.contortus* L3 from immune sheep (Miller *et al.*, 1983; Jackson *et al.*, 1988). A similar mechanism might also be responsible

for the rejection of adult worms during "self cure" (Stoll, 1929; Stewart, 1952; Soulsby and Stewart, 1959; Mulligan, 1968; Rothwell, 1989).

Experimental studies have shown that adult sheep acquire immunity following repeated infection with *H.contortus* and *O.circumcincta* (Hong *et al.*, 1987). Thus, they are able to resist the accumulation of pathogenic burdens, but seldom remove all the parasites (Allonby and Urquhart, 1973, 1975). Lambs, on the other hand, do not develop immunological reactivity until the age of 7 to 8 months (Manton *et al.*, 1962; Urquhart *et al.*, 1966; Smith and Angus, 1980; Monsell *et al.*, 1984).

The reason for non-responsiveness in young lambs is not known. Several theories have been put forward to explain this phenomenon. For example, Tavernor (1989) proposed that exposure to larval antigens of *Haemonchus*, either through ingestion or through colostrum induced the production of T-suppressor cells in the neonate. Another theory is the one proposed for the *Nippostrongylus brasiliensis* in the rat, whereby, rats less than 6 weeks old are unable to expel primary infections. This is thought to be due to poor expression of MHC Class I antigens on the enterocytes (Mayrhofer *et al.*, 1983).

However, partial immunity to challenge against infections with *H.contortus* can be adoptively transferred between monozygotic twin lambs by using lymphocytes from immune lambs to naive lambs, 1 to 5 days after challenge of immune donor (Smith *et al.*, 1984); both a local mastocytosis and IgA response were observed in the recipient animals.

Faecal egg output is increased in the periparturient period (Urquhart *et al.*, 1987). The increase may be due to a temporary depression in immunity in ewes in late pregnancy and during

lactation (Courtney *et al.*, 1984, 1985a,b, 1986). The decreased immunity has been associated with hormonal changes in steroids and prolactin (Flemming and Conrad, 1989; Jeffcoate *et al.*, 1990). A similar effect has been produced by the administration of corticosteroids to immune sheep (Adams and Davies, 1982).

The effector mechanisms against gastric nematodes that have so far been proposed, include: thickening of the gastric mucosa (Miller, 1984); antibody production, especially locally-produced IgA (Smith, 1977; Duncan *et al.*, 1978;) and the IgE-mediated mast cell responses (Jarrett and Miller, 1982; Presson *et al.*, 1988). Also considered important is the mast cell and globule leukocyte hyperplasia that has been reported following nematode infection (Murray *et al.*, 1968; Salman and Duncan, 1984; Douch *et al.*, 1984; Gill, 1991).

1.5 CONTROL OF GASTRIC NEMATODES IN SHEEP

The most commonly used method to control gastric nematodes involves a combination of grazing management (Herd, 1988; Uriate and Valderrabano, 1990), and anthelmintic treatment (Armour and Coop, 1991; Armour and Bogan, 1982).

1.5.1. Grazing management

Controlled grazing aims at minimising the chances of the host coming into contact with the infective stages of the parasite, in particular the highly susceptible groups, including young animals and pregnant ewes (Armour and Coop, 1991). For example, rotational grazing (Uriate and Valderrabano, 1990) of animals on pasture ensures that the highly susceptible lambs are allowed to graze the pasture first, followed by the older and relatively immune animals (Mitchell, 1984). This method of control works well in areas where the land tenure

system allows individuals, groups of people or companies to own relatively large tracts of land which can be subdivided into small paddocks. In the tropics and in the arid and in semi-arid areas, land is often communally owned and grazing management is seldom applicable. In these areas, the practice is to concentrate grazing areas around scarce watering points; this leads to overcrowding and increases the risk of transmission of infection (Schillhorn van Veen, 1978). Likewise, in areas where sheep production is intensive, recommended pasture management practices are not applicable.

1.5.2. Chemotherapy

The first broad-spectrum anthelmintic, thiabendazole, was introduced in the early 1960s (reviewed by Armour and Bogan, 1982). Since then a wide range of broad-spectrum drugs have become available (Armour and Bogan, 1982). These drugs are highly effective against both immature and adult worms and control practices rely heavily upon their use in either a curative and preventive role. To obtain maximum benefits animals should be treated at the time of maximum risk of exposure, maximum parasite arrest, or during certain critical physiological stages, such as pregnancy when the immune status of the ewe is relatively low.

The benefits of anthelmintic treatment are two-fold: treatment minimises the effects of disease and, at the same time, reduces pasture contamination (Armour and Coop, 1991).

However, there are increasingly serious problems on the continued reliance on the use of drugs. These include, 1) the increasing incidence of resistance in nematode parasites to the most commonly used anthelmintics (Waller, 1990); 2) the high development costs involved in identifying new anthelmintics; 3) the stringent

government regulations involved in the registration of new drugs; 4) the increasing community awareness of environmental issues, and consumer demand for animal products and pastures free of chemical residues (Windon, 1990). Therefore, it is necessary to investigate alternative methods of parasite control which could help reduce the current reliance on chemotherapy.

1.5.3. Vaccination

The initial attempts to vaccinate against gastric nematodes in ruminants suggested that effective vaccination would soon be possible. For example, the use of gamma-irradiated L3 vaccines was shown to provide significant protection against *H.contortus* in lambs older than 6 months (Urquhart *et al.*, 1966; Smith and Angus, 1980).

Several reviews have been written on the progress that has been made on the development of vaccines against nematode parasites (Miller, 1986; Sivanathan *et al.*, 1988; Emery and Wagland, 1991). However, to date there is only one helminth vaccine, against the cattle lungworm, *Dictyocaulus viviparus*, that is commercially available (Jarrett *et al.*, 1959). Vaccination against the dog intestinal hookworm, *Ancylostoma caninum* was also effective, but was not commercially viable (reviewed by Miller, 1986).

In recent years, one approach that has been receiving increased attention is that of vaccination against "concealed" antigens (Smith and Munn, 1990; Emery and Wagland, 1991; Willasden *et al.*, 1993; Smith and Smith, 1993). These are antigens that do not normally stimulate an immune response during natural infestation because of their physical location, but despite this, if the host is vaccinated with the antigen, the parasite can be damaged by the resultant immunological response. This approach has been elegantly

demonstrated for *H.contortus*. Thus, protection was conferred by vaccination of sheep with extracts enriched in contortin, a helical structure loosely associated with the luminal surface of the plasma membrane of the parasite intestinal epithelium - the concealed antigen. Following vaccination, a significant reduction in both faecal egg counts and worm burdens was observed (reviewed by Willasden *et al.*, 1993).

However, field vaccines are still awaited and there is still a need for alternative strategies of controlling gastric nematodes of sheep. One that is now being given increasing attention is the utilisation of genetic variation within sheep populations to increase flock resistance to nematode parasites.

1.6 GENETIC VARIATION IN RESISTANCE OF SHEEP TO GASTRIC NEMATODES

Evidence for genetic variation in resistance of sheep to gastric nematodes comes from both between-breed and within-breed comparisons (Barger, 1989; Albers and Gray, 1987; Gray *et al.*, 1987; Piper, 1987). Resistance in this case refers to the ability of sheep to suppress the establishment and/or subsequent development of gastric nematode parasites once they have been infected. Resistance has been measured by faecal egg counts, PCV following infection, erythrocyte potassium content or serum iron concentration (Piper, 1987). Resilience refers to the ability of sheep to maintain production levels in the presence of gastric nematode infection. Production measures, include, wool growth, average fibre diameter and body weight change. Piper (1987) defined resilience as the difference in production recorded (e.g., wool growth, fibre diameter, body weight change) during infected and uninfected periods.

1.6.1. Between-breed variation in resistance to *Haemonchus contortus*

In Kenya, the indigenous Red Maasai breed of sheep would appear to be more resistant to *H.contortus* infection than local Merinos (Preston and Allonby, 1978), with recent findings showing that Red Maasai infected with *H.contortus* are able to maintain relatively higher PCVs than the Somali Blackhead, Dorper and Romney Marsh breeds (Onyango-Abuge *et al.*, 1993). In the United States of America, certain breeds such as the Florida Native are relatively more resistant than their Rambouillet counterparts to infection with *H.contortus* (reviewed by Gray, 1987). Recently, Gamble and Zajac (1992) have also reported that St. Croix lambs were relatively more resistant than Dorset lambs to both experimental and natural infections with *H.contortus*.

1.6.2. Within-breed variation in resistance to *H.contortus*

Differences in resistance to *H.contortus* infection within the Merino (Allonby and Urquhart, 1976), Scottish-Blackface and the Finnish-Dorset breeds of sheep have been reported (Altaif and Dargie, 1978a). For example, sheep which were homozygous for haemoglobin type A had fewer worm burdens after infection than their homozygous B or heterozygous counterparts. Similarly, Whitlock (1958) examined offspring from parents selected for increased resistance, offspring from unselected parents and offspring from parents selected for decreased resistance to *H.contortus*. The offspring of parents selected for increased resistance to *H.contortus* had lower faecal egg counts and higher PCVs following infection, demonstrating the existence of within-breed genetic variation.

1.6.3. Between-breed variation in resistance to *Ostertagia circumcincta*

Compared to *H.contortus*, there is less information on genetic variation in resistance to *O.circumcincta* in sheep (Piper, 1987). One possible reason is that, faecal egg counts during *O.circumcincta* infection are not necessarily correlated with worm burden (Jackson and Christie, 1984; Piper, 1987), with the result that there is no easy method to assess resistance to *O.circumcincta* infection.

Nevertheless, variation between breeds in resistance to natural infections with *O.circumcincta* has been reported (Stewart *et al.*, 1937; Scrivner, 1967). For example, Scrivner (1967) studied resistance of purebred and crossbred lambs of Dorset Horn, fine wool Merino, strong wool Merino and Corriedale breeds to natural infections of *O.circumcincta*. He reported that faecal egg counts in crossbreds were significantly lower than in purebred animals.

1.6.4. Within-breed variation in resistance to *O.circumcincta*

Scrivner (1967) compared the resistance of offspring of a resistant ram with that of equivalent offspring of two susceptible rams and was able to show that resistance to *O.circumcincta* was heritable. Altaif and Dargie (1978b) reported that Scottish-Blackface sheep with haemoglobin type A showed milder biochemical and pathophysiological changes than their HbB counterparts. At the same time, sheep with haemoglobin type A had relatively smaller numbers of adult worms and more inhibited larvae at necropsy, 16 days after a primary infection with 100,000 *O.circumcincta* third-stage larvae.

1.7 GENETIC MARKERS ASSOCIATED WITH RESISTANCE OF SHEEP TO GASTRIC NEMATODES

1.7.1 Haemoglobin

There are three types of haemoglobin commonly found in sheep, with the order of occurrence HbAA > HbAB > HbBB (Jain, 1986). Earlier investigators reported that sheep with HbAA were more resistant to infections with *H.contortus* than those with HbAB or HbBB (Evans and Blunt, 1961; Evans *et al.*, 1963). Allonby and Urquhart (1976) observed that in Merino sheep in Kenya, sheep of Hb type AA had higher PCV and haemoglobin concentration values than those of the HbBB type, following infection with *H.contortus*. Those of Hb type AB were intermediate. Similar findings were reported by Preston and Allonby (1979). Altaif and Dargie (1978b) observed similar results for infections with *O.circumcincta*. HbA has a higher affinity for molecular oxygen than HbB because of differences in dissociation rates (Jain, 1986). This may be one of the possible reasons for the higher Hb values and PCVs reported in sheep with HbAA type (Evans and Whitlock, 1964). However, Albers *et al.* (1984) failed to find any association between haemoglobin type and resistance to haemonchosis. Rifkin and Yong (1979) also failed to find any association between Hb type with resistance to mixed infections of *Trichostrongylus columbriformis* and *O.circumcincta*.

1.7.2. Putative major gene

The possible presence of a major gene for resistance to *H.contortus* was suggested by the findings of Albers *et al.*, (1987). They reported that the progeny of one of 61 sires had an extremely high level of resistance, as assessed by faecal egg output. However, to date the putative carrier gene has not yet been identified.

1.7.3. Major histocompatibility complex

Resistance to disease has been associated with genes at the major histocompatibility complex (MHC) (Wakelin, 1985; Kennedy, 1989; Tomlinson *et al.*, 1989; Stear *et al.*, 1990).

There are certain ways by which the MHC may influence resistance to gastric nematodes of sheep. Resistance to infection with gastric nematodes of sheep is immunologically-mediated and correlated with the levels of parasite-specific antibody, especially IgG and IgA (Smith and Christie, 1978), and eosinophilia (Salman and Duncan, 1984; Gill, 1991). The MHC controls antibody production through specific antigen recognition and presentation to antibody-producing cells (Tomlinson *et al.*, 1989; Kennedy *et al.*, 1990). At the same time, MHC has been reported to control eosinophilia in mice (Wakelin, 1985). It is possible that a similar situation may exist in sheep.

Outteridge *et al.*, (1984, 1985, 1986) have carried out a series of studies to evaluate the association between MHC class I ovine lymphocyte antigen (OLA) phenotypes, and acquired resistance to *T.colubriformis*. They established a panel of typing sera which was then used to identify OLA determinants within three lines of sheep: a high responder (HR) line of sheep which had been selected for low faecal egg counts following vaccination with irradiated *T.colubriformis* larvae and subsequent challenge with infective larvae; a low responder (LR) line which had been selected for high faecal egg counts following vaccination and challenge, and a randomly-bred line. They reported that two particular OLAs (SY1 and SY2) which were present at intermediate frequencies (48% and 38.6%, respectively) in the unselected animal population, occurred at very different frequencies in animals which were classified either as

high or low responders in the parenteral and early generations of the HR and LR lines. SY1 increased in HR animals (72.2% in rams, 65.7% in ewes), while in the LR animals it was decreased (21.9% in rams, 33.5% in ewes). The opposite pattern was observed for the SY2, whose frequency was decreased in HR and increased in LR. Further analysis on the effects of the presence or absence of these antigens suggested that there were effects on the levels of faecal egg counts. The association has, however, been criticised because of the small number of sires and the possibility of chance sampling effects (Piper, 1987). However, studies by Douch and Outteridge (1989) have given further indications for the association between faecal egg production and OLAs.

Cooper *et al.*, (1989) in their preliminary analysis failed to find any evidence for an association between OLA types with minimum PCV and maximum faecal egg counts following *H.contortus* infection, although the lack of any association was possibly due to the small number of dams and sires used.

1.8 SELECTION CRITERIA FOR RESISTANCE TO NEMATODES

1.8.1. Selection for resistance to haemonchosis

So far, the most commonly used parameters used to measure resistance of sheep to gastric nematodes have been faecal egg counts and PCV values. The primary lesion of haemonchosis is blood loss. The amount of blood lost is linearly related to the number of adult worms (Le Jambre *et al.*, 1971), hence the use of PCV decline and faecal egg counts as a selection criteria for resistance to *H.contortus*.

Substantial progress has been achieved in attempts to select sheep for resistance to *H.contortus* (reviewed by Piper, 1987). For example, investigations at the Pastoral Research Laboratory,

Armidale, New South Wales used faecal egg counts following artificial infection of 5 to 6 month old Merino lambs grazing on natural pastures. Significant differences in faecal egg counts between LR and HR groups of animals were observed after the fifth year. The heritability was estimated at 0.33±0.03 (Piper, 1987). Still, another report came from work based at the University of New England, Armidale, where resistance and resilience were measured in the progeny of 61 sires following artificial infection while on natural pasture. Resilience had a low heritability which did not differ significantly from zero (Piper, 1987). In contrast, heritability for resistance was 0.34±0.10 (Piper, 1987; Albers and Gray, 1987; Albers *et al.*, 1984). The estimated genetic correlation between resistance measurements (faecal egg counts and PCV) were high (>0.75), while those between resistance and resilience traits were moderate, between 0.3 to 0.7. These results confirmed that it should be possible to breed for increased resistance to *H.contortus* without producing unfavourable genetic changes in economically important traits.

1.8.2. Selection for resistance to ostertagiasis

There has been less progress in selection of sheep that are genetically resistant to *O.circumcincta*. Two selection programmes have been reported which are directed against naturally acquired infections of predominately *Trichostrongylus* and *Ostertagia* spp. In New Zealand, divergent lines of Romney sheep have been established on the basis of faecal egg count during two sampling periods which were separated by anthelmintic treatment (Baker *et al.*, 1990). Response to selection was reported to have been greatest when based on the faecal egg counts following the second infection, suggesting the involvement of genetic regulation in the acquirement of resistance. Another selection study

is being carried out in Australia using lymphocyte responsiveness to parasite antigens and, together with data on faecal egg counts, to breed Merino sheep that are genetically resistant to natural infections (Cummins *et al.*, 1991).

One common feature of most selection studies reported so far is the lack of a negative correlation between measures of resistance and production (Windon *et al.*, 1984, 1987). Thus, selection for resistance would not influence production in worm free animals, but during infection lambs selected for superior resistance would have increased production. Woolaston *et al.*, (1990) have shown that selection for susceptibility adversely affects fertility of animals.

The heritability estimates for resistance to gastric nematodes are remarkably consistent (0.2-0.4) and the results from selection programmes suggest that relatively rapid genetic gains can be achieved (Piper, 1987). Simulation studies which compared responses to four different breeding strategies have also shown that progress will be most rapid if selection were to be based on the presence of a major gene. Nevertheless, even in the absence of a major gene Piper and Barger (1988) have estimated that income per ewe could be increased by 10% per year in a moderately infected environment, by including parasite resistance in a selection index from which superior animals can be chosen.

1.9 EOSINOPHILIA AND RESISTANCE OF SHEEP TO GASTRIC NEMATODES

The preferential recruitment and accumulation of eosinophil leukocytes at the site of infection with gastric nematodes is one of the characteristic responses of all host species studied, including, guinea pigs (Rothwell, 1975; Handlinger and Rothwell, 1981) and sheep

(Salman and Duncan, 1985; Douch *et al.*, 1986; Befus *et al.*, 1986; Gill, 1991).

1.9.1. Eosinophil production

The major site of eosinophilopoiesis is the bone marrow (Jain, 1986). Eosinophil precursors migrate from the bone marrow to peripheral blood, before finally lodging in tissues. Once in the tissues, eosinophils do not normally re-enter the circulation (Jain, 1986). The survival time in tissue cultures is 8 to 12 days while the total life span, from bone marrow, to blood, to tissues, is about 8 to 15 days.

The production and localisation of eosinophils is selectively regulated by T-lymphocytes (Rand *et al.*, 1991) via granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3, and IL-5 (Chihara *et al.*, 1990) which not only help to expand eosinophil progenitors but also stimulate activity in mature eosinophils.

Studies in rats have shown that the highest concentration of eosinophils is in four main sites: bone marrow, subcutaneous tissues, gastrointestinal tract and lungs (Rytooma, 1960). In cattle and pigs, the highest concentration of eosinophils is found in the small intestine (0-1500 mm^{-2}), with moderate concentrations in the stomach, lymph nodes and the conjunctiva (50-200 mm^{-2}) (Jain, 1986). Butterworth (1977) also observed relatively high concentrations of eosinophils in the reproductive tract especially during the oestrous cycle. In the guinea pigs, there are approximately 300 eosinophils in the tissues per single eosinophil cell in the peripheral circulation.

The mechanism of genetic control of eosinophilia has been investigated in mice. For example, Wakelin (1985) reported that in mice infected with *Trichinella spiralis*, the extent of eosinophilia was higher in mice with particular alleles in the MHC.

The migration of eosinophils into tissues is directed by chemotactic factors which act in concert with leukocyte adhesion molecules expressed by endothelial cells at the site of inflammation (Walsh *et al.*, 1990). Chemotactic activity for eosinophils has been isolated from certain nematode species (Ishinda and Yashimura, 1990). Klesius *et al.*, (1989), using monoclonal antibodies, have demonstrated the accumulation of eosinophil chemotactic factor in the intestinal cells and lateral hypodermal cords of developing fifth stage larvae of *O.ostertagia* in the abomasal tissue. Klesius *et al.*, (1986) have also shown the presence of eosinophil chemotactic factor in soluble extracts and in excretory-secretory products of *O.ostertagi* third stage larvae. Similarly, several products of host tissues have the capacity to induce eosinophil chemotaxis *in vitro*, including C5a fragment of activated complement, leukotriene B4, platelet-activating factor (PAF) (Topper *et al.*, 1992), GM-CSF (Wang *et al.*, 1987) and IL-5 (Wang *et al.*, 1990). The source of PAF includes mast cells and basophils.

1.9.2. Peripheral blood eosinophilia

The reference values for peripheral blood eosinophil concentration in sheep range from 1 to 1000 (mean = 400) cells per ul of blood (Jain, 1986). Peripheral eosinophil counts show diurnal variation commonly associated with changes in cortisol levels in the blood (Butterworth, 1977; Jain, 1986; McEwen, 1992). For example, in healthy men, the normal range of peripheral blood eosinophil counts (40-440 ml^{-3}) is subject to considerable diurnal variation, with the lowest counts occurring in the morning (10 a.m. to noon) and the highest at night (midnight to 4 a.m.) (Dacie and Lewis, 1984). Fulkerson (1975) reported that cortisol levels in sheep had a circadian rhythm. It is

likely that diurnal variation in peripheral blood eosinophilia in sheep, will also be influenced by the cortisol levels.

Other factors that might influence the distribution of eosinophils in peripheral blood include, stress due to parasite infection (Khansari *et al.*, 1990), handling during the time of blood collection, parturition and nutritional status (Jain, 1986).

1.9.3. Receptors on eosinophils

Eosinophils have receptors for complement factors and immunoglobulins (Anwar and Kay, 1977; Capron *et al.*, 1981a,b; Capron *et al.*, 1985).

Following IgE receptor binding, eosinophil peroxidase, PAF, major basic protein (MBP) and eosinophil-derived neurotoxin are released (Capron *et al.*, 1985; Capron and Jouault, 1986). IgG receptor binding triggers liberation of eosinophil cationic protein (ECP) without the release of eosinophil peroxidase (Capron and Jouault, 1986). The IgG Fc receptor subset, identified on human eosinophils (Hartnell *et al.*, 1990) is thought to mediate eosinophil antibody-dependent cell cytotoxicity (Fanger *et al.*, 1989).

Several reports have suggested the presence of receptors for IgA (Ghazaleh *et al.*, 1988) and IgM (De Simone *et al.*, 1982) on eosinophils but these have not been fully documented.

1.9.4. Structure of eosinophils

Eosinophils are polymorphonuclear cells with cytoplasmic granules that stain characteristically pinkish red with eosin dyes (Jain, 1986). Compared to mature neutrophils, the nucleus of an eosinophil is smoother and less segmented with two or three lobes while the nucleus of a mature neutrophil has three to five lobes. At the same

time, the granules of the eosinophil are loosely packed within the cell, and their size and shape vary among different animal species and sometimes within the same species of animal. For example, the granules of equine eosinophils are the largest in the domestic animals, while feline eosinophil granules are usually small and rod-shaped (Jain, 1986). Ovine eosinophil granules are pear- or cigar-shaped (Murray, 1968).

Occasionally, morphological abnormalities have been observed in eosinophils and these have been associated with certain hereditary and acquired disorders. Hyposegmentation, vacuolation (Saran, 1973) and abnormally large granules due to various causes has been reported in several animal species (Jain, 1986).

Eosinophils contain several cationic proteins and many lysozymal enzymes (Jain, 1986). The characteristic staining of eosinophil granules is due to their highly basic cationic protein commonly referred to as the major basic protein.

Peripheral blood eosinophil cells are heterogeneous in their density, and they have been classified into normal density (normodense) and light density (hypodense) eosinophils (Winqvist *et al.*, 1982; Fukuda and Gleich, 1989). The terminology 'normodense' and 'hypodense', however, imply that there are two distinct subsets of eosinophils, while in fact, there is a spectrum of densities. The density of eosinophils would appear relate to their state of activation (Fukuda and Gleich, 1989).

1.9.5. Function of eosinophils

Eosinophils are thought to play an important role in host resistance to helminth infections by killing larval stages of the parasite (Rothwell, 1989).

Eosinophil functions are influenced by their state of activation (Silberstein and David, 1987). Activation occurs in response to chemotactic agents, cytokines and microbial factors. Activated cells have distinctive physical, morphological, biochemical and functional properties compared to non-activated cells (Fukuda and Gleich, 1989). For example, the activated eosinophils more readily release their granules together with lipid mediators and reactive oxygen metabolites.

The cytokines IL-1 and TNF can stimulate cultured endothelial cells to acquire adhesive properties for various peripheral blood leukocytes, including eosinophils, (Silberstein and David, 1987). Unlike the neutrophils, eosinophils and basophils are capable of binding vascular cell adherence molecules expressed locally on endothelial cells and this may represent a novel mechanism by which endothelium may directly promote the recruitment of these cells without influencing the neutrophil (Walsh *et al.*, 1990).

Eosinophils express CD4 which binds MHC class II antigens (Lucey *et al.*, 1989; Rand *et al.*, 1991) and consequently enhances the avidity of interaction between CD4+ lymphocytes and accessory cells presenting antigens with MHC class II (Rand *et al.*, 1991).

Eosinophils can ingest particulate antigens, including, bacteria, immune complexes, yeast, protozoa and mast cell granules (Sabesin, 1963). It is not clear the extent to which eosinophils can phagocytose parasites, but compared to neutrophils they are less well equipped for this role (Litt, 1964). They have a lesser microbicidal capacity than neutrophils (Beeson and Bass, 1977). However, MBP is highly toxic to larval stages of certain parasites such as *Schistosoma mansoni* (MacKenzie *et al.*, 1977), *T.spiralis* (Wassom and Gleich, 1979),

Fasciola hepatica (Duffus *et al.*, 1980) and *Onchocerca volvulus* (Brattig *et al.*, 1991).

The tendency to discharge granular products extracellularly (Henderson *et al.*, 1983), combined with an absence of O_2 dependent bactericidal enzymes, suggest that the main role of the eosinophil is to defend against tissue invasion by parasites.

1.10 OBJECTIVE OF THE STUDY

There is a need to find markers that can be used to identify sheep that are resistant to economically important gastric nematodes of sheep. The current reliance on faecal egg counts and PCV values is limited to certain parasite species or stages of infection.

Most of the immunological responses to infection with gastric nematode are under genetic control (Wakelin, 1985; Kennedy, 1989; Sewell and Vadas, 1983; Tomlinson *et al.*, 1989). An understanding of the cellular and humoral responses associated with resistance to infection with gastric nematodes might, therefore, help to identify single or polygenes that could be used as genotype markers for resistance to gastric nematodes of sheep. One advantage of selection based on the presence or absence of particular genes is that lambs could be selected at birth without having to undergo prior infection with the parasite.

The objective of this study was to investigate the association between the eosinophil and resistance of Scottish Blackface sheep to gastric nematodes in order to evaluate whether eosinophilia might help identify markers for resistance.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

2.1.1. Source, breed, age and sex

The animals used in all the experiments were purebred Scottish-Blackface sheep (Figure 2.1). The source, age, and sex of the animals differed according to the design of experiments and are described in the appropriate chapter.

2.1.2. Management of experimental animals

Following selection and assignment to particular experiments, the animals were identified with a numbered plastic ear tag. The type of management of the animals differed according to the design of the experiment and is described in the appropriate chapters.

Experimental animals were weighed once a week using a weighbridge.

2.1.3. Clinical examination

Experimental animals were observed regularly for any clinical abnormality during the course of all experiments.

2.2 HAEMATOLOGY

2.2.1. Collection of blood

Blood samples were collected from experimental animals with minimum restraint in order not to cause any physical discomfort. Bleeding was done on two or three occasions each week, between 8.30 and 9.30 a.m., before the morning feeding.

Ten ml of blood were collected by jugular venepuncture of animals using 19 gauge needle into vacutainer or monovet tubes containing (EDTA).

Figure 2.1

A Scottish Blackface ewe with her twin lambs

2.2.2. Haematology parameters

The red cell counts, haemoglobin concentration, haematocrit, mean cell volume, mean corpuscular haemoglobin concentration, mean haemoglobin concentration were analysed by the electronic impedance method using an automatic veterinary cell counter (ABX-Minos, Roche Diagnostics, Welwyn Garden City, England).

Packed red cell volume: blood was drawn into a capillary tube. The tube was sealed at one end with Cristaseal (Hawkeley and Son Ltd., UK) and spun at 12,000 g for 5 minutes on a microhaematocrit centrifuge. The percentage of the packed red blood cells was measured on a haematocrit reader.

Total white blood cell counts were measured by the electronic impedance method using an automatic veterinary cell counter (ABX-Minos, Roche Diagnostics, Welwyn Garden City, England). Blood smears for differential white blood cell counts were stained with May Grunwald-Giemsa (Dacie and Lewis, 1984). At least 200 white cells were counted and the percentage of each cell type calculated.

2.2.3. Enumeration of peripheral blood eosinophils

Ten ul of whole blood (EDTA) were added to 90 ul of phloxine dye-eosinophil diluting fluid in an eppendorf tube to make a 1/10 dilution (Dacie and Lewis, 1984). The preparation was vortex mixed and then left standing for 2 minutes. The two chambers of the haemocytometer were filled by Pasteur pipette and then examined under phase contrast microscopy using a x10 objective lens. Eosinophils stain red, while dead cells (non-eosinophilis) appear black. The total number of eosinophils in both chambers were counted using a tally counter and converted into number of cells per ml of blood.

2.2.4. Plasma separation

Whole blood was centrifuged at 1200 g for 25 minutes (Dacie and Lewis, 1984). The plasma layer was then drawn using a Pasteur pipette and transferred to bijou bottles. The plasma was stored at -20°C.

2.3 PATHOLOGY

2.3.1. Post-mortem procedures

Animals were killed using a captive bolt pistol and exsanguinated. The abomasum was removed immediately after slaughter and opened along the greater curvature. The mucosa of the abomasum was washed with tap water to remove any worms and adherent food debris, and the contents collected in a bucket (Salman, 1980).

Samples for histology were taken from the fundus and pylorus and fixed in 10% neutral buffered formal saline (NBF) and in Carnoy's fluid (Bancroft and Stevens, 1982). The latter were transferred into absolute alcohol after 4 hours, while NBF fixed tissues were kept until the time of processing.

2.3.2. Histological techniques

Tissues were fixed for 24 hours in 10% NBF with 24 hours secondary fixation in buffered formaldehyde sublimate. Processing to paraffin wax was carried out using a Tissue Tek Vacuum Impregnation Processor (VIP) from Bayer Diagnostics using a 16 hour cycle. Tissues fixed in Carnoys for 4 hours were transferred directly to the VIP or to absolute alcohol for short time storage. Sections were then cut at 3 to 5 μm on a 1512 Leitz rotary microtome and stained with Haematoxylin and Eosin, Martius Scarlet Blue, Giemsa or Carbal Chromotrope (Bancroft and Stevens, 1982). Other sections were cut at

5 to 6 μm and stained with Astra Blue/Safranin O and Toluidine Blue (Bancroft and Stevens, 1982).

2.3.3. Immunocytochemical demonstration of IgA plasma cells

Two sections were cut at 3 to 5 μm for use with the Avidin-Biotin Complex peroxidase method (ABC) (Hsu *et al.*, 1981) to demonstrate IgA plasma cells; one section was used as a negative control. The sections were de-paraffinised, rehydrated and immersed in Lugals iodine (Bancroft and Stevens, 1982) followed by immersion in 5% sodium thiosulphate (HYPO) to remove mercuric chloride pigment. Protocol is as follows:

Fixation: formalin fixed minimum 24 hours, post fixed in mercuric chloride.

Sections: paraffin sections ut at 3 to 5 μm.

1.	Sections to water via iodine and 5% Sodium thiosulphate	
2.	Block endogenous peroxidase (0.5% hydrogen peroxide in methanol)	30 minutes
3.	Wash in Tris buffer (x 3 changes)	
4.	Trypsin solution at 37°C (0.1% Trypsin, 0.1% Calcium chloride in Tris, preheated)	30 minutes
5.	Wash in Tris buffer (x 3 changes)	
6.	1% normal rabbit serum	20-30 minutes
7.	Shake off excess rabbit serum and apply primary antibody (rat-anti IgA) diluted in 0.1% BSA, 0.01% Na azide in Tris, overnight at 4°C at optimal dilution. (1.250)	
8.	Wash in Tris buffer (x 3 changes)	

9.	Rabbit anti-rat biotinylated IgG (Vectastain Rat Kit Cat.No. pK4004) Make up ABC complex	30 minutes
10.	Wash in Tris buffer (x 3 changes)	
11.	ABC Complex (Vectastain Kit)	45-60 minutes
12.	Wash in Tris buffer (x 3 changes)	
13.	DAB solution (0.05g in 100ml Tris + 3% hydrogen peroxide)	5 minutes
14.	Wash in water	
15.	Counterstain with Haematoxylin, differentiate, in 1% acid alcohol and blue in STWS.	
16.	Wash in water.	
17.	Dehydrate, clear and mount in DPX	

Results: Sites of IgA - *brown*
Nuclei - *blue*

ABC	Avidin Biotin Complex
BSA	Bovine serum albumin
DAB	Diaminobenzidine tetrachloride
STWS	Scotts Tap Water Substitute
DPX	This is a synthetic mounting medium

2.4 PARASITOLOGY

2.4.1. Faecal egg counts

Faeces were collected from the rectum and examined by a modified McMaster technique (Gordon and Whitlock, 1939). Briefly, 3 gm of faeces were added to 42 ml of tap water. This was mixed thoroughly and passed through a sieve (60 meshes per inch); 15 ml samples of the filtrate were centrifuged in flat bottomed test tubes for 2 minutes at 12000 g and the supernatant poured off. The sediment was resuspended in saturated salt (NaCl) solution, the test tube being

inverted several times; using a pipette, both chambers (volume 0.15ml) of a McMaster worm counting slide (Hawksley and Sons, London, England) were filled with the suspension. The number of eggs in both chambers were multiplied by 50 to give the number of eggs per gm of faeces.

2.4.2. Total worm counts

The total worm burdens were estimated by counting the worms in the abomasal contents and mucosa digest (Ministry of Agriculture, Fisheries and Food Reference Book 418, 1986). The abomasal contents were made up to a total volume of 2,000 ml with tap water. After stirring thoroughly, the number of worms in 200 ml of suspension were counted. The dilution was corrected for and the total worm burden in the abomasal contents calculated.

Half of the abomasal mucosa was scraped gently with a knife for digestion using 1% pepsin in 3% HCl for 6 hours to release immature stages. The digested mucosa was then treated in the same way as the abomasal contents to estimate the number of worms present.

Worms were differentiated morphologically into three groups, namely, adults (females and males), mature L4 (parasites at the fourth moult) and immature L4 (Ministry of Agriculture, Fisheries and Food Reference Book 418, 1986).

2.4.3. Larval culturing and differentiation

A faecal bag was used to collect the total daily output of faeces. Larvae were cultured and differentiated using the method of Keith (1953). Briefly, 20 gm of faecal pellets were cultured in disposable cartons at 23^{o}C for 14 days after which the larvae were harvested by

soaking the faeces in warm water for 3 hours. The larvae were then concentrated by sedimentation to a final volume of 10 ml.

2.5 BIOCHEMISTRY

2.5.1. Pepsinogen concentration

The method developed by Paynter (1992) was used with modification. Briefly, two sets of 75 ul of pepsinogen substrate (3.2% 0.2 glycine, pH 1.6) were incubated with 50ul of plasma. One sample was incubated for 30 minutes at 37°C, and the other for 210 minutes at 37°C before the reaction was stopped with 250ul 10% perchloric acid. All samples were centrifuged for 10 minutes at 12000g. Quadruplicate samples were then taken from each for protein estimation by the BCA method. After incubating four samples with 200ul Pierce BCA reagent for 30 minutes at 37°C, the optical density was estimated at 560nm on a Dynatech MR5000 microplate reader. The difference in protein concentration before and after incubation was estimated by comparison with a standard curve of tyrosine concentration.

2.5.2. Haemoglobin typing

The electrophoretic separation of fast(A) and slow(B) forms of sheep haemoglobin was made with a Paragon agarose gel electrophoresis kit at pH 8.6 (Beckman) (Jain, 1986).

2.6 STATISTICAL ANALYSIS

The means, standard deviations, standard error and quartile ranges for various parameters were determined using the appropriate commands on the MINITAB statistical package (Ryan *et al.*, 1985).

Faecal egg counts and peripheral blood eosinophil counts were overdispersed and were log transformed before analysis of variance. The Univariate procedure on Statistical Analysis System (SAS) was

used to estimate the degree of normality of log transformed peripheral blood eosinophil counts and faecal egg counts.

Differences among animals or groups in unbalanced experimental data were estimated from a mixed model analysis of variance on the SAS statistical package. Groups and sex were fitted as fixed effects and animal was fitted as a random effect. The effects of time and of interactions, were also included where appropriate.

Repeatability is the correlation between repeated measurements on the same animal and estimates the proportion of the phenotypic variance that is due to genetic and environmental effects (Falconer, 1981). It was estimated from a mixed model analysis of variance according to Becker (1984) and was calculated as the ratio of between sheep variance to the total of between-sheep variance and random error (Falconer, 1981).

Gene frequencies were estimated by gene counting. In a closed genetic locus with two autosomal codominant alleles, gene counting gives a maximum likelihood solution (Cavalli-Sforza and Bodmer, 1975).

Statistical tests have been carried out using 5% significance levels.

CHAPTER 3

EOSINOPHIL RESPONSES IN SCOTTISH BLACKFACE SHEEP FOLLOWING NATURAL MIXED NEMATODE INFECTIONS, PREDOMINANTLY *OSTERTAGIA (TELADORSAGIA) CIRCUMCINCTA*

3.1 INTRODUCTION

Several investigators have reported that sheep grazing on natural pasture show wide variations in resistance or susceptibility to infections with nematode parasites (Piper, 1987; Barger, 1989; Gruner, 1991). There are several possible reasons for this, but the general consensus is that part of the variation in host resistance is under genetic control (Wakelin, 1992).

In order to have immediate and widespread application a suitable method for identifying the most resistant animals for breeding and the least resistant for culling should be operable under field conditions. Unfortunately, under many sheep husbandry systems, the age and breeding histories of sheep are often not known and animals are exposed to variable and unquantified levels of infection which may be routinely treated with various anthelmintic regimes; and in this respect, it is known that the use of routine anthelmintic drugs can influence resistance status by leading to the development of acquired immunity (Urquhart *et al.*, 1987; Doenhoff *et al.*, 1991).

Ostertagia circumcincta is the most pathogenic and economically important gastric nematode of sheep in the temperate countries (Urquhart *et al.*, 1987). There is sufficient evidence for genetic variation in resistance to *O.circumcincta* (Chapter 1). However, unlike *Haemonchus contortus*, there is currently no method of identifying sheep which are resistant or susceptible to infection with *O.circumcincta* (Chapter 1).

Eosinophilia has long been associated with nematode parasite infection. Previously, eosinophilia during infection with helminth parasites was thought of as merely an indicator of parasite infection, but recently, several reports have suggested that the eosinophil may

have an effector role against gastrointestinal nematode infections in sheep (Rothwell, 1989; Dawkins *et al.*, 1989; Gill, 1991). However, the association between eosinophilia and other traits used to measure resistance to nematode parasites has not been fully investigated.

The purpose of this study was to investigate the natural variation of peripheral blood eosinophilia and its association with resistance, as measured by faecal egg counts and plasma pepsinogen, and resilience, measured as change in body weight, during natural (field) mixed infections of sheep with nematode parasites.

3.2 MATERIALS AND METHODS

3.2.1. Sheep

Purebred Scottish-Blackface sheep were sampled from a commercial hill farm in the west of Scotland.

3.2.2. Husbandry

The purebred commercial Scottish Blackface flock consisted of 800 ewes which lambed on an upland area in March or April. Ninety-two ewes with twin lambs were moved on to an area approximately 100 acres in size within one week of parturition. The fields on which these animals were kept had been used for this purpose for at least 20 years and lambs on these pastures had been routinely treated for gastrointestinal parasitism during each of the previous 10 years.

All 92 ewes and 184 lambs were gathered at monthly intervals between June and August 1990 and on these occasions blood and faecal samples were collected from all animals. All of the lambs were treated with an anthelmintic (Rycoben 2.5%, Youngs Animal Health Ltd). The anthelmintic was given at the recommended dose rate of 5mg/kg bodyweight, based on the bodyweight of the heaviest lamb in

the group at the time of treatment. During the study the numbers of lambs grazing the 100 acre area decreased as animals were sold.

3.2.3. Sampling

The lambs were examined clinically in June, July and August 1990 and rectal faecal samples were collected. Blood samples were taken into EDTA monovette tubes

Haematology, plasma pepsinogen assays, faecal nematode egg counts, larval culture and differentiation, and haemoglobin typing were performed as described in Chapter 2.

3.2.4. Statistical analysis

The mean, standard deviation, Spearman correlation coefficient (r) and analysis of variance were obtained by using the appropriate commands on MINITAB statistical package (Ryam *et al.*, 1985).

The Univariate procedure on the SAS statistical package was used to estimate skewness and kurtosis (Snedecor and Cochran, 1980).

Faecal egg counts did not follow a normal distribution and the data were transformed to logarithm (count+10) in order to stabilise the variance prior to analysis of variance.

The repeatability was estimated from a mixed-model analysis of variance (Becker, 1984). Animal was fitted as a random effect and sex was fitted as a fixed effect.

3.3 RESULTS

3.3.1. Clinical signs

A small number of the sheep had soft faeces or mild diarrhoea at each sampling. No other abnormalities were observed and the majority of lambs were in good condition.

3.3.2. Faecal egg counts

The mean (± SD) of faecal egg counts was 31.95±54.14, 193.40±269.4, 146.2±224.8 for the months of June, July and August 1990, respectively (Table 3.1). Figure 3.1 shows that the faecal egg counts were overdispersed and significantly different from a normal distribution ($p<0.001$). There was a weak, positive correlation between faecal egg counts in the second and the third samples ($r=0.039$), but not in the first.

The repeatability of faecal egg counts was 0.06 which is not significantly different from zero.

Females had significantly lower egg counts than males. There were 57 males and 88 females. Two-way analysis of variance on log transformed faecal egg counts showed that, after fitting the effect of sample date, males had 160% more nematode eggs in their faeces than females.

3.3.3. Larval differentiation

Faecal cultures from the three samples were predominantly *O.circumcincta* (81%, 94% and 85% in the June, July and August, respectively, with smaller numbers of *Trichostrongylus* spp. (18%, 6% and 14%, respectively) (Figure 3.2). *Haemonchus contortus* larvae accounted for less than 1% in the June and August samples, and were not found in the July sample.

3.3.4. Haematology

Table 3.2 presents means and standard deviations for red blood cell counts, platelet counts, total white blood cell counts and the number of lymphocytes, monocytes, neutrophils and basophils. Values for different sampling dates were significantly different from each other

Table 3.1

Faecal egg counts of Scottish Blackface lambs in June, July, August 1990

Month	n	mean	S.D.
June 1990	169	31.95	54.14
July 1990	174	193.40	269.40
August 1990	146	146.20	224.80

n = number of animals sampled

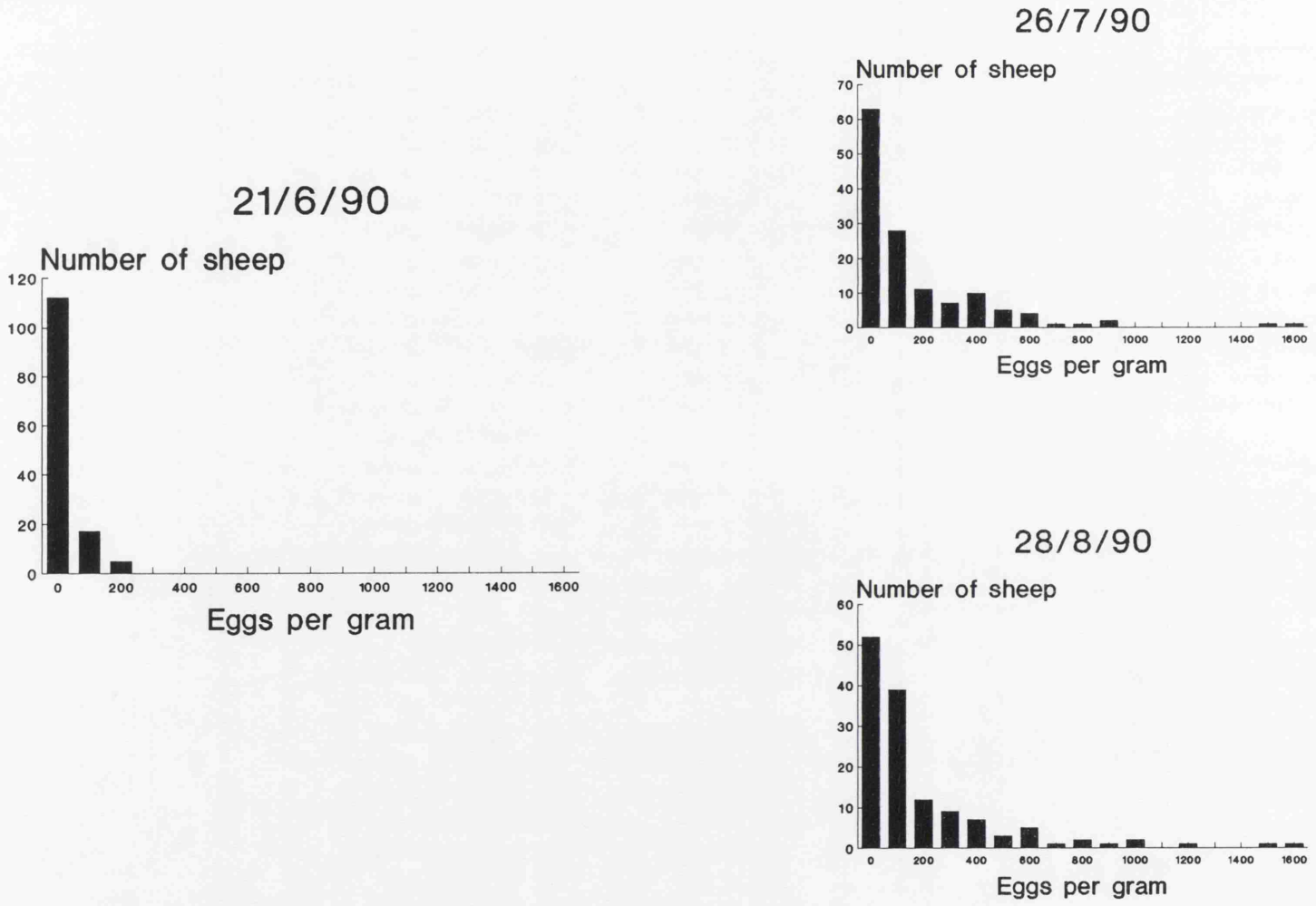
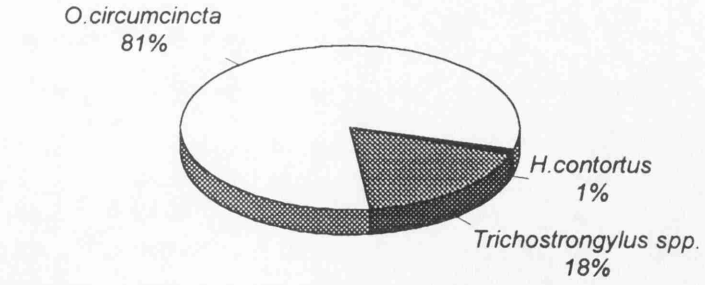
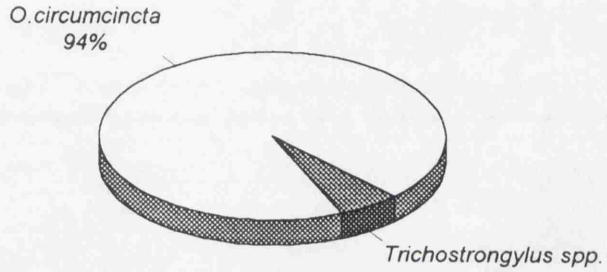


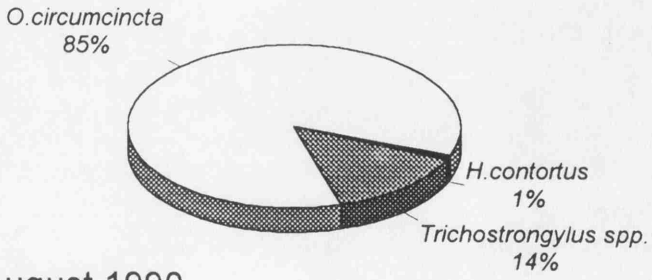
Figure 3.1 Distribution of faecal egg counts in Scottish Blackface lambs in June, July and August 1990



June 1990



July 1990



August 1990

Figure 3.2 Distribution of larvae of different nematode parasites in Scottish Blackface lambs in June, July and August 1990

Table 3.2

Haematological values for Scottish Blackface lambs in June, July and August 1990

	June (n=183)	July (n=177)	August (n=143)
Red blood cell count (10^9 ml^{-1})	12.0±0.9	12.7±0.9	13.0±0.8
Platelet count ($10^6 ml^{-1}$)	365.5±67.5	283.7±68.5	262.7±63.1
White blood cell count ($10^6 ml^{-1+}$)	11.7±3.3	13.9±3.2	13.0±3.1
Lymphocytes	8.9±2.7	9.6±2.4	8.1±2.4
Monocytes	0.15±0.13	0.21±0.16	0.26±0.19
Neutrophils	2.3±1.3	0.12±0.20	0.14±0.20
Basophils	0.08±0.08	0.09±0.09	0.07±0.09

mean + standard deviation

n = number of animals samples

($p<0.05$) and have been presented separately. Values for haemoglobin, haematocrit, MCV, MCH and MCHV are not shown. The values for all the haematological variables fell within the ranges reported by Jain (1986), except for white blood cell counts which were slightly higher in our sheep.

3.3.5. Peripheral blood eosinophilia

The distribution of peripheral blood eosinophil counts during the three sampling periods are given in Table 3.3. Figure 3.3 shows that eosinophil counts during the three sampling dates were overdispersed. They were positively skewed and strongly kurtotic. The repeatability of eosinophil counts monitored three times at monthly intervals was moderate at 0.25 ($p<0.001$).

Of the haematological variables, only eosinophil number showed a significant correlation with faecal egg count; the correlation was negative and significant at the third sampling (Spearman's rank correlation test = -0.173; $p<0.05$) (Table 3.4). Animals with very high faecal egg counts had low eosinophil counts while animals with very high eosinophil counts had low faecal egg counts. There were no animals with high faecal egg counts and high eosinophil counts.

3.3.6. Weight changes

The mean±SD of the lamb weights were 17.3+2.2, 25.4+2.8 and 30.0+2.9 kg at the June, July, August dates, respectively (Table 3.5). The weights were normally distributed at each sampling date (Figure 3.4). Analysis of covariance showed that animal ($p<0001$), sex ($p<0.01$), sample date ($p<0.001$) and the log-transformed faecal egg count ($p<0.05$) were all associated with differences in body weight.

Table 3.3

Peripheral blood eosinophil counts (10^3 cells per ml^{-1}) in Scottish Blackface lambs in June, July and August 1990

Month	n	mean	SD
June 1990	183	8.46	16.04
July 1990	177	12.29	20.06
August 1990	144	14.43	19.91

n = number of animals samples

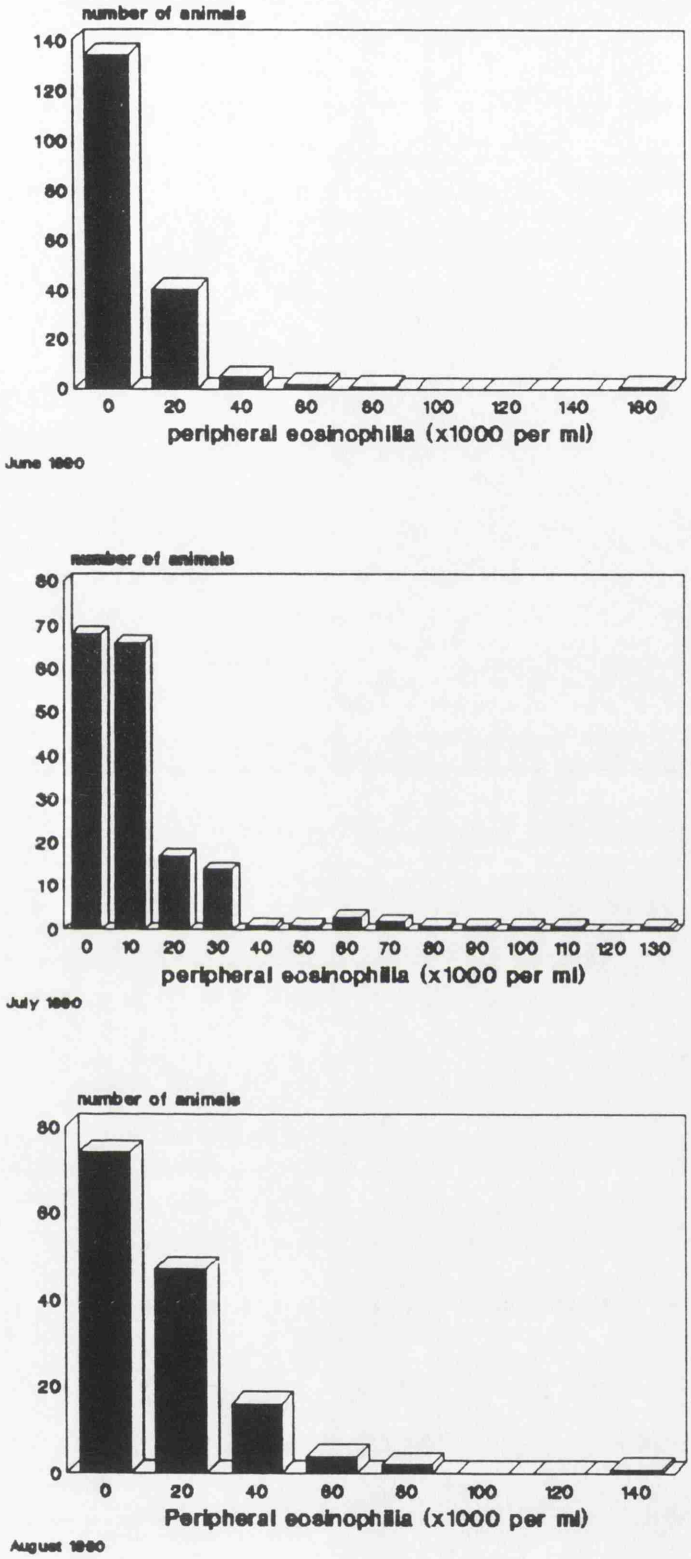


Figure 3.3 Distribution of peripheral blood eosinophilia in Scottish Blackface lambs in June, July and August 1990

Table 3.4

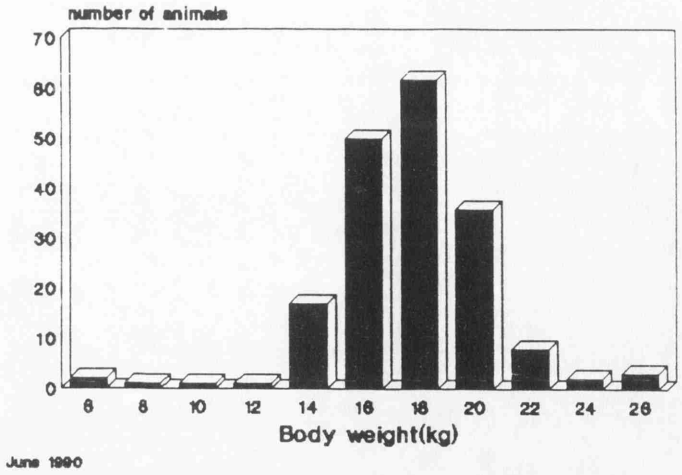
The Spearman rank correlation coefficient (r) between faecal egg counts and peripheral blood eosinophilia in June, July and August 1990.

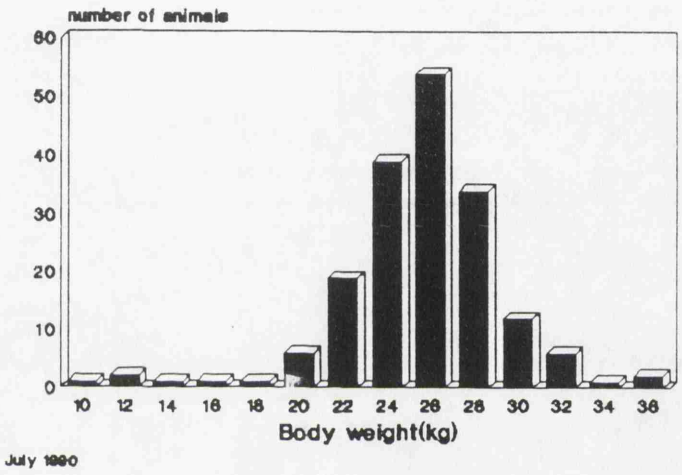
Month	df	r	Remarks
June	124	-0.006	NS
July	173	-0.090	NS
August	143	-0.173	($p<0.05$)

Table 3.5

Body weights of Scottish Blackface lambs (kg) in June, July and August 1990.

Month	n	mean	SD
Juene 1990	183	17.29	2.79
July 1990	179	25.26	3.67
August 1990	145	29.98	3.57





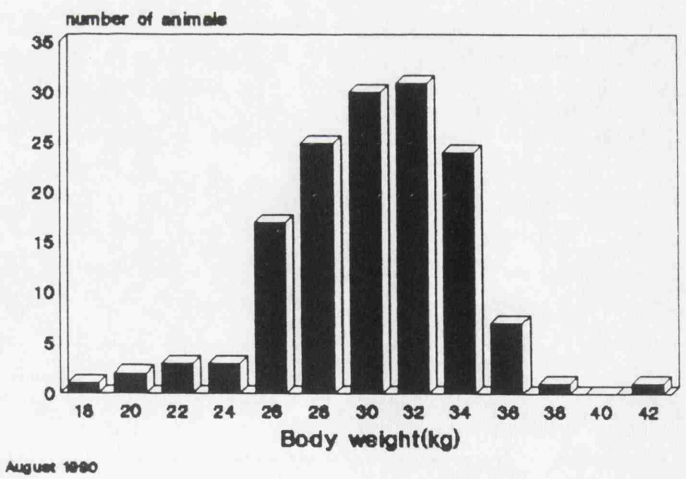


Figure 3.4 Distribution of body weight in Scottish Blackface lambs in June, July and August 1990

3.3.7. Plasma pepsinogen concentration

Plasma pepsinogen concentrations were only estimated at the second and third sample dates. They were significantly different from a normal distribution; they were positively skewed and kurtotic. They ranged from 0 to 9.5 with a mean±SD of 1.8+2.4 international units for the second sample date and from 0 to 16.1 with a mean+SD of 3.9+3.2 international units for the third sample date. The repeatability of plasma pepsinogen concentration was 0.24. There was a weak negative correlation between faecal egg counts and plasma pepsinogen value ($r = 0.16$) in the third set of samples ($0.05<p<0.10$). Animals with high faecal egg counts tended to have low plasma pepsinogen values and animals with high plasma pepsinogen values tended to have low faecal egg counts.

3.3.8. Haemoglobin typing

Of the 114 lambs tested, 92 were homozygous for A and 22 were heterozygous for AB. There were no lambs homozygous for B. The maximum likelihood estimates of gene frequency±standard error are 0.90±0.02 for allele A and 0.10±0.02 for allele B. There was no significant association between haemoglobin type and log-transformed faecal egg count by analysis of variance after allowing for the effects of sample date and sex.

3.4 DISCUSSION

Four parameters show promise as indicators of resistance to naturally acquired, predominantly *O.circumcincta* infection: faecal nematode egg count, peripheral eosinophilia, plasma pepsinogen and body weight.

Faecal nematode egg counts were overdispersed in the population at each of the three sample dates and the negative binomial distribution provided a satisfactory description of the observed distribution of counts. Negative binomial distributions have been fitted to faecal nematode egg counts from Scottish hill sheep following natural mixed infection (Hunter and Quenouille, 1952), from Australian sheep naturally infected with *Nematodirus* spp. (Donald, 1968) and from Australian sheep naturally infected with *H.contortus* (Roberts and Swan, 1982). Worm counts of the genera *Haemonchus, Ostertagia, Trichostrongylus* and *Nematodirus* in naturally infected lambs were also found to follow negative binomial distributions (Barger, 1985). The indices of overdispersion reported in this study were 0.69±0.21, 0.55±0.07 and 0.48±0.07. They all fall within the range 0.42 to 1.45, reported for Scottish hill sheep by Hunter and Quenouille (1952). Interestingly, the three estimates of the index of overdispersion are not significantly different from each other. A common index of overdispersion allows a more meaningful comparison of means in different samples. Also, the proportion of sheep with egg counts above a threshold value will depend upon the mean and the index of overdispersion. The proportion of individuals above a certain value can be obtained by expansion of the expression $(q-p)^{-k}$ where p = mean/k and q = 1+p (Bliss and Fisher, 1953). A mean value of 146.2 nematode eggs per gram and an index of overdispersion of 0.483 were observed in the third sample. These values imply that 65% of lambs would be expected to have egg counts of 100 eggs per gram or less, yet 10% of lambs would be expected to have egg counts of 500 eggs per gram or greater. Also, in these data a mere 10% of the lambs were responsible for 46% of the eggs counted.

One question that becomes relevant in the identification and selection of sheep for genetic resistance to parasites is whether animals with relatively high or low faecal egg counts during one of infection behave the same way following subsequent infections. The repeatability of a trait, such as faecal egg count (or peripheral blood eosinophilia) refers to the proportion of the trait that is expressed at different times in the life of the same individuals. It indicates what proportion of the total phenotypic variance for the trait is attributable to stable differences among individual animals. The repeatability is greater than heritability (h^2), and can be used to set an upper limit for heritability.

There was only a weak correlation between egg counts in the second and third samples. The repeatability of faecal egg count was only 0.06. As the heritability cannot be higher than the repeatability, the heritability of faecal egg count in our data must be less than 0.06. This result contrasts with the moderate (0.2 to 0.4) heritabilities and repeatabilities for faecal egg count following mixed natural infections in Australian sheep (Piper, 1987; Piper and Barger, 1978; Cummins *et al.*, 1991), New Zealand sheep (Watson *et al.*, 1986; Baker *et al.*, 1990) and Australian cattle (Stear *et al.*, 1990). The results imply a greater influence of non-genetic factors on our data, which could be due to larger variances in egg counts due to smaller numbers of eggs being counted; a weaker relationship between worm burden and egg count for *O.circumcincta* than for other species; a greater contribution of non-genetic factors on *O.circumcincta* infection and establishment rates; or to the influence of anthelmintic treatment. Clearly single egg counts are of little value in differentiating sheep which are resistant or susceptible to *O.circumcincta* but serial egg counts which are consistently low or consistently high show more promise. Animals at

the extremes of the distribution should have the best (or worst) combination of genetic and non-genetic influences. Additionally, it is the animals at the extreme ends of the distributions which would be required for breeding, or culling, and which would also be the most informative in a study of the mechanisms of resistance to infection; these animals also provided most information on the relationship between faecal egg count and peripheral eosinophilia and plasma pepsinogen concentration.

Faecal nematode egg counts were higher in males than females. Faecal egg counts were also reported by Stear *et al.* (1990) to be higher in male than in female calves following mixed, natural infection. The reason for lower egg counts in females may be a consequence of sexual dimorphism in humoral and cell mediated immune responses (Grossman, 1985).

Peripheral blood eosinophil counts showed substantial variation, but were within the wide range of 0 to 1000 $x10^3$ cells ml^{-1} quoted by Jain (1986). The counts for the three sampling dates were overdispersed and not normally distributed. Dawkins *et al.* (1989) observed that lambs infected with *Trichostrongylus colubriformis* which were selected for low faecal egg counts had significantly higher peripheral blood eosinophil counts compared with lambs which were selected for high faecal egg counts. The negative correlation between faecal egg counts and peripheral blood eosinophilia suggests that peripheral blood eosinophilia is a measure of resistance rather than merely an indicator of infection. There are various other reports that peripheral blood eosinophilia is markedly increased during infection with other nematode parasite species in different host species (Rothwell and Love, 1975; Butterworth and David, 1981; Sugane and Matsura, 1982; Sugane, 1988).

The mechanism of genetic control of eosinophil production in sheep has not been investigated. In mice, the production of eosinophil cells has been shown to be controlled by the MHC (Sewell and Vadas, 1983; Wakelin, 1985). If the level of peripheral eosinophilia is inheritable in sheep, peripheral eosinophilia might be a phenotypic marker for resistance to nematode parasites. However, because of the wide natural variation in peripheral blood eosinophil counts, there is a need for further studies to investigate the optimum number and time of sampling, especially during peak peripheral blood eosinophilia counts following secondary, and subsequent challenge infections, and during the peri-parturient period (Agyei *et al.*, 1991).

The effector role of eosinophil in resistance to *O.circumcincta* has not been investigated. Butterworth (1984) in his review has discussed the function of eosinophils, including, chemotaxis and antibody-mediated adherence to the parasite, and degranulation of eosinophils resulting in damage or death to the parasite. It is not clear whether a similar mechanism operates for *O.circumcincta*. Investigations similar to those carried out on *Schistosomes* might help to clarify the role of eosinophils during *Ostertagia* spp. infections.

Plasma pepsinogen values showed a weak, negative correlation with faecal egg count, as with peripheral eosinophilia, suggesting that a high value indicates a protective response to infection rather than the presence of a heavy infection. It is likely that high plasma pepsinogen values result from increased mucosal permeability, possibly as a result of a strong inflammatory response in the abomasum, and a strong inflammatory response is at least partially protective (Armour *et al.*, 1979). Yakoob *et al.*, (1983) suggested that increased plasma pepsinogen values might be associated with increased protein losses into the gut and impaired production but

there was no association between plasma pepsinogen values and body weight in our data. However, a more sophisticated study under experimental conditions would be necessary to resolve more subtle effects.

This study has shown that single faecal egg counts and peripheral blood eosinophil counts are of little value in differentiating sheep which are resistant or susceptible to *O.circumcincta*. However, serial counts of nematode eggs or eosinophils which are consistently low or high might be more promising.

In conclusion, under field conditions, Scottish Blackface sheep, exposed to predominantly *O.circumcincta* infection, showed marked variation in resistance and resilience. Furthermore, those animals with consistently low faecal egg counts, had high peripheral blood eosinophil numbers, high plasma pepsinogen concentrations and higher body weight gains. In contrast, in sheep with high faecal egg counts, the opposite was the case. Thus, peripheral blood eosinophilia might be considered as a phenotypic marker for resistance of sheep to *O.circumcincta*. However, before any attempt is made to select sheep on the basis of peripheral blood eosinophilia it will be necessary to investigate the genetic basis of the trait so that the likely response to selective breeding can be estimated.

In this study, there was no association between haemoglobin type and resistance to natural infections, predominantly *O.circumcincta* as measured by faecal egg counts, in spite of the relatively large number of animals tested. This is in contrast with the findings of Alttaif and Dargie (1978b) who reported an association between resistance and haemoglobin types following *H.contortus* infections in sheep. It is possible that haemoglobin type is important only in blood sucking parasites (Jain, 1986).

CHAPTER 4

DIURNAL VARIATION IN PERIPHERAL BLOOD EOSINOPHILIA IN SCOTTISH-BLACKFACE SHEEP FOLLOWING EXPERIMENTAL INFECTION WITH *OSTERTAGIA (TELADORSAGIA) CIRCUMCINCTA*

4.1 INTRODUCTION

The results from our studies have suggested that peripheral blood eosinophilia following infection of sheep with gastric nematodes might serve as a useful phenotypic marker for selecting sheep which are genetically resistant to the parasites (Chapters 3 and 5). This is because of the moderately repeatable, overdispersed distribution of eosinophil counts in blood following natural, mixed predominantly *Ostertagia circumcincta* infection (Chapter 3), and the positive correlation between peripheral eosinophilia and tissue eosinophilia in parasitised abomasum following infection with *Haemonchus contortus* (Chapter 5). Eosinophilia in the abomasal tissue was negatively correlated to worm burdens following experimental infection with *H.contortus* (Chapter 5).

In most of the animal species studied, including man (Dacie and Lewis, 1984), horses (McEwan, 1992) and sheep (Jain, 1986), eosinophil counts in peripheral blood are subject to diurnal variation. Jain (1986) has reported that, in sheep, the peak level of peripheral blood eosinophilia occurred at midnight and the lowest at noon. The extent to which the diurnal variation in peripheral blood eosinophil counts influences the wide variation in eosinophil counts observed following natural infection with nematode parasites (Chapter 3) is not known.

In order to determine the optimum time for sampling peripheral blood eosinophils, an experiment was designed to investigate the diurnal rhythm of eosinophil counts in peripheral blood of sheep after experimental infection with the gastric nematode *O.circumcincta*.

4.1 MATERIALS AND METHODS

4.2.1 Experimental animals

Purebred Scottish-Blackface female lambs which had been exposed to a natural mixed, gastrointestinal parasite infection, predominantly *O.circumcincta*, were housed in worm-free conditions at the Glasgow University Veterinary School. They were treated with albendazole at the rate of 5 mg/kg bwt. Eleven animals were then experimentally infected on two occasions with 50,000 *O.circumcincta* L3, 266 days apart.

4.2.2 Sampling

The first assessment for diurnal variation in peripheral blood eosinophilia was made on days 250 and 251 after the first infection. A second assessment was carried out on days 8 and 9 after the second infection, at the time of the first peak in peripheral blood eosinophilia following reinfection.

The animals were bled for 48 hours during the two sampling periods at intervals of 4 hours, at 0100hours, 0500hours, 0900hours, 1300hours, 1700hours and 2100hours.

Peripheral blood eosinophil counts were performed using the phloxine dye eosinophil dilution method described in Chapter 2.

4.2.3 Statistical analysis

The means and standard deviations (SD) of the peripheral blood eosinophil counts were determined using the appropriate commands on the MINITAB statistical package (Ryan *et al.*, 1985).

Peripheral blood eosinophil counts are overdispersed (Chapter 3). Thus, the counts were log transformed and then, the SAS mixed model analysis of variance was used to test the log transformed

peripheral blood eosinophil counts for differences between animals and over time. In this case, animal was fitted as a random effect and time as a fixed effect.

4.3 RESULTS

The mean peripheral blood eosinophil count on days 250-251 after the first infection was 54 ± 38 x 10^3 cells per ml^{-1}, and 634 ± 481 x 10^3 cells per ml^{-1} on days 8-9 after the second infection (Tables 4.1 and 4.2). There was a marked variation in peripheral blood eosinophil counts among animals (Table 4.1). On both samplings, differences in peripheral blood eosinophil counts among animals were highly significant (Tables 4.3 and 4.4).

On days 250-251 after the first experimental infection, time of sampling had significant effects on the mean of eosinophil counts in peripheral blood (Table 4.3). However, on days 8 and 9 after the second experimental infection, time of sampling had no significant effects on eosinophil counts in peripheral blood (Table 4.4).

Figure 4.1 and Table 4.2 show that the highest peripheral blood eosinophil counts following the first experimental infection were observed at 0900hours and the lowest around midnight. The highest peripheral blood eosinophil count following the second experimental infection occurred in the early morning hours (0100 to 0500 hrs) and the lowest counts at 0900hours and 1700 and 2100hours (Figure 4.2 and Table 4.2).

Table 4.1

Peripheral blood eosinophilia (10^3 cells per ml^{-1}) during the two sampling periods following experimental infection of Scottish Blackface lambs with *Ostertagia circumcincta*.

	250-251 days after first experimental infection			8-9 days after second experimental infection	
Animal number	n	mean	SD	mean	SD
620	12	35.86	16.59	431.6	166.6
625	12	87.52	34.97	432.9	104.4
672	12	33.73	18.75	127.2	57.8
675	12	65.75	34.57	699.7	118.8
722	12	21.35	24.39	689.5	240.5
735	12	49.52	20.20	578.1	141.1
738	12	44.83	18.33	1708.5	407.6
744	12	32.02	16.67	1211.6	342.5
755	12	28.60	11.06	335.1	131.4
760	12	106.70	45.3	384.7	166.2
773	12	89.65	34.35	355.2	153.5
		54.14	37.98	633.6	481.1

n = number of samples analysed

Table 4.2

Peripheral blood eosinophil counts ($x10^3$cells per ml^{-1}) done at 4 hour intervals, following the experimental infection of Scottish-blackface lambs with *Ostertagia circumcincta.*

	250-251 days after first experimental infection			8-9 days after second experimental infection	
Time	n	mean	SD	mean	SD
0100	22	49.70	28.12	695.0	556.0
0500	22	50.96	42.43	686.0	495.0
0900	22	59.70	44.12	586.1	385.3
1300	22	57.27	40.58	685.0	550.0
1700	22	50.71	29.33	600.0	481.0
2100	22	53.73	39.82	576.0	484.0

n = number of samples analysed

Table 4.3

Mixed model analysis of variance of log-transformed peripheral blood eosinophil counts done 12 times at 4 hour intervals over 48 hours on days 250 and 251, following experimental infection of Scottish Blackface lambs with *Ostertagia circumcincta*.

Dependent Variable: Log Transformed Eosinophil Counts

Source of variance	df	Sum of squares	Mean square	F value	Probability
Animal	10	5.115	0.512	19.95	<0.0001
Time	11	1.127	0.102	3.92	<0.0001
Error	120	3.077	0.026		

Table 4.4

Mixed model analysis of variance of log-transformed peripheral blood eosinophil counts done 12 times at 4 hour intervals over 48 hours on days 8 and 9, following experimental infection of Scottish Blackface lambs with *Ostertagia circumcincta*.

Dependent Variable: Log Transformed Eosinophil Counts

Source of variance	df	Sum of squares	Mean square	F value	Probability
Animal	10	12,162	1.216	47.93	<0.0001
Time	11	0.325	0.029	1.o7	0.3920
Error	120	3.019	0.025		

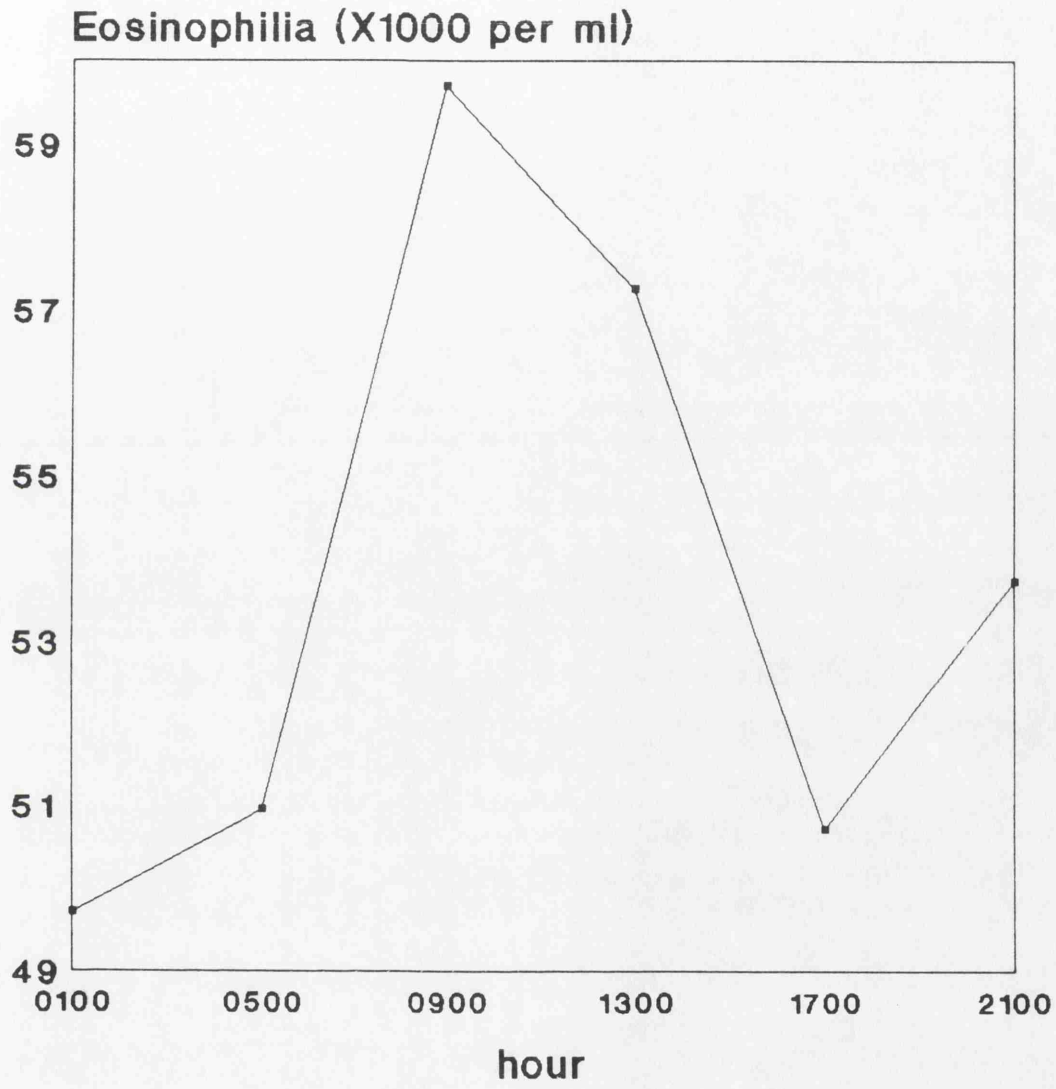


Figure 4.1 Peripheral eosinophilia in Scottish Blackface lambs 250 to 251 days after the first experimental infection with *Ostertagia circumcincta*

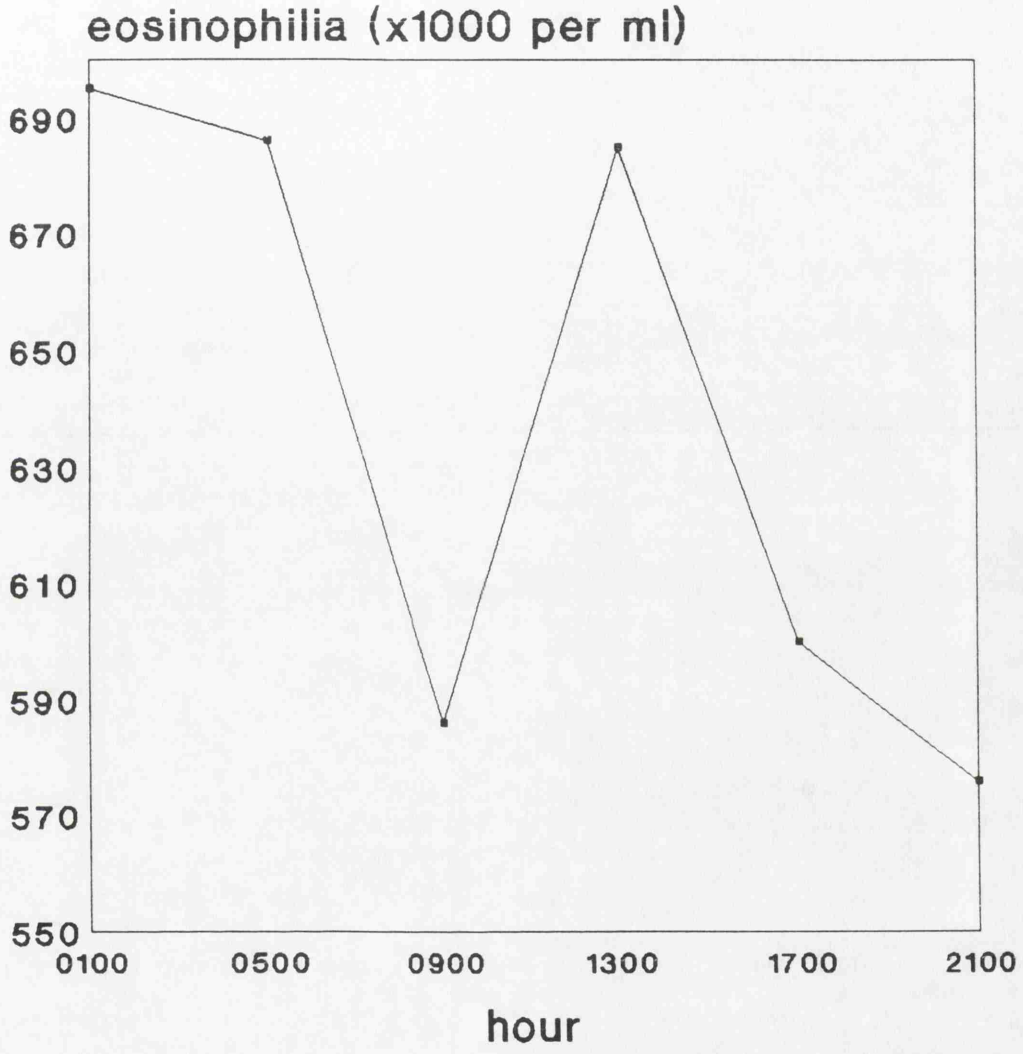


Figure 4.2 Peripheral eosinophilia in Scottish Blackface Lambs 8 to 9 days after the second experimental infection with *Ostertagia circumcincta*

4.4 DISCUSSION

This study confirms the existence of a diurnal rhythm in peripheral blood eosinophil responses in sheep 250-251 days after the first experimental infection with *O.circumcincta*. However, in our study the peak eosinophil count occurred at 09.00hours while Jain (1986) found the peak occurred around midnight.

The diurnal variation in peripheral blood eosinophilia is commonly associated with the synchronous episodic release of cortisol (Fulkerson, 1975) into the blood (Butterworth, 1977; Jain, 1986: McEwen, 1992). Separately, radioimmunoassays of cortisol levels in peripheral blood revealed a significant association between changes in eosinophil levels and changes in cortisol levels (unpublished data). It is possible that other factors which have direct influence on cortisol levels in blood, such as adrenalin and insulin (Benjamin, 1962), might have some indirect effects on the levels of peripheral blood eosinophilia. An increase in adrenalin levels in blood might occur during handling of animals (Jain, 1986). High glucose levels in blood have a positive effect on insulin production and this might cause a decrease in peripheral blood eosinophil count (Benjamin, 1962). Thus, the differences in diurnal variation between our study and that of Jain (1986) might possibly be due to factors such as the time and level of feeding.

In contrast, despite an undulating pattern on days 8 to 9 after the second experimental infection, diurnal variation was not apparent. This was at a time when the eosinophilia was an order of magnitude greater than in the first experimental infection.

In conclusion, the time of sampling is an important factor to consider when eosinophil counts in peripheral blood are low to moderate, but less so during the peak infection period when eosinophil

counts are high. Thus, during subclinical nematode parasite infections repetitive sampling for peripheral blood eosinophil counts should be done at the same time of the day, preferably when the counts are relatively high, in order to avoid the fluctuations attributable to diurnal rhythm.

CHAPTER 5

EOSINOPHIL RESPONSES IN SCOTTISH BLACKFACE LAMBS FOLLOWING EXPERIMENTAL INFECTION WITH *HAEMONCHUS CONTORTUS*

5.1 INTRODUCTION

The most commonly used parameters for measuring resistance to *Haemonchus contortus* are faecal egg counts and packed red cell volumes (Piper, 1987; Albers *et al.*, 1987). There is a high positive correlation between faecal egg counts and worm burdens following infections with *H.contortus* in sheep (Roberts and Swan, 1981). At the same time, *H.contortus* is a voracious blood sucker and the degree of anaemia has been reported to correlate well with parasite burden (Piper, 1987).

These traits are useful markers for resistance during the clinical stage of the infection when the adult parasites are residing in the abomasum. However, they are less likely to be reliable when infections are subclinical or when arrested larval development (inhibition) has occurred (Urquhart *et al.*, 1987).

Therefore, it is necessary to identify other traits that measure host resistance, particularly to migrating or inhibited larvae of *H.contortus*. Such additional markers would help cross-check between other markers of resistance, and at the same time, they could be used as additional selection criteria, thereby, increasing the efficiency of selecting sheep that are genetically resistant to *H.contortus*.

Peripheral blood eosinophilia and tissue eosinophilia are markedly increased during infection with gastrointestinal nematode infections of sheep (Rothwell, 1989; Dawkins *et al.*, 1989). Dawkins *et al.*, (1989) analysed eosinophil responses in sheep selected for high and low responsiveness following vaccination with *Trichostrongylus colubriformis*. After challenge with the same parasite, they observed that lambs which were selected for low faecal egg counts had significantly higher peripheral blood eosinophilia compared with

lambs which were selected for high faecal egg counts, suggesting that peripheral blood eosinophilia was heritable.

This study reports the results of an investigation on the association between eosinophilia (peripheral blood and abomasal tissue) and resistance, as measured by worm burden, faecal egg counts and PCV values, and the association between eosinophilia and resilience, as measured by body weight, in Scottish Blackface lambs following experimental infection with *H.contortus*.

5.2 MATERIALS AND METHODS

5.2.1. Experimental animals

Twenty four male and female Scottish Blackface lambs, aged 7 to 8 months, were raised worm-free and were transferred to Glasgow University Veterinary School where they were kept under parasite-free conditions for 10 days before the first experimental infection.

5.2.2. Experimental design

Twenty lambs were infected orally with 10,000 third stage larvae of *H.contortus*. A similar second and a third experimental infection were given 8 and 16 weeks later, i.e., on days 56 and 112. Four animals became progressively weak and were sacrificed for abomasal histology and worm counts on days 64, 92, 92 and 140. These were classified as susceptible animals.

The sixteen lambs that survived the three infections were classified as 'resistant'. These animals were sacrificed 8 weeks after the third infection, i.e. on day 168. The four lambs that were not infected (controls) were sacrificed at the same time.

5.2.3. Parasitological techniques

Faecal egg counts were carried out by the method of Gordon and Whitlock (1939). Total worm burdens were estimated by counting the worms in the abomasal contents and mucosal digest (Ministry of Agriculture, Food and Fisheries, 1986).

5.2.4. Haematology

Blood samples were taken into EDTA monovet tubes.

Peripheral blood eosinophil counts were carried out using the phloxine dye eosinophil dilution method (Dacie and Lewis, 1984) as described in Chapter 2.

Haematocrit was analysed by the electronic impedance method (ABX-Minos, Roche Diagnostics, Welwyn Garden City, England).

5.2.5. Pathology

Post-mortem examination was carried out according to the method of Murray *et al.* (1983). Tissue samples were processed as described by Bancroft and Stevens (1982).

Tissue eosinophil counts were performed by a modification of the method used by Salman (1980). Briefly, the distribution of eosinophils in the region of the lamina propria was estimated by systematic movement of the microscope stage. A total of 20 random graticule fields measuring 0.7 x 1.05 mm^2 each were examined on each sample from the abomasal fundus and the pylorus. Cell counts were carried out with x 100 objective.

5.2.6. Statistical analysis

The mean, standard deviation (SD) and the Spearman's rank correlation between faecal egg counts (EPG), packed red cell volume

(PCV), peripheral blood eosinophil counts (PBEOS), body weight changes (BWT), abomasal worm burden and abomasal tissue eosinophils were determined using the appropriate commands on the minitab statistical package (Ryan *et al.*, 1985).

The differences between the means of each variable were estimated from a mixed-model analysis of variance using SAS system. Animal was fitted as a random effect and group as a fixed effect. Newman-Keuls Multiple Range Test was used to calculate the least significant range for the means.

The association between blood and tissue eosinophilia and worm burdens was examined by regression analysis of the transformed eosinophil counts (logarithm to the base 10 plus one) at the time of slaughter on the transformed worm burden, with the GLM program on the SAS statistical package.

5.3 RESULTS

5.3.1. Clinical, parasitological, haematological and pathological findings

There was a marked difference in the response to experimental infection to *H.contortus* among the 20 Scottish Blackface lambs. When assessed by survival, 16 lambs were classified as resistant as they survived the three infections, while 4 lambs were classified as susceptible because they succumbed. On the basis of faecal egg output, and PCV as well as survival, the group of 20 lambs could be further classified into four groups, highly resistant, resistant, moderately resistant and susceptible (Table 5.1; Figures 5.1 to 5.20). The highly resistant group was classified on the basis of complete 'self cure' (disappearance of eggs in faeces) following the second infection, and failure of the third infection to become established, as assessed by

Table 5.1

Clinical outcome and resistance rating following three experimental infections of 10,000 L_3 *Haemonchus contortus* parasites at 8 week intervals in Scottish Blackface lambs.

Sheep ID No	Resistance rating	Remarks on second and third infections
FR 51	xxx	Self-cure, re-infected, maintained high PCV
FR 52	xxxx	Complete self-cure, maintained high PCV
FR 53	xxx	Self-cure, re-infected, maintained high PCV
FR 54	xx	Some self-cure, maintained low PCV
FR 55	xxxx	Complete self-cure, no re-infection, high PCV
FR 56	xx	Self-cure, re-infected, maintained low PCV
FR 57	xx	Self-cure, re-infected, maintained low PCV
FR 58	xxxx	Complete self-cure, maintained high PCV
FR 59	x	Some self-cure, low PCV, weak, died
FR 60	xxxx	Complete self-cure, maintained high PCV
FR 61	xxxx	Complete self-cure, maintained high PCV
FR 62	xxx	Self-cure, re-infected, self-cured, high PCV
FR 63	xx	Some self-cure, re-infected, maintained low PCV
FR 64	xx	Some self-cure, re-infected, maintained low PCV
FR 65	xx	Self-cure, re-infected, high PCV
FR 66	xxxx	Complete self-cure, maintained high PCV
FR 67	x	Some self-cure, maintained low PCV, died
FR 68	xxxx	Complete self-cure, maintained high PCV
FR 69	x	Some self-cure, re-infected, low PCV, died
FR 70	x	Some self-cure, low PCV, died

Key to resistance rating

xxxx	High resistant
xxx	Resistant
xx	Moderate resistant
x	Susceptible

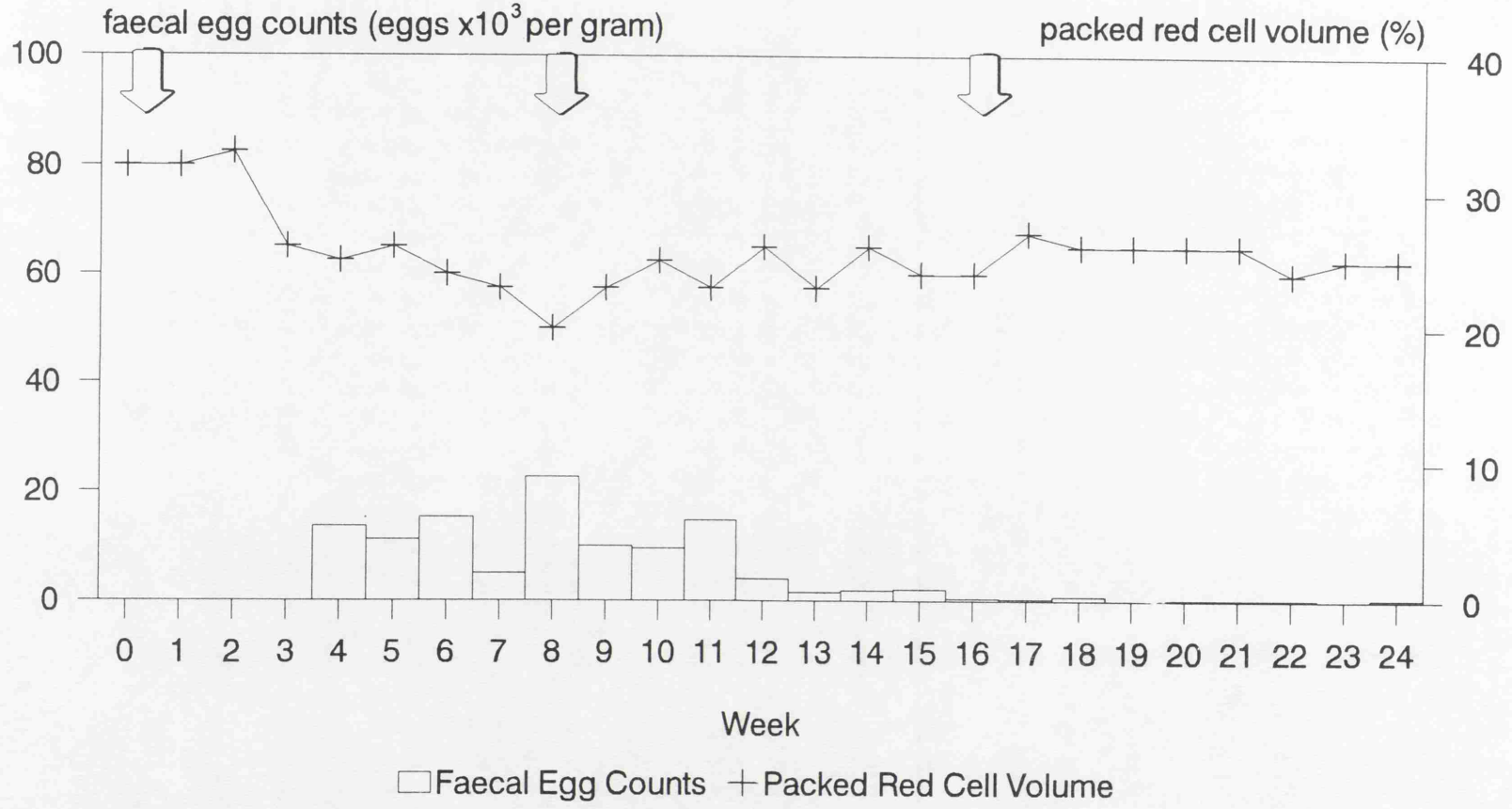


Fig. 5.1: Faecal egg counts and packed red cell volume of animal no. FR 51.

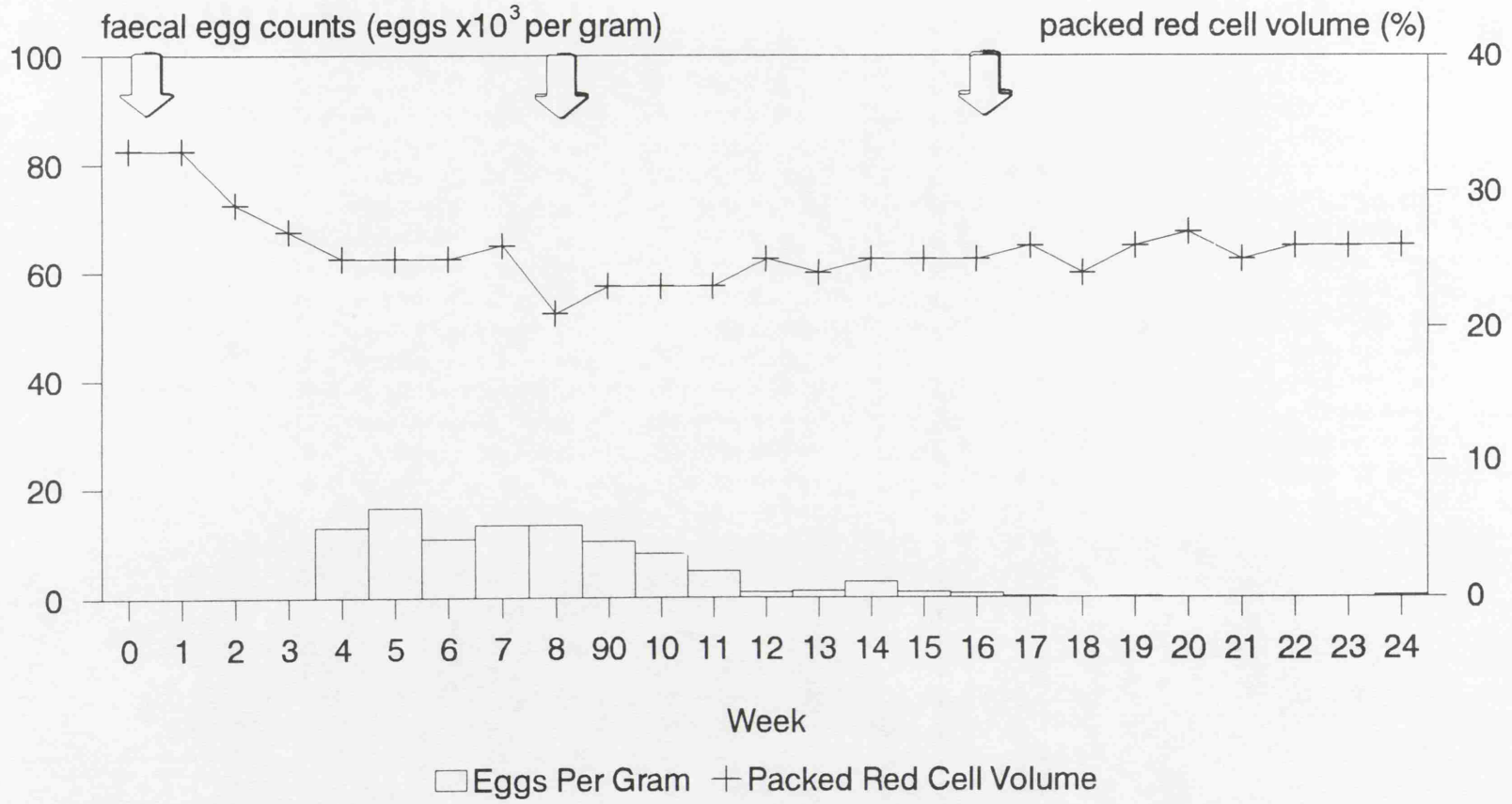


Fig. 5.2: Faecal egg counts and packed red cell volume of animal no. FR 52.

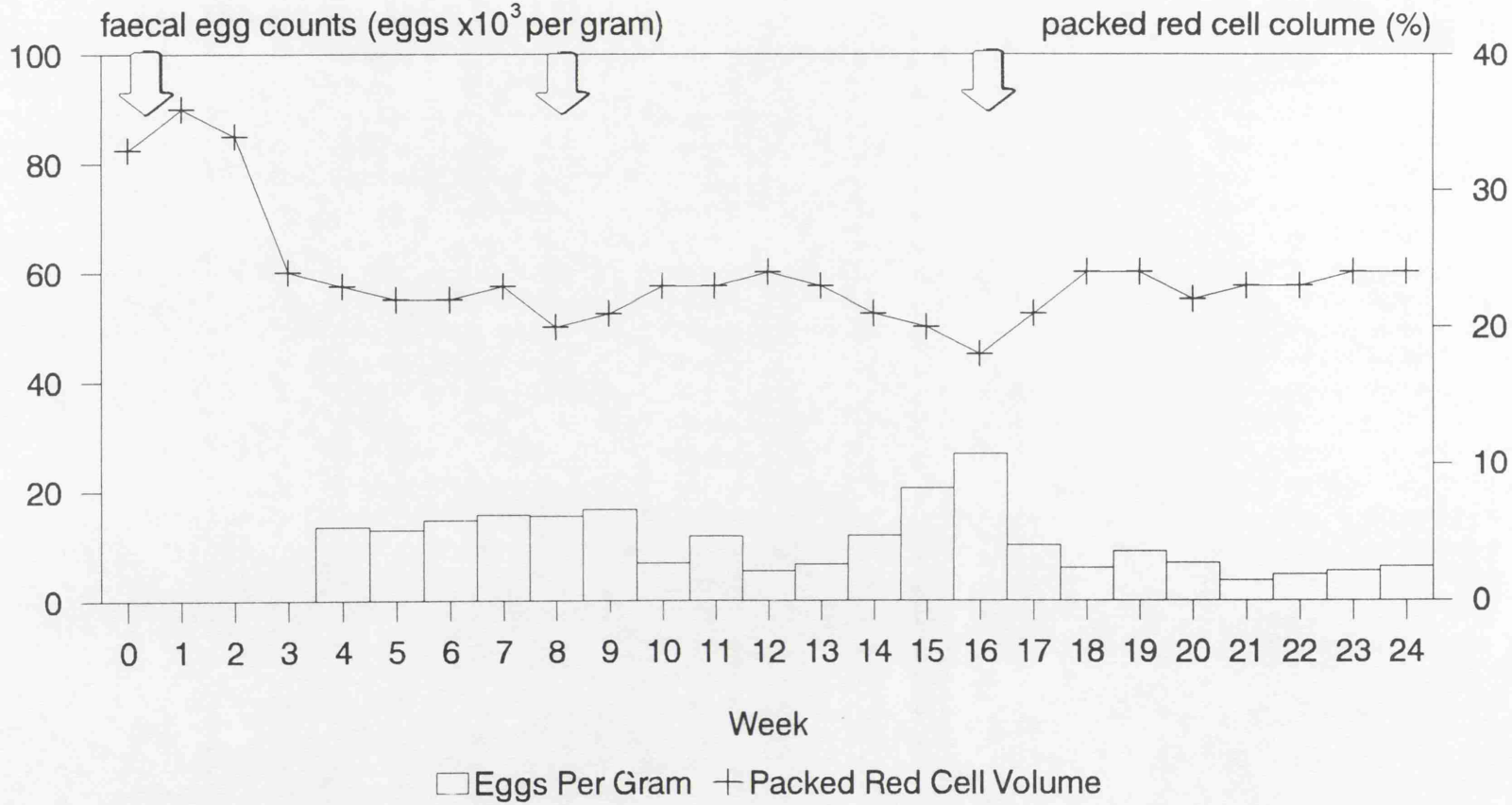


Fig. 5.3: Faecal egg counts and packed red cell volume of animal no. FR 53.

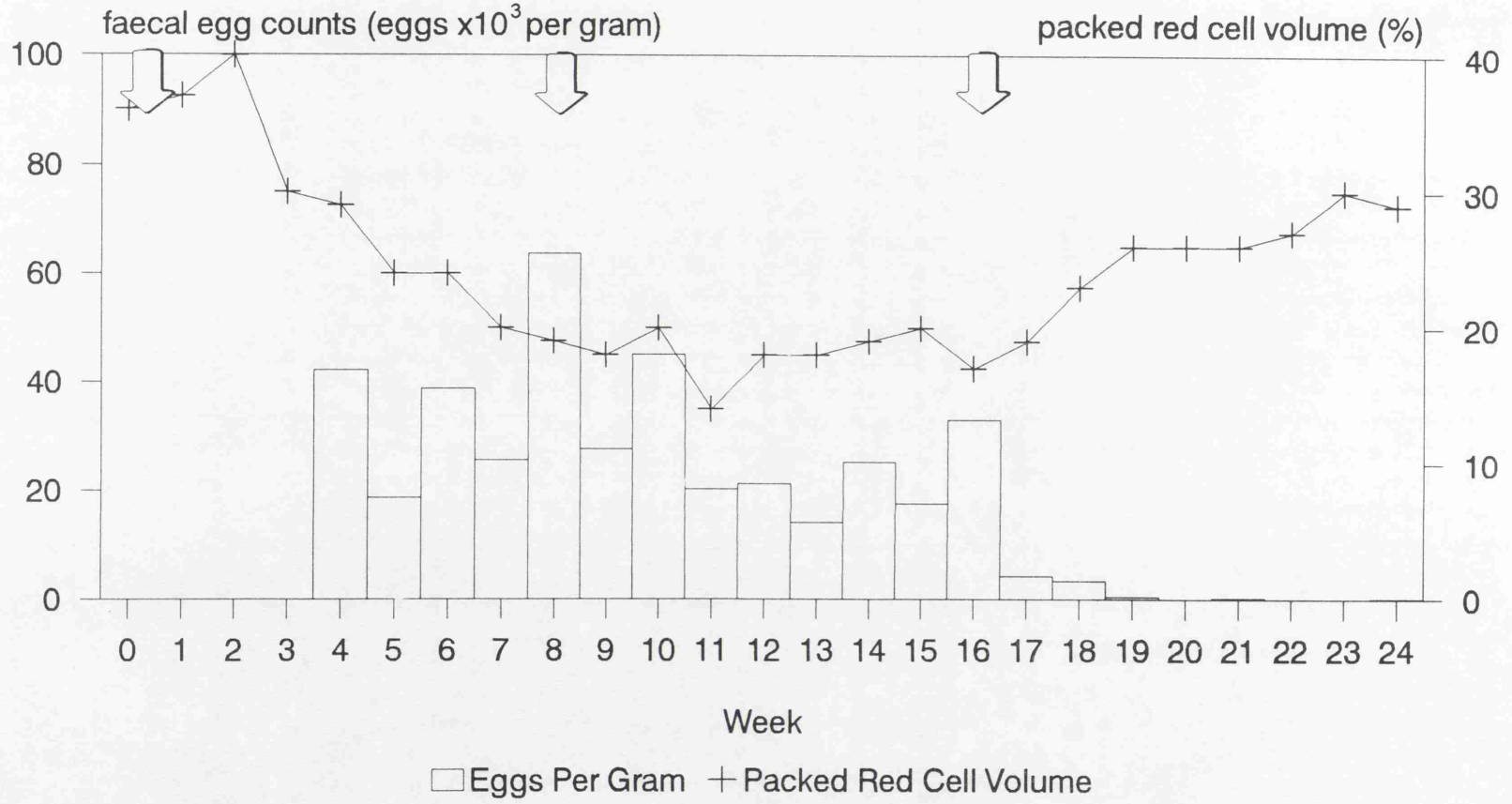


Fig. 5.4: Faecal egg counts and packed red cell volume of animal no. FR 54.

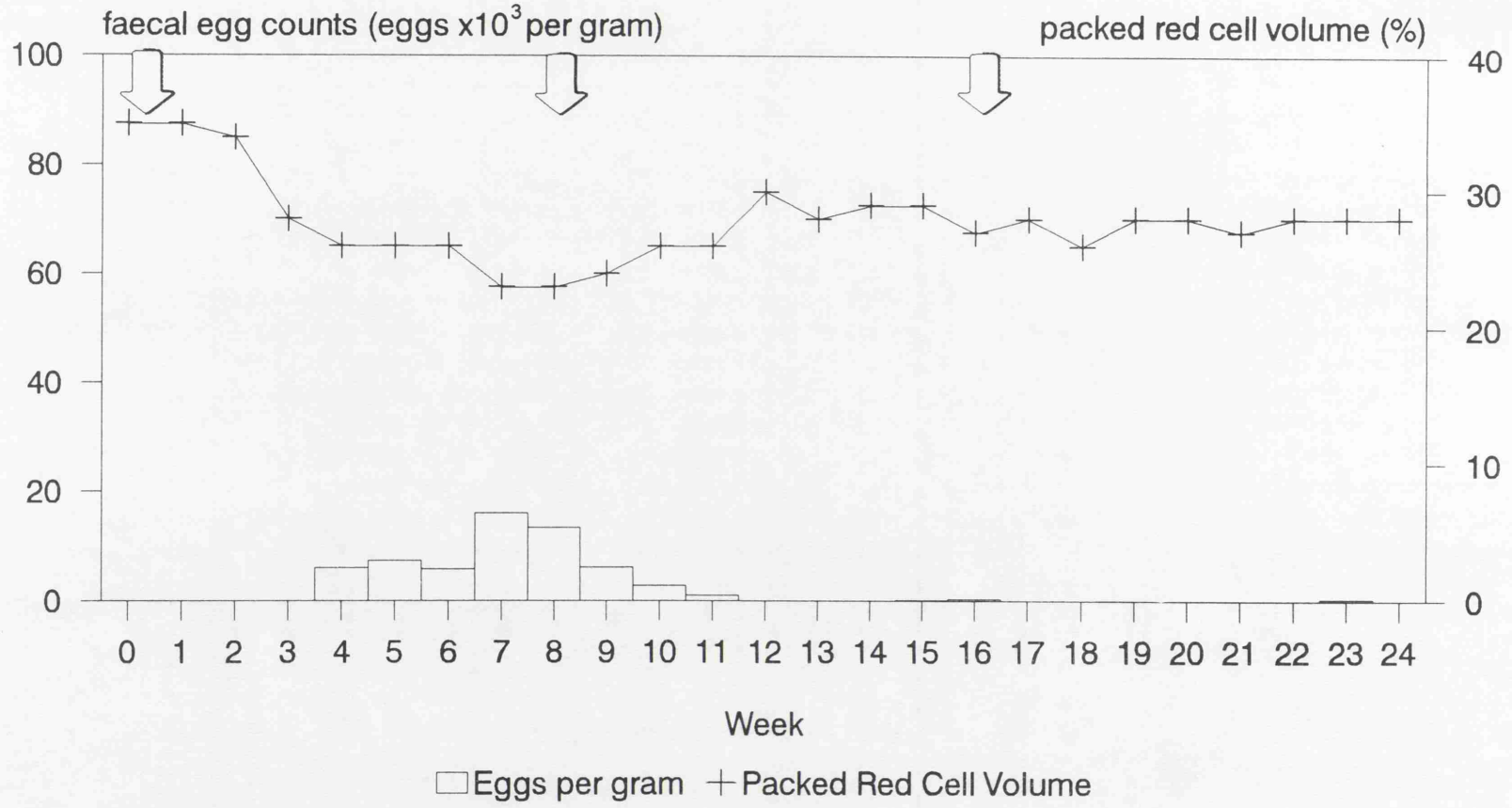


Fig. 5.5: Faecal egg counts and packed red cell volume of animal no. FR 55.

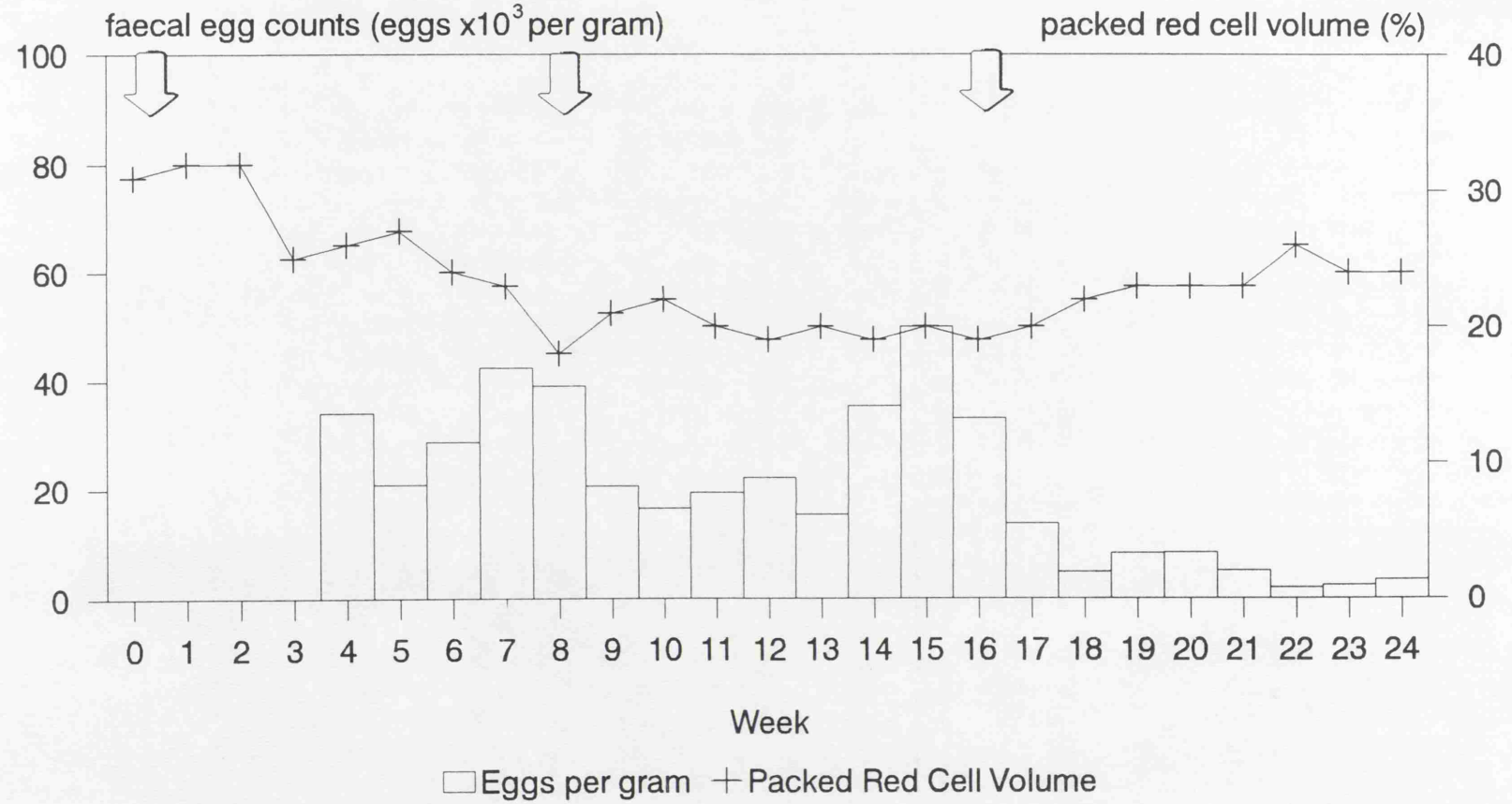


Fig. 5.6: Faecal egg counts and packed red cell volume of animal no. FR 56.

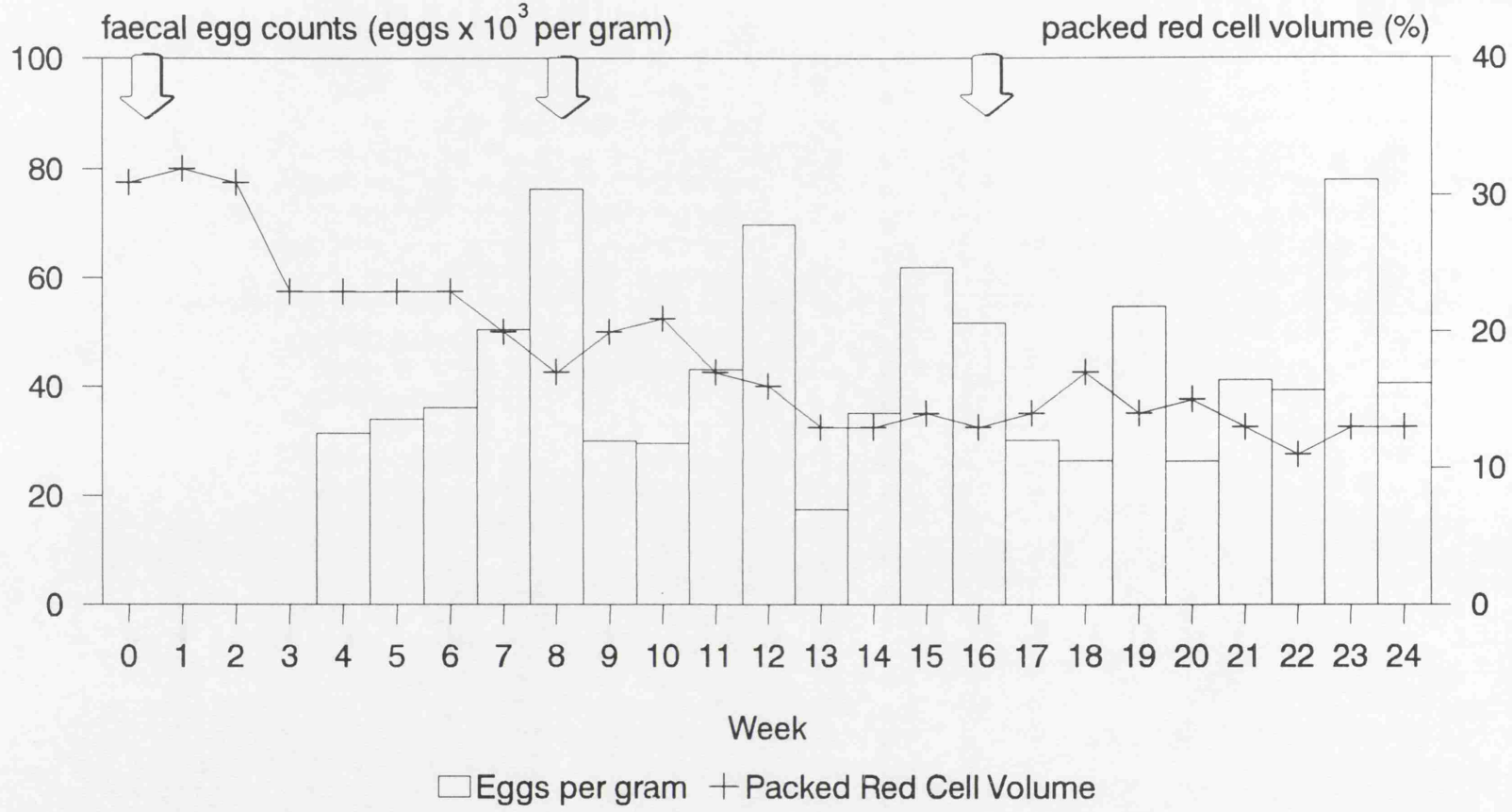


Fig. 5.7: Faecal egg counts and packed red cell volume of animal no. FR 57

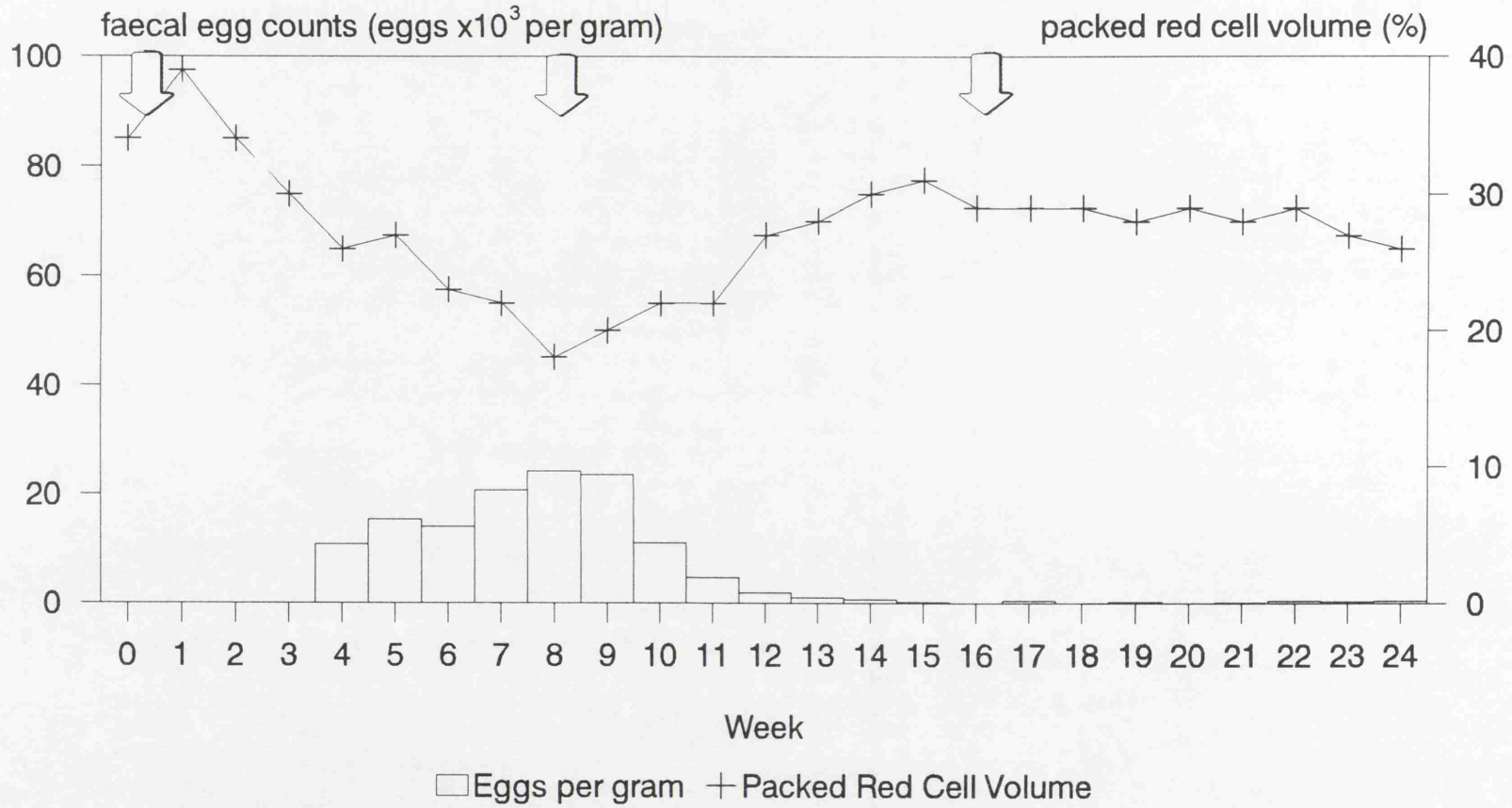


Fig. 5.8: Faecal egg counts and packed red cell volume of animal no. FR 58.

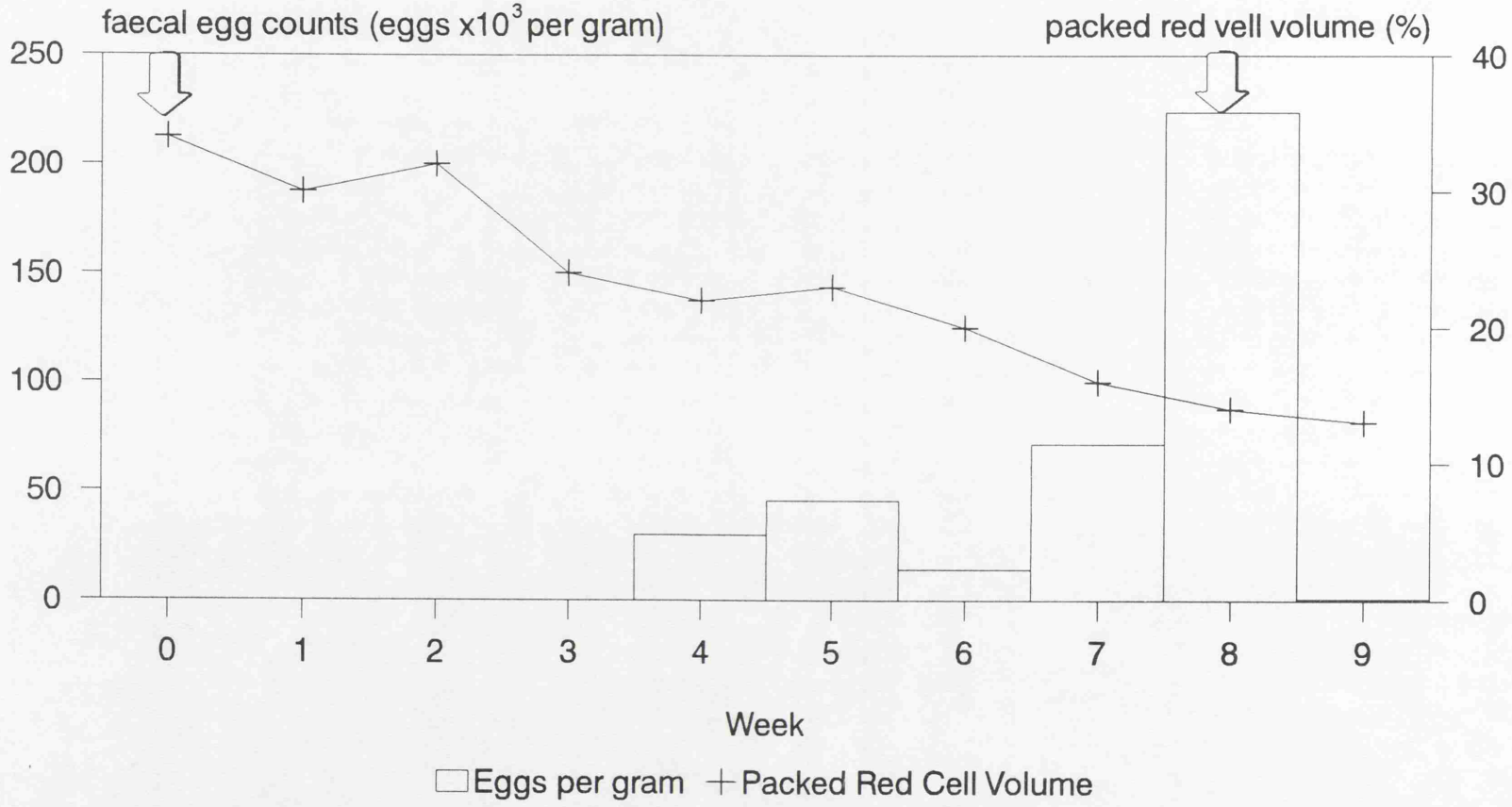


Fig. 5.9: Faecal egg counts and packed red cell volume of animal no. FR 59.

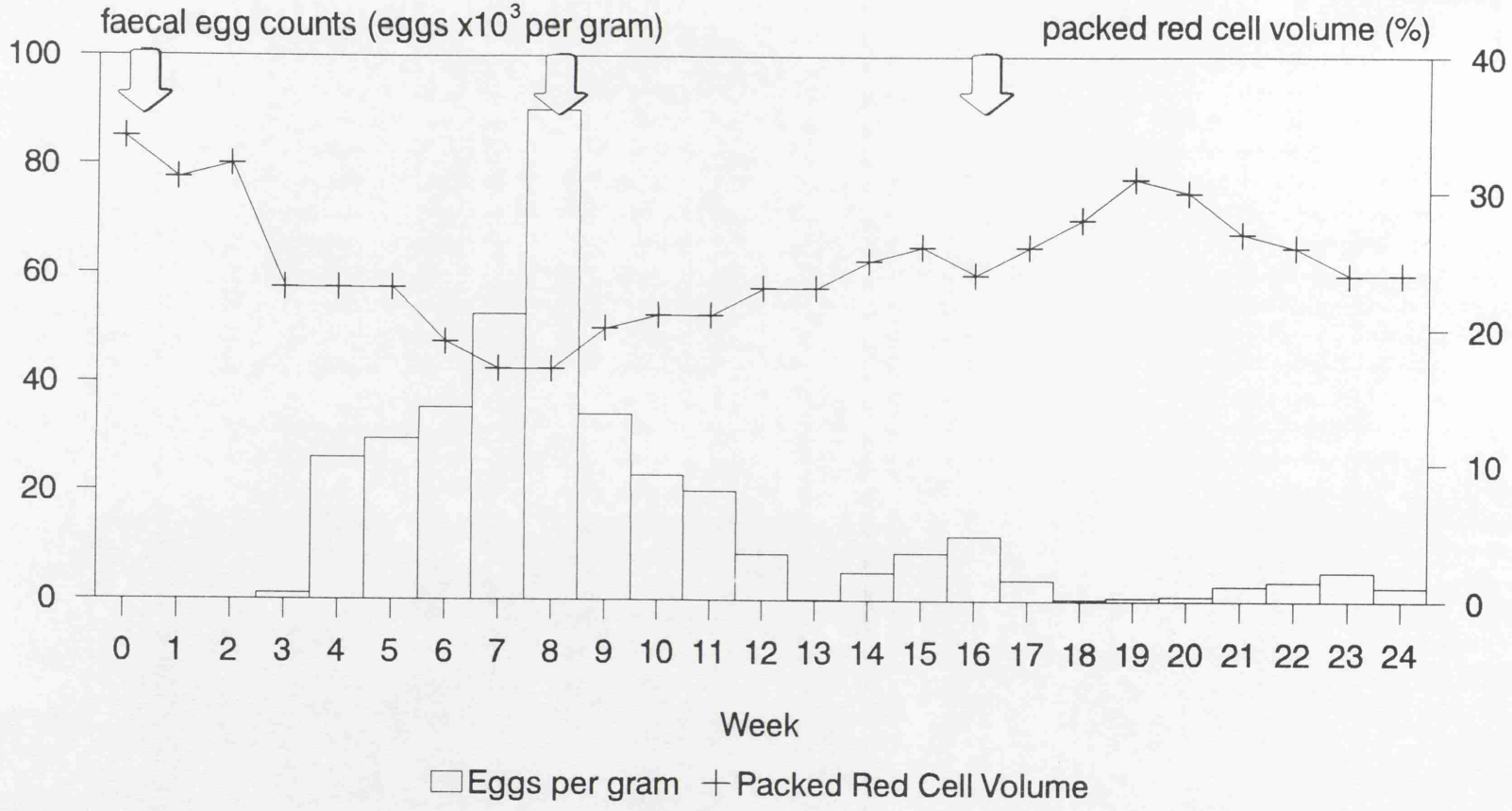


Fig. 5.10: Faecal egg counts and packed red cell volume of animal no. FR 60.

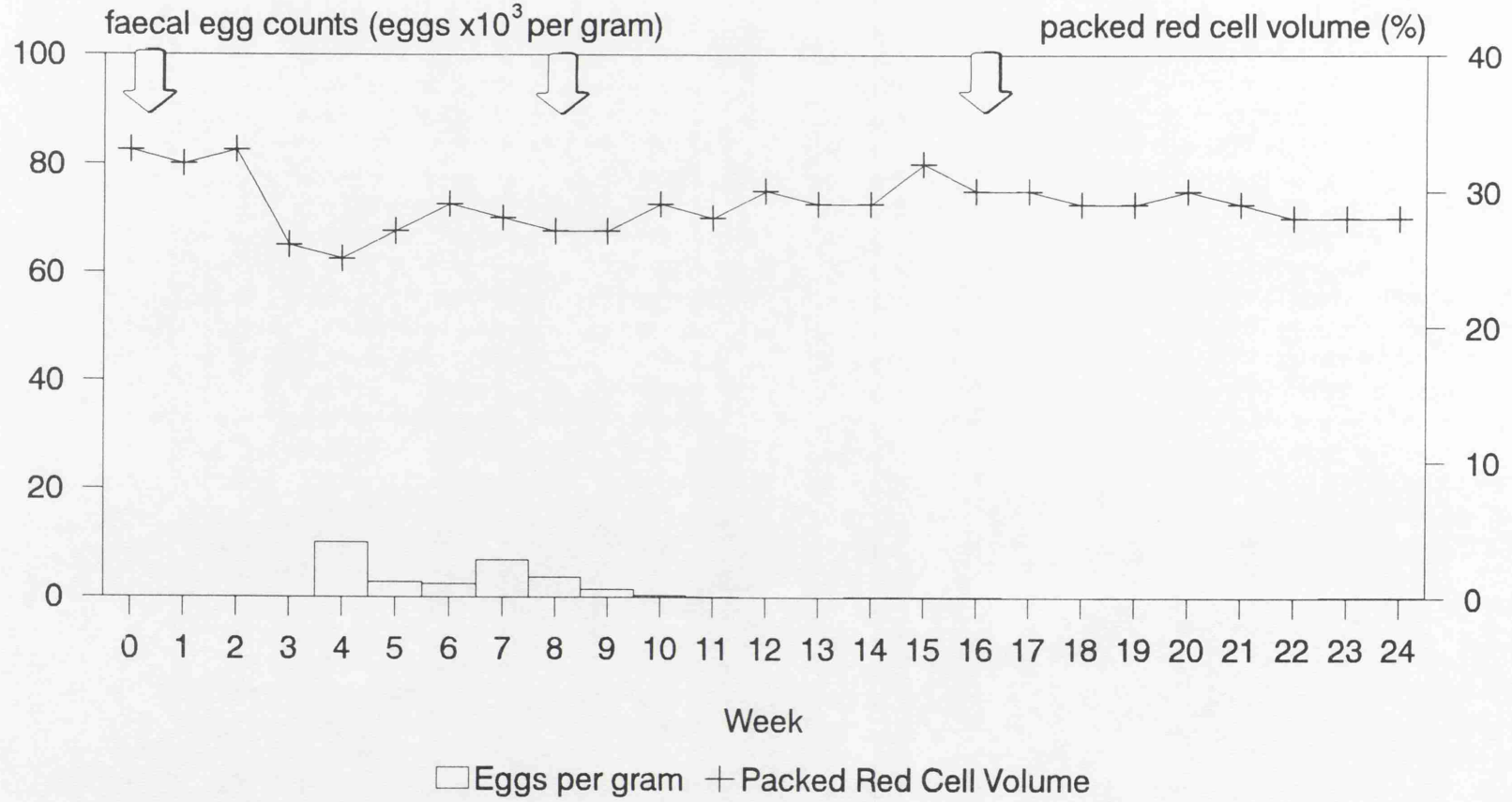


Fig. 5.11: Faecal egg counts and packed red cell volume of animal no. FR 61.

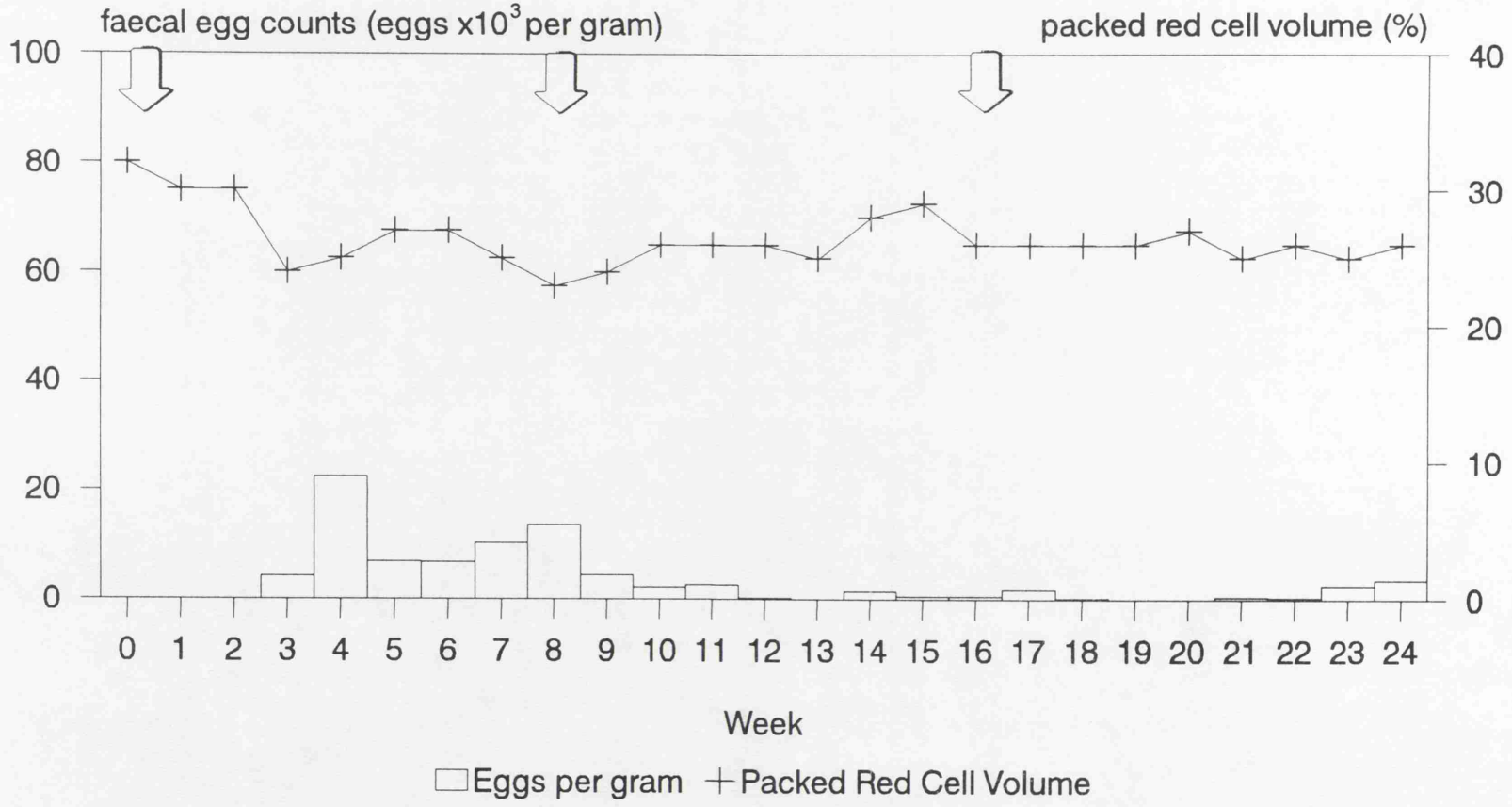


Fig. 5.12: Faecal egg counts and packed red cell volume of animal no. FR 62.

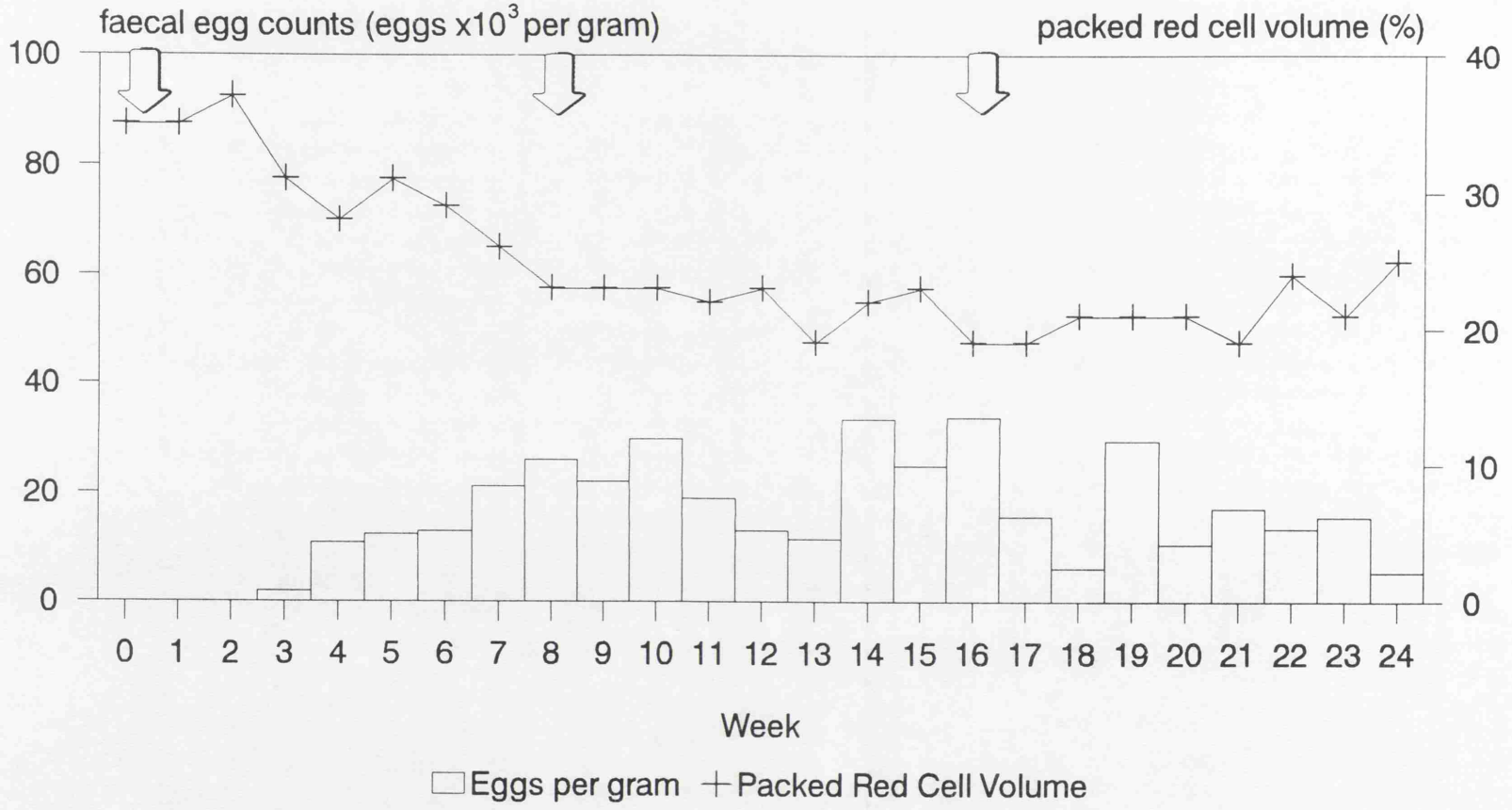


Fig. 5.13: Faecal egg counts and packed red cell volume of animal no. FR 63.

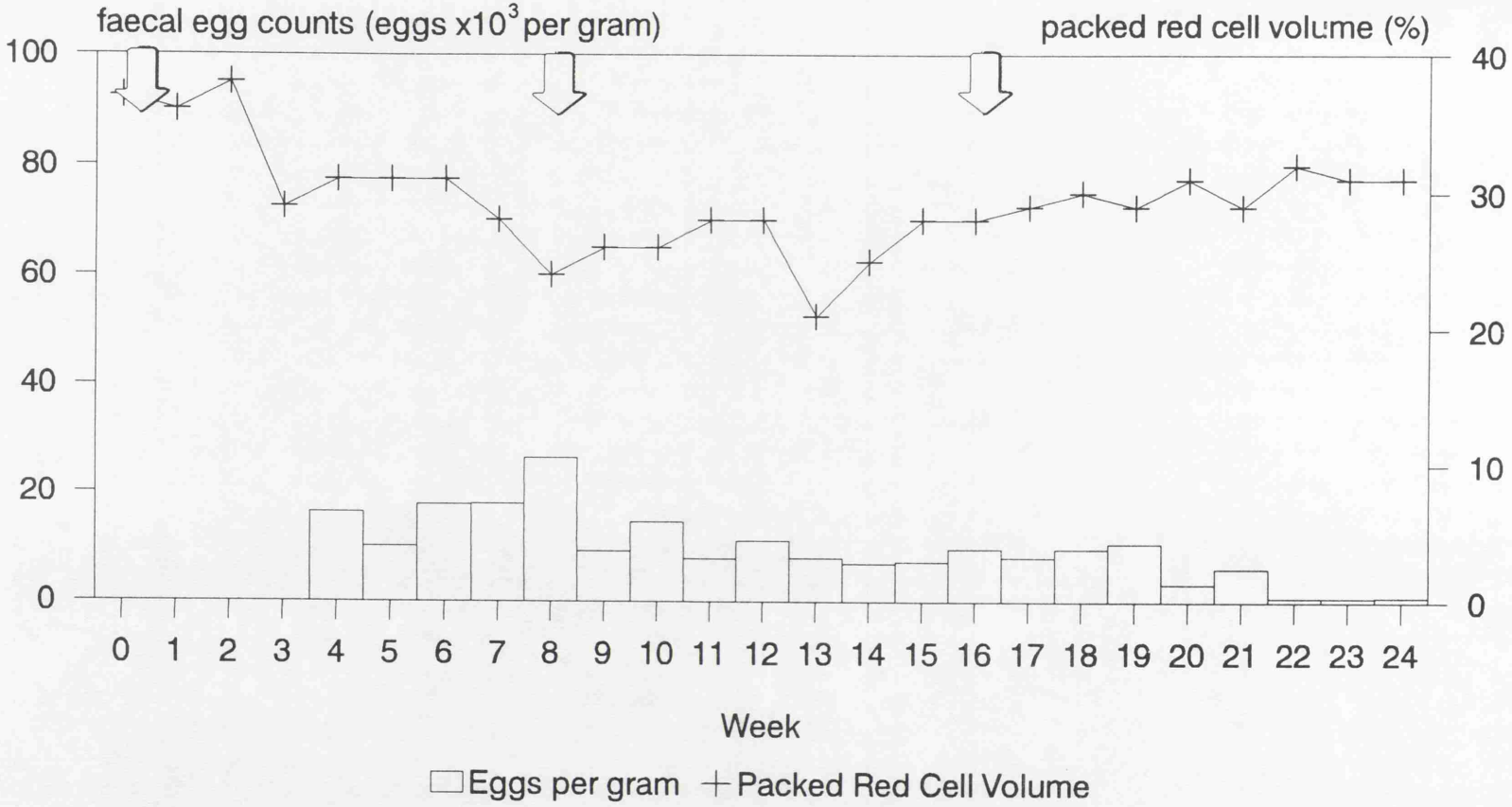


Fig. 5.14: Faecal egg counts and packed red cell volume of animal no. FR 64.

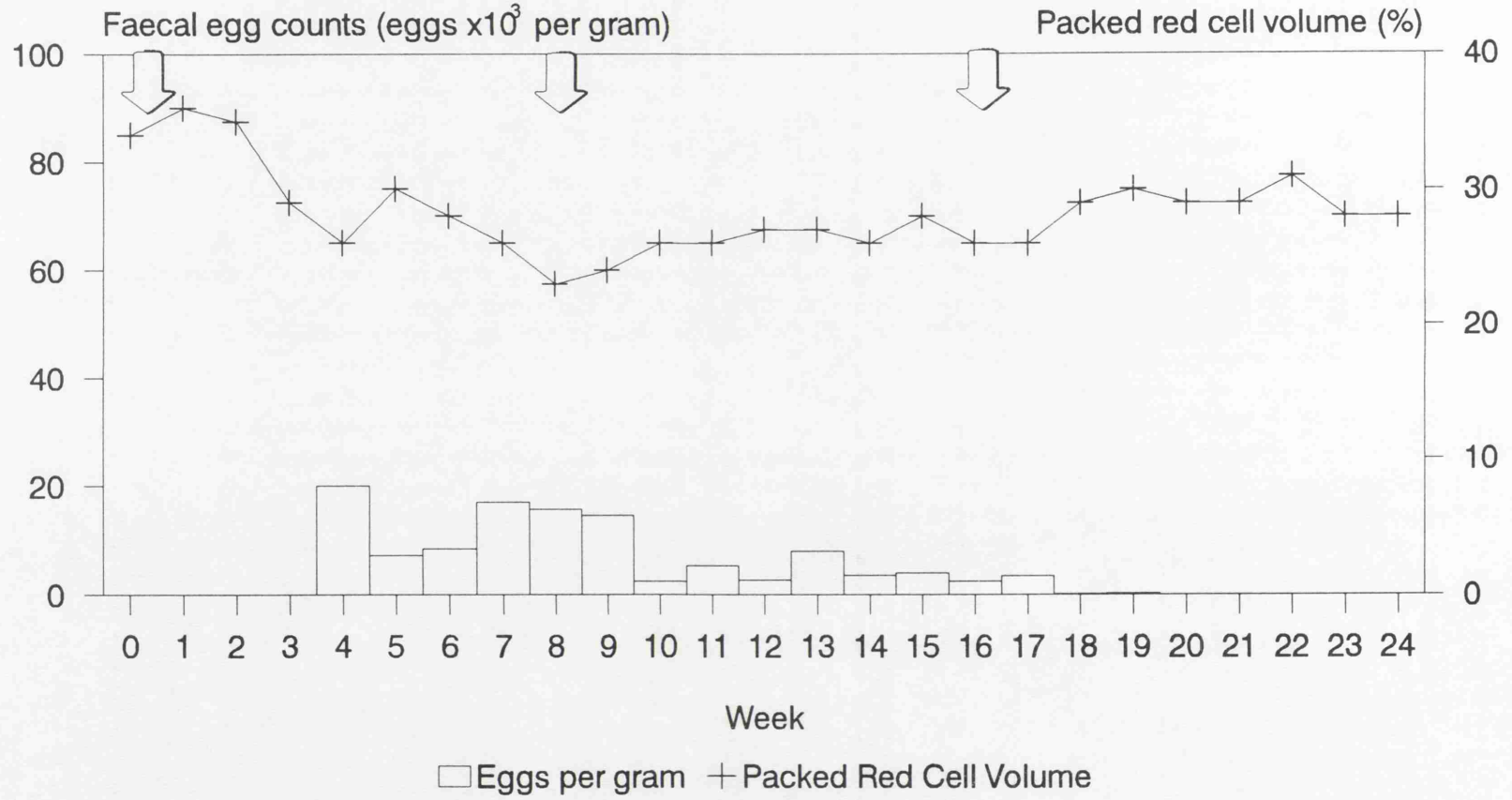


Fig. 5.15: Faecal egg counts and packed red cell volume of animal no. FR 65.

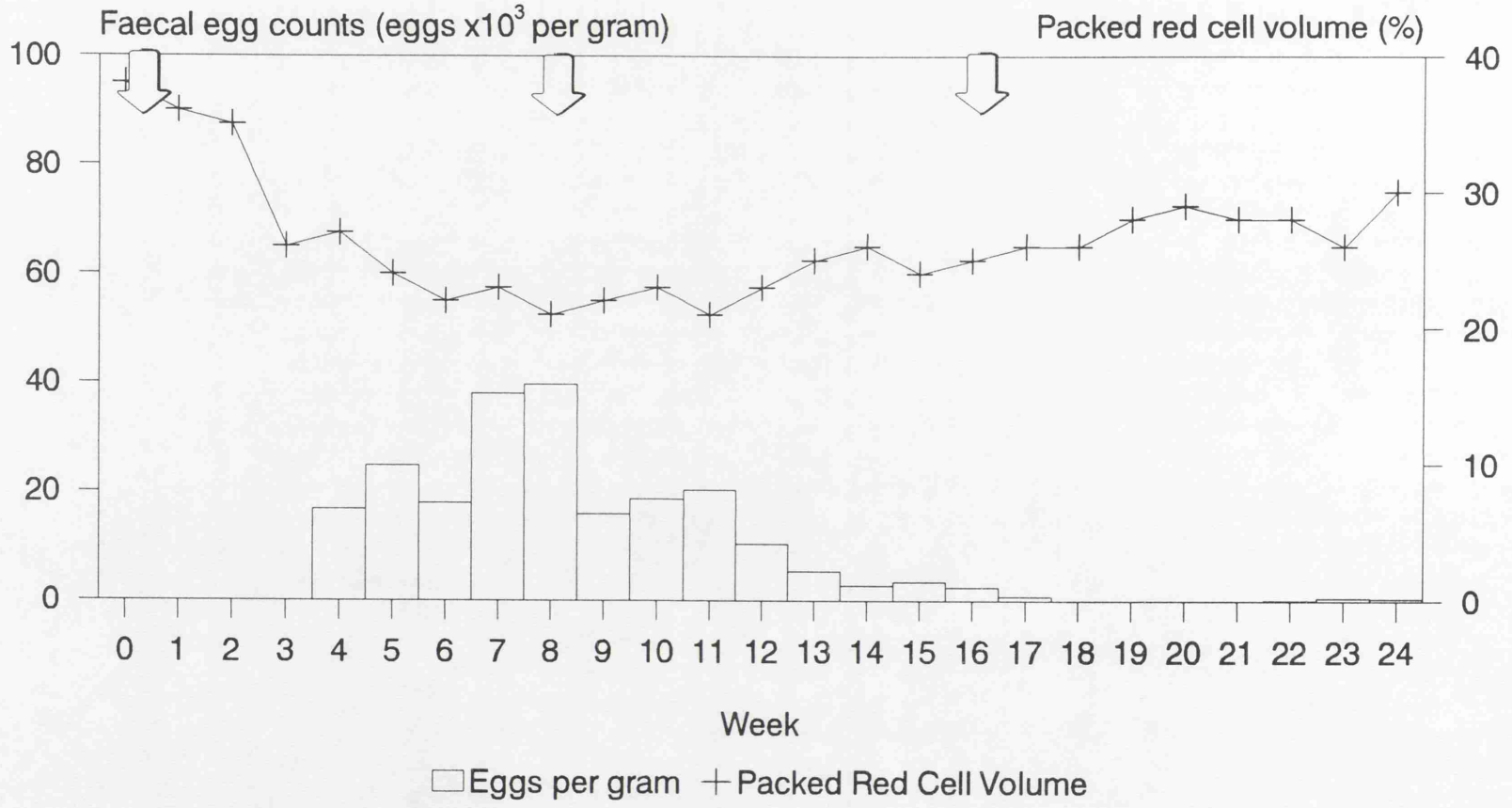


Fig. 5.16: Faecal egg counts and packed red cell volume of animal no. FR 66.

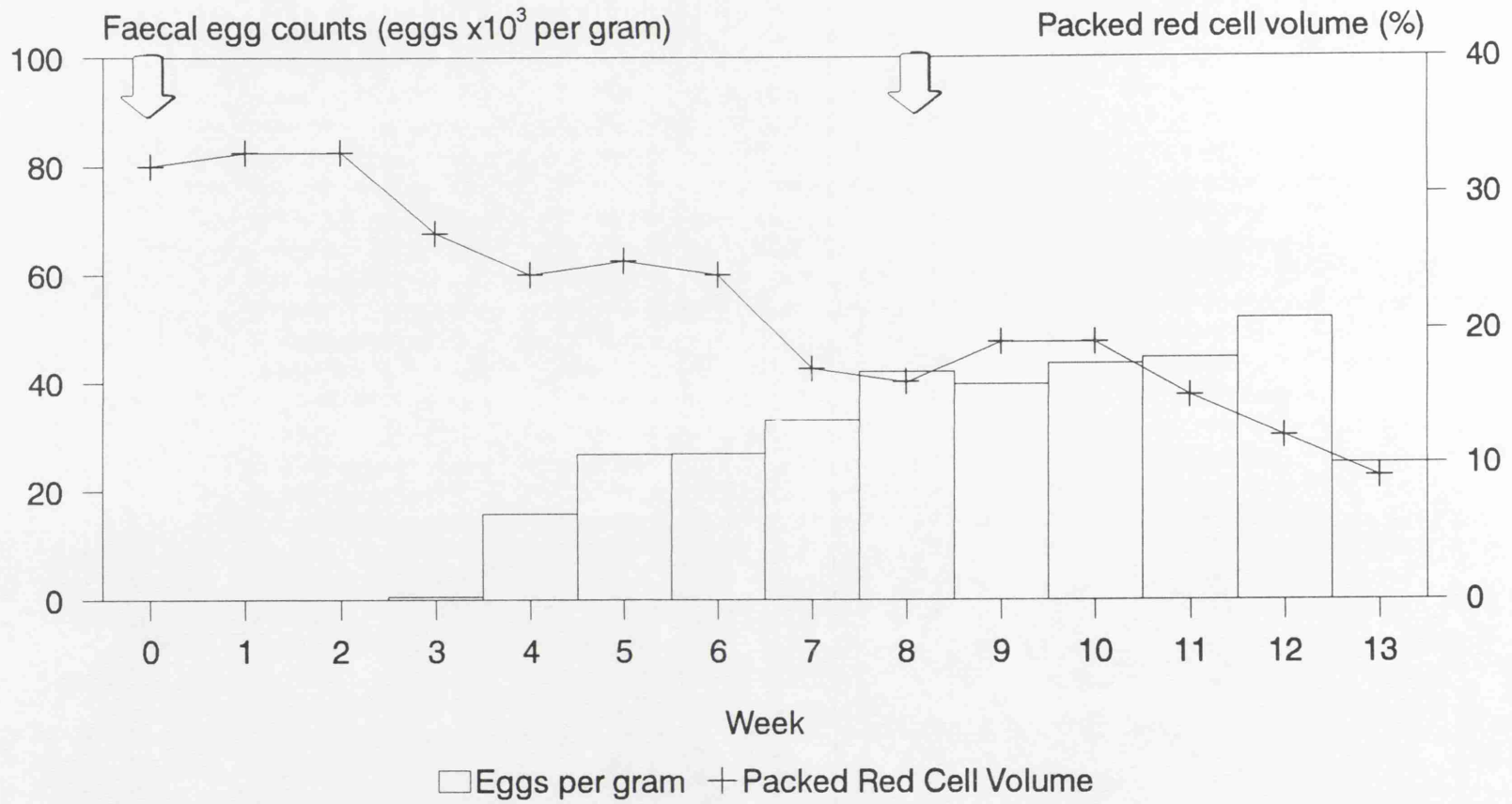


Fig. 5.17: Faecal egg counts and packed red cell volume of animal no. FR 67.

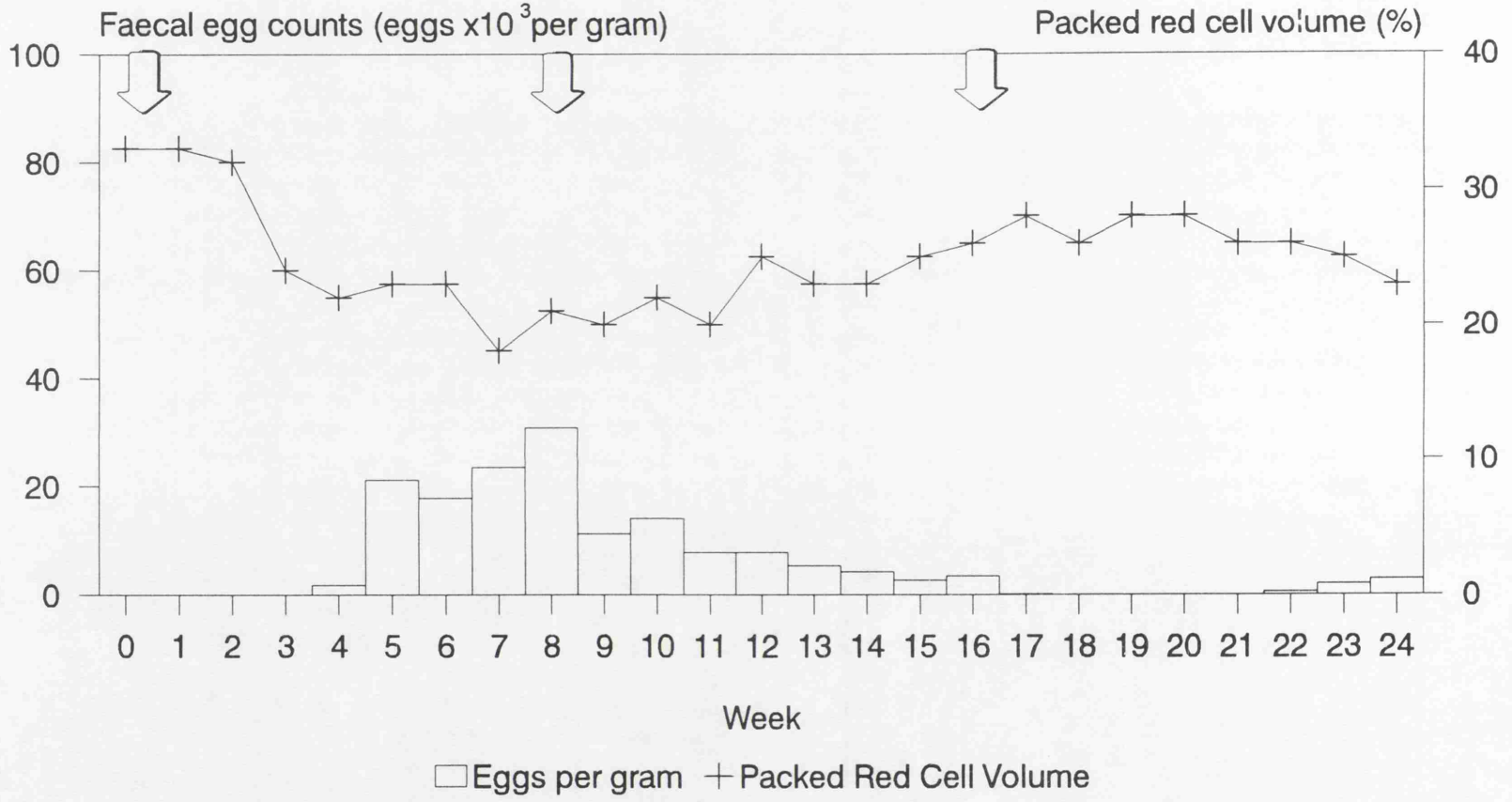


Fig. 5.18: Faecal egg counts and packed red cell volume of animal no. FR 68.

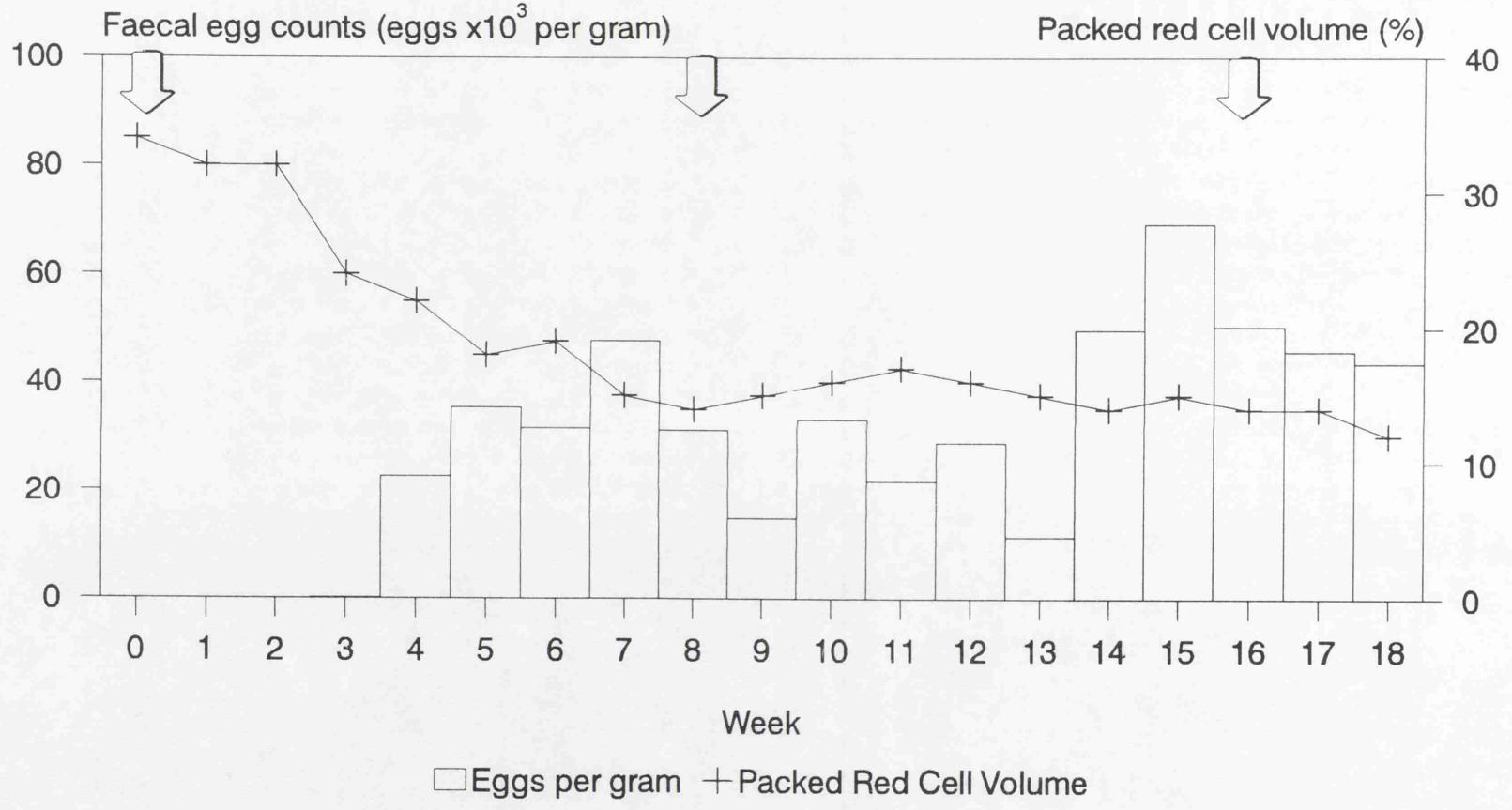


Fig. 5.19: Faecal egg counts and packed red cell volume of animal no. FR 69.

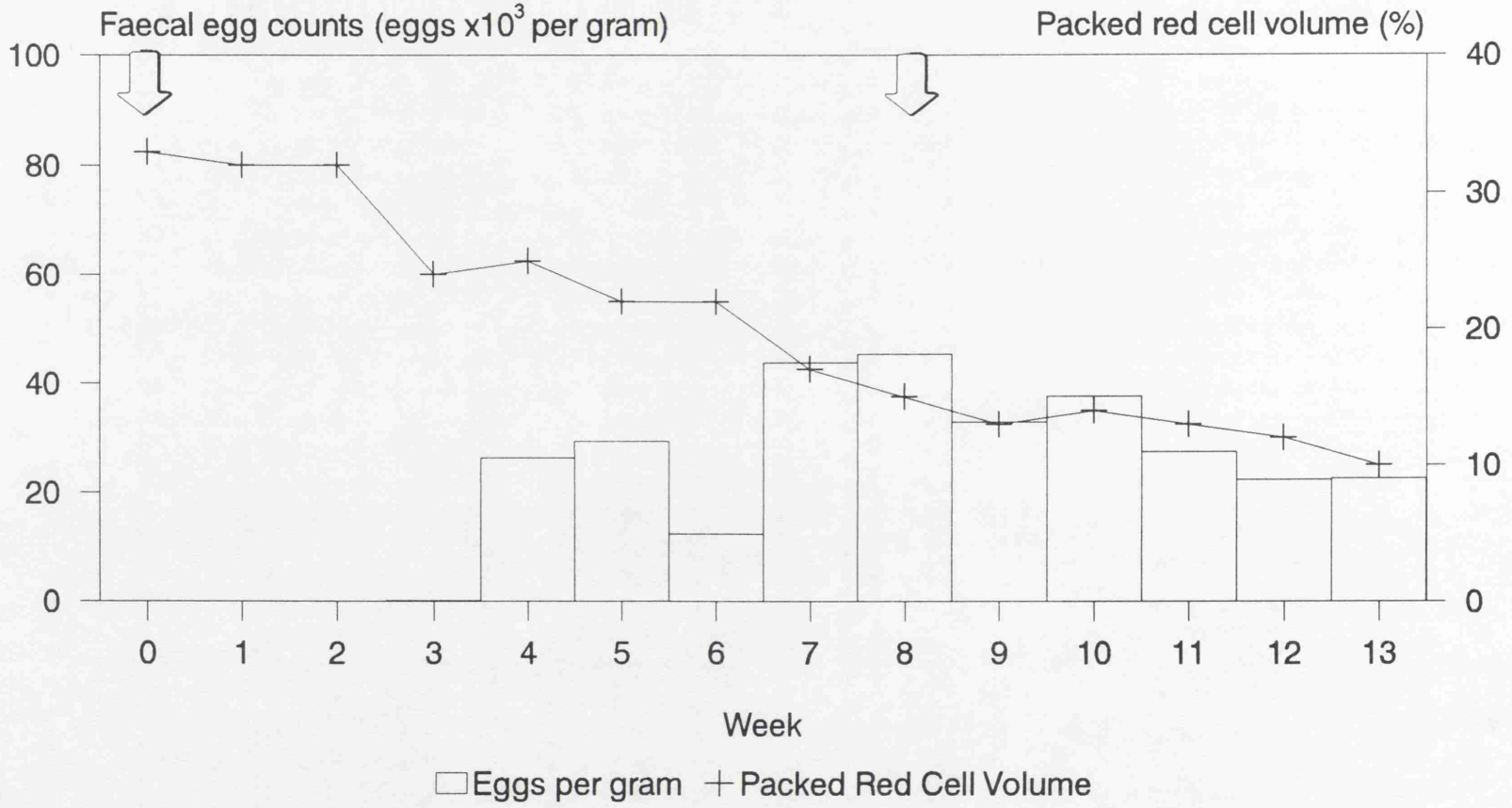


Fig. 5.20: Faecal egg counts and packed red cell volume of animal no. FR 70.

absence of eggs in faeces; at the same time, the lambs in this group were able to maintain a reasonably high PCV. Seven animals were in this group, FR52, FR55, FR58, FR60, FR61, FR66 and FR68. Three lambs were classified as resistant, FR51, FR53 and FR62. These animals showed some degree of self cure following the second infection but were reinfected following the third infection and then 'self-cured'; they were able to maintain a relatively high PCV through the second and third infection periods. Five lambs were classified as moderately resistant, FR54, FR56, FR57, FR63 and FR64. These animals showed some degree of "self cure" during both the second and third infections, but were unable to maintain their PCV values. Four lambs were classified as susceptible and were sacrificed in the terminal stage of disease, FR59 on day 64, FR67 and FR70 on day 92, all in the second infection, and FR69 on day 140 during the third infection. All four animals had high faecal egg counts and had developed very low PCV values.

Figures 5.1-5.20 show the changes in faecal egg counts and PCV values of the 20 lambs following the first, second and third infections with *H.contortus*.

Following the first infection, all the animals allowed the establishment of larvae that developed into adult parasites, which then started laying eggs producing patent infections. The highest peak in faecal egg count following the first experimental infection was 44±48.8 (x1000) epg on day 54, the large standard deviation reflecting the marked variation among the lambs (Table 5.2). Figures 5.1-5.20 show that all 20 lambs experienced a drop in PCV values. The mean PCV value on day 54, the week of the peak faecal egg count during the first infection was 19.9±3.5% (Table 5.3). The mean peripheral blood eosinophil count during the same period was 566±525 (10^3 cells per

Table 5.2

Peak faecal egg counts in the first, second and third infections of Scottish Blackface lambs with *Haemonchus contortus*.

Period of infection	No.of animals sampled	Day of peak faecal egg count	Mean±SD(x1000) eggs per gram
First	20	54	44.0±48.8
Second	17	68	17.3±13.9
Third	16	118	7.5±12.6

Table 5.3

Packed red cell volume before infection and in the first, second and third infections of Scottish Blackface lambs with *Haemonchus contortus* at the peak of faecal egg counts.

Period	No.of animals sampled	Day of peak faecal egg count	Mean±SD PCV %
Before infection	20	-	33.7±1.9
First	20	54	19.9±3.5
Second	17	68	23.5±5.1
Third	16	118	23.0±5.2

ml^{-1}) (Table 5.4). The mean body weights dropped from 30.0±5.8 (pre-infection weight) to 25.4±4.9 kg by day 54 (Table 5.5).

Following the second infection, there were again marked differences in both faecal egg count and PCV responses among the lambs. The highest mean faecal egg count was 17.3±13.9 (x1000) epg on day 68 (Table 5.2). At the same time, mean PCV value was 23.5±5.10% (Table 5.3); the mean peripheral blood eosinophil count was 566±525 (10^3 cells per ml^{-1}) (Table 5.4) and the mean body weight was 26.7±5.5 kg (Table 5.5). FR59, FR67 and FR70 were sacrificed on days 64, 92 and 92, respectively, because of severe anaemia and deterioration in their clinical condition.

After the third experimental infection, marked variation was once again observed in the response of the lambs. The highest mean faecal egg count was 7.5±12.6 (x1000) epg on day 118 (Table 5.2), while the mean PCV value on day 118 remained relatively stable at 23.0±5.2% (Table 5.3). At the same time, the mean of peripheral blood eosinophil counts on day 118 was 790±657 (10^3 cells per ml) (Table 5.4). The mean body weight increased to 30.9±6.5 kg on day 118 (Table 5.5). Animal number FR69 was sacrificed during this period on day 140 due to a deterioration in clinical condition and severe anaemia.

As stated, four lambs became progressively ill during the course of infection. Just before slaughter, the animals were weak, inappetent and recumbent with PCV values just above 10%. FR59 was sacrificed on day 64; FR67 and FR70 were sacrificed on day 92, while FR69 was sacrificed on day 140.

At necropsy, the carcasses of all four were emaciated and the visible mucous membranes were pale. A blood smear revealed signs of anaemia, including, anisocytosis, poikilocytosis and polychromasia.

Table 5.4

Peripheral blood eosinophilia in the first, second and third infections of Scottish Blackface lambs with *Haemonchus contortus* at the peak of faecal egg counts.

Period of infection	No.of animals sampled	Day of peak faecal egg count	Mean±SD ($x10^3$cells/ml$^-$)
First	20	54	512±527
Second	17	68	566±525
Third	16	118	790±657

Table 5.5

Body weights before infection, in the first, second and third infections of Scottish Blackface lambs with *Haemonchus contortus* at the peak of faecal egg counts.

Period	No.of animals samples	Day of peak faecal egg count	Mean±SD kg
Before infection	20	-	30.0±5.8
First	20	54	25.4±4.9
Second	17	68	26.7±5.5
Third	16	118	30.9±6.5

On opening the carcass, the significant findings were pale mucous membranes, and gelatinisation of subcutaneous fat. In the abomasum, there was severe oedema of abomasal folds, adult parasites, petechial and ecchymotic haemorrhages, and enlargement of the gastric lymph nodes.

On histopathological examination, there was marked infiltration of the abomasum with inflammatory cells resulting in the displacement of glandular tissue in the lamina propria. The main cells were lymphocytes, macrophages, eosinophils (Figure 5.21), globule leucocytes (Figure 5.22) and a few mast cells (Figure 5.23). Immunoperoxidase staining using monoclonal rat anti-sheep IgA showed widespread distribution of IgA-contained plasma cells in the abomasal lamina propria (Figure 5.24).

5.3.2 Association between major parameters and eosinophilia

The key characteristic in considering genetic resistance to disease is the ability of animals to gain weight despite infection. Hence, in presenting the results, in the first instance the association between the various parasitological and haematological parameters, and body weight has been considered.

5.3.2.1. Faecal egg counts and body weights

The correlation coefficient between faecal egg counts and body weight during the first infection was very weak ($r = 0.007$) while the correlation during the second infection was negative but weak ($r = -0.198$). However, during the third infection animals with the highest faecal egg counts lost more weight ($r = -0.540$; $0.01 < p > 0.02$) (Table 5.6)

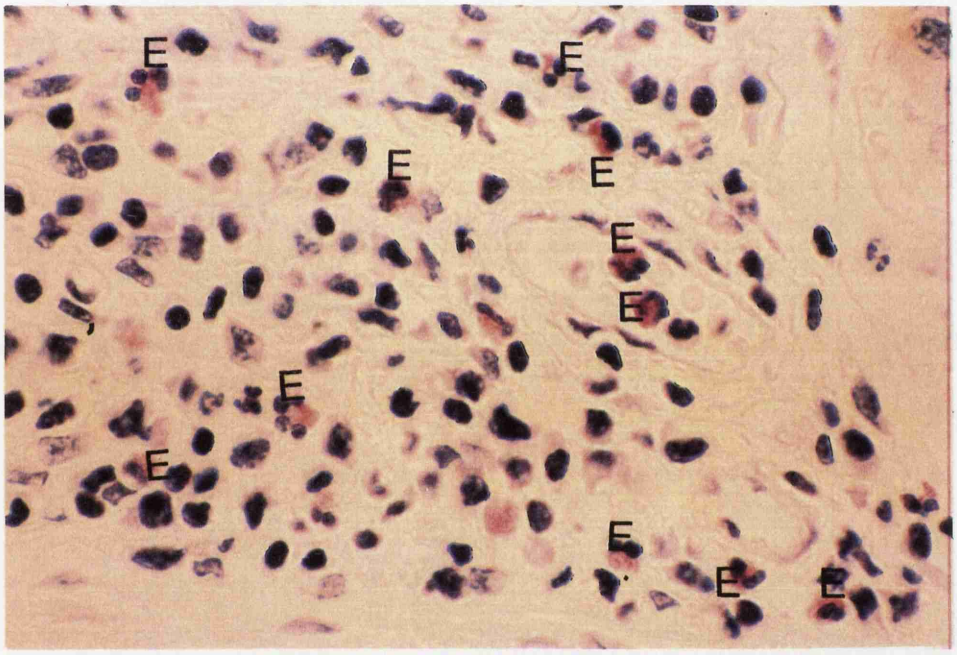


Figure 5.21

Eosinophil (E) infiltration into the abomasal lamina propria of a sheep following infection with *Haemonchus contortus*. Carbol Chromotrope.x400

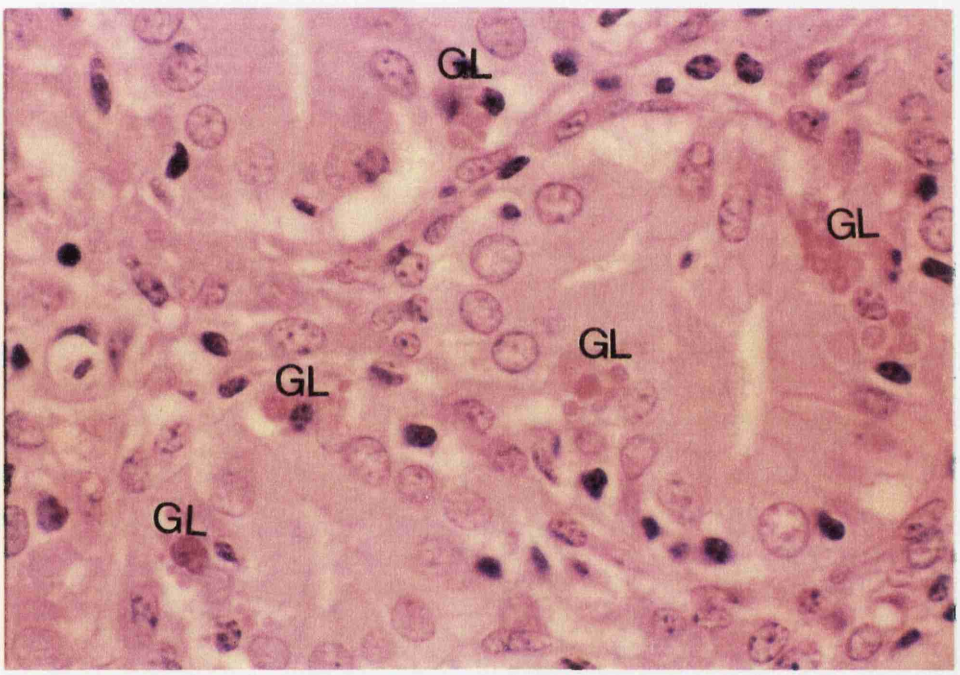


Figure 5.22
Globule leucocytes (GL) in the glandular epithelium of the abomasal mucosa of a sheep infected with *Haemonchus contortus*.
Haematoxylin and Eosin stain.x1000

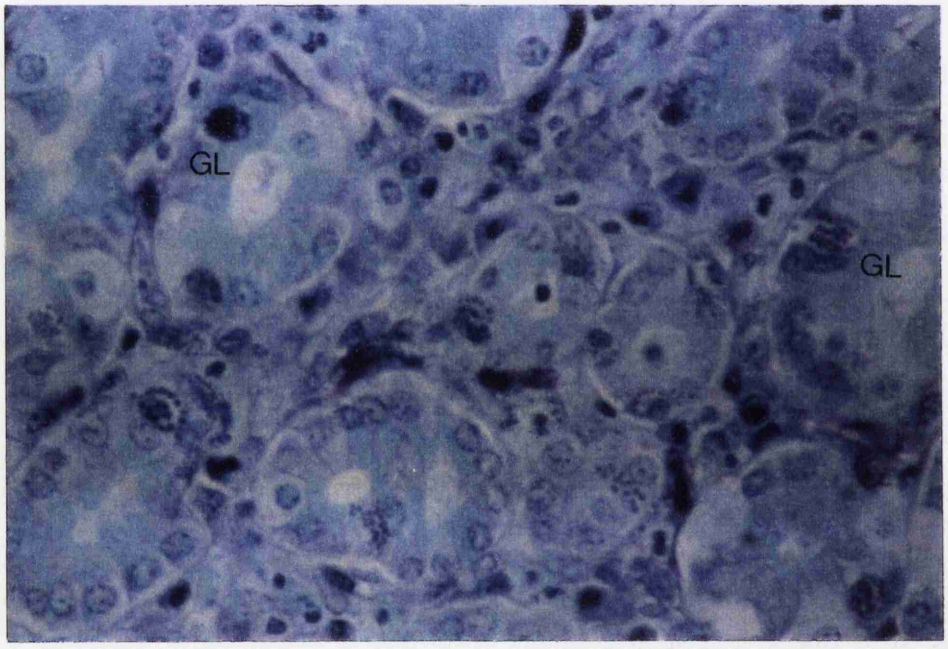


Figure 5.23

Mast cells in the abomasal lamina propria and globule leukocytes (GL) in a sheep infected with *Haemonchus contortus*. Toludine Blue.x1000

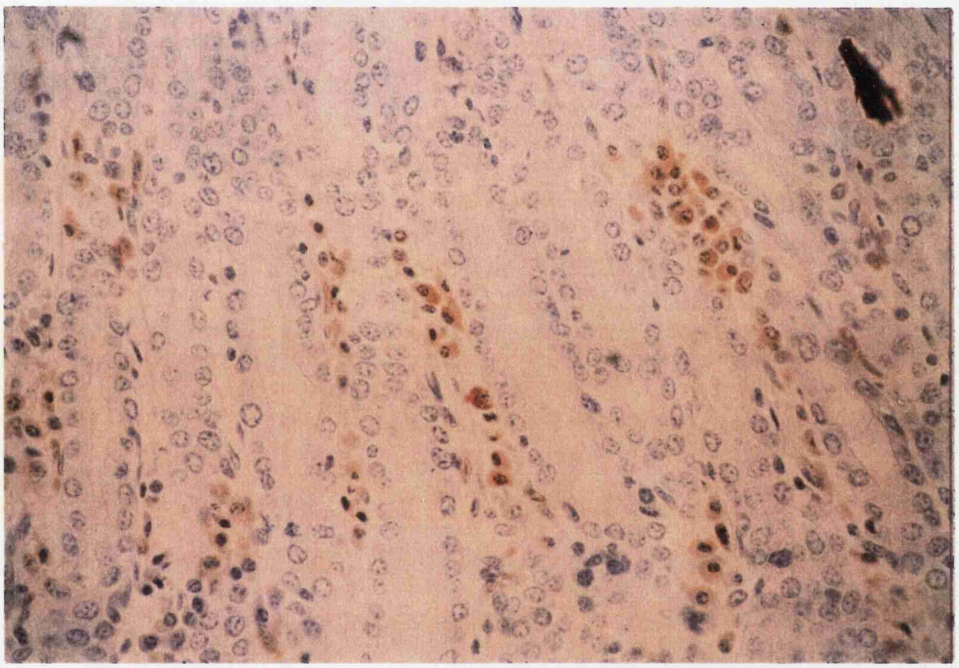


Figure 5.24
IgA plasma cells in the abomasal mucosa of a sheep infected with *Haemonchus contortus*. Peroxidase stain.x400

Table 5.6

Spearman rank correlation coefficient (r) between faecal egg counts and body weight in the first, second and third infections* of Scottish-Blackface lambs with *Haemonchus contortus*.

Period of infection	r	Remarks
First	0.007	not significant
Second	-0.198	not significant
Third	-0.540	0.01 <p <0.02

* on days 54, 68 and 118, respectively

5.3.2.2. Packed red cell volume and body weight

The correlation coefficient between PCV values and body weight was positive during the first ($r = 0.297$) and second infections ($r = 0.102$) but only significant during the third ($r = 0.536$; $0.02 < p < 0.05$). Thus, animals with high PCVs during the third infections gained more weight (Table 5.7).

5.3.2.3. Faecal egg counts and packed red cell volumes

The correlation coefficient between faecal egg counts and packed cell volume was negative and strong during the first ($r = -0.643$; $p < 0.01$), second ($r = -0.886$; $p < 0.01$) and third ($r = -0.712$; $p < 0.01$) infections. The correlation was strongest during the second infection and weakest during the first infection (Table 5.8).

5.3.2.4. Peripheral blood eosinophilia and body weights

The correlation coefficient between peripheral blood eosinophilia and body weight was positive and significant during the first and the second infection ($r = 0.544$ and 0.535), respectively ($0.01 < p < 0.020$) in both cases. However, the correlation was positive but not significant during the third infection ($r = 0.330$; $p > 0.05$) (Table 5.9).

5.3.2.5. Peripheral blood eosinophilia and faecal egg counts

The correlation coefficient between peripheral blood eosinophilia and faecal egg counts was highest during the second infection ($r = -0.802$, $p > 0.01$). The correlation coefficient between the two was also high and negative during the third infection ($r = -0.723$, $p < 0.01$). However, the correlation coefficient between the two was weak during the first infection ($r = -0.201$) (Table 5.10).

Table 5.7

Spearmans rank correlation coefficient (r) between packed red cell volume and body weight in the first, second and third infections* of Scottish-Blackface lambs with *Haemonchus contortus.*

Period of infection	r	Remarks
First	0.297	not significant
Second	0.102	not significant
Third	0.536	$0.02 < p < 0.05$

* on days 54, 68 and 118, respectively

Table 5.8

Spearman rank correlation coefficient (r) between faecal egg counts and packed cell volume in the first, second and third infections* of Scottish-Blackface lambs with *Haemonchus contortus*.

Period of infection	r	Remarks
First	-0.643	$p<0.01$
Second	-0.886	$p<0.01$
Third	-0.712	$p<0.01$

* on days 54, 68 and 118, respectively

Table 5.9

Spearman rank correlation coefficient (r) between peripheral blood eosinophilia and body weight in the first, second and third infections* of Scottish-Blackface lambs with *Haemonchus contortus*.

Period of infection	r	Remarks
First	0.544	$0.01 < p < 0.02$
Second	0.535	$0.01 < p < 0.02$
Third	0.330	not significant

* on days 54, 68 and 118, respectively

5.3.2.6. Peripheral blood eosinophilia and packed red cell volume

The correlation coefficient between peripheral blood eosinophilia and PCV was positive during the first ($r = 0.47$; $p>0.05$), second ($r = 0.728$; $p <0.01$) and third ($r = 0.648$; $p <0.01$) infections but was only significant during the second and third infections (Table 5.11).

5.3.2.7. Peripheral blood eosinophilia, abomasal tissue eosinophilia and worm burdens

For both peripheral blood eosinophilia and abomasal tissue eosinophilia, there was a significant ($p <0.01$) and negative association with worm burdens, i.e., animals with higher eosinophil counts had lower worm burdens. At the same time, there was a significant ($p <0.01$) and positive association between peripheral blood eisnophilia and abomasal tissue eosinophilia (Table 5.12).

Table 5.10

Spearman rank correlation coefficient (r) between peripheral blood eosinophilia and faecal egg counts in the first, second and third infections* of Scottish-Blackface lambs with *Haemonchus contortus*.

Period of infection	r	Remarks
First	-0.201	not significant
Second	-0.802	$p>0.01$
Third	-0.723	$p<0.01$

*on days 54, 68 and 118, respectively

Table 5.11

Spearman rank correlation coefficient (r) between peripheral blood eosinophilia and packed red cell volume in the first, second and third infections* of Scottish-Blackface lambs with *Haemonchus contortus*.

Period of infection	r	Remarks
First	0.470	not significant
Second	0.728	$p < 0.01$
Third	0.648	$p < 0.01$

*on days 54, 68 and 118, respectively

Table 5.12

Peripheral blood eosinophilia ($x10^3$ cells ml^{-1}), abomasal eosinophilia, abomasal worm counts in Scottish Blackface lambs on day 168 following three sequential infections with *Haemonchus contortus*, compared with non-infected lambs.

	Eosinophilia					Abomasal worm burdens	
	peripheral blood			abomasal tissue			
	n	mean	SD	mean	SD	mean	SD
Uninfected control	4	172.1	122.9	2.7	3.06	0	0
Infected survivors	16	251.5	174.5	42.1	70.5	1641	2927

n = number of animals sampled

5.4 DISCUSSION

There was marked variation in the degree of resistance to *H.contortus* exhibited by the 20 Scottish Blackface lambs following three experimental infections with 10,000 L3. Four lambs succumbed to severe haemonchosis, while the rest survived, with 7 animals showing a remarkable degree of resistance that was also reflected in body weight gains, or resilience. The marked variation in resistance was also reflected by wide variations in faecal egg counts, the ability to maintain stable PCV values, eosinophilia (both peripheral blood and abomasal tissue), in worm burdens and in weight body gains. There were also marked differences in the ability to undergo self-cure, as assessed by faecal egg output. Similar findings were reported for 2 year old Merino wethers, all of which had been previously exposed to natural infection of *H.contortus*, following two experimental infections with same parasite (Allonby and Urquhart, 1975). Allonby and Urquhart, (1975) observed that following challenge infections some of the animals showed self-cure and protection, while others showed some degree of self-cure but allowed re-establishment of infection. Still others showed a temporary suppression of egg production followed by hyperinfection. Allonby and Urquhart (1975) also noted that some of the wethers neither lost the existing infection, nor did they contract new infections. There were still others which lost most of the worms from the initial infection but were susceptible to reinfection.

As in previous studies with *H.contortus* (reviewed in Chapter 1), there were significant correlations between body weight changes, and faecal egg counts and PCV values, and significant correlations between faecal egg counts and PCV values. What was exciting and interesting about the findings was that eosinophilia, as assessed both

in the peripheral blood and in the abomasal tissue, was significantly correlated to worm burden, faecal egg counts, PCV values and body weight changes, i.e., to both resistance and resilience parameters. Similar observations on the association between peripheral blood eosinophilia and faecal egg counts have been made in Scottish Blackface sheep infected with *O.circumcincta* (Chapter 3) and in Merino sheep infected with *T.colubriformis* (Dawkins *et al.*, 1989).

Resistance to *H.contortus* is thought to operate at different levels, namely, prevention of larval establishment, also called immune exclusion (Miller *et al.*, 1983; Miller, 1987); destruction and expulsion of established parasites, also called self-cure (Stoll, 1929; Soulsby and Stewart, 1959). The relative importance of each of these and the effector mechanisms involved are not clear. The effector mechanisms that have been suggested, include, thickening of the abomasal mucosa (Nicholls and Lee, 1989; Newlands *et al.*, 1990), antibody production, especially locally produced IgA (Duncan *et al.*, 1978), the IgE-mediated mast cell responses (Murray *et al.*, 1971; Murray, 1972; Presson *et al.*, 1988) and the antibody-mediated eosinophil adherence leading to parasite death through intoxication (Butterworth, 1984).

Miller, (1984) has suggested that inflammatory cells such as macrophages, mast cells, basophils, eosinophils and neutrophils, in the presence of appropriate sensitising antibodies, e.g., IgA, IgE or IgG or complement, are capable of generating a variety of toxic mediators which cause damage to the tegument or cuticle of the parasite. Thus, immature, migratory nematode parasites are probably eliminated from the host through the action of anaphylactic antibodies or complement in conjunction with inflammatory cells (Miller, 1984). However, for lumen dwelling parasites such as adult *H.contortus*, direct contact with inflammatory cells is unlikely. T cell mediated

recruitment of mast cells and eosinophils to the mucosa (Miller, 1990) is associated with the generation of lipid mediators and, possibly, free radicals which may directly affect the motility or the orientation of the parasites. Thus, substances such as leukotrienes have been implicated in causing paralysis of the parasites (Douch *et al.*, 1984). Additionally, the levels of parasite-specific IgA are raised in the mucus, for example, in *O.circumcincta* infections (Smith *et al.*, 1982a) and it is possible the same applies to *H.contortus* infections (Smith and Christie, 1978, 1979). It is possible that IgA is the factor that causes stunting of the adult worms, and reduced fecundity of the female parasite, as has been shown for *T.columbriformis* following treatment with serum IgG from immune animals (Bone and Klesius, 1986). Similarly, leakage of plasma proteins, including IgG, into the superficial mucus as a consequence of mast cell-mediated permeability changes (Murray *et al.*, 1969), may alter the quality of superficial mucus so that it traps and eliminates the parasite (Miller *et al.*, 1981), or acts as a barrier to the establishment of incoming nematode larvae into the host. Mucus may also retain secreted lipid mediators, such as leucotrienes (Douch *et al.*, 1984), thereby inhibiting parasite motility (Miller, 1987).

During helminth infections, the histamines released from mast cells and possibly basophils, are chemotactic to the eosinophils (Rothwell, 1989). Experimental models have also shown that the production and localisation of eosinophils in tissues is selectively regulated by T-lymphocytes (Muranoto *et al.*, 1988) through the three lymphokines with colony-stimulating activity, including, GM-CSF, IL-3, and IL-5 (Rand *et al.*, 1991). These lymphokines not only help in the expansion of the eosinophil progenitors but also stimulate the function of mature eosinophils (Rand *et al.*, 1991).

Eosinophils have been shown to have receptors for various immunoglobulins, including, IgG (Fanger *et al.*, 1989), and IgE (Capron *et al.*, 1981, 1984, 1986). IgA response in the abomasal tissue is one of major local antibody responses but there is a lack of sufficient evidence for its receptors on the eosinophil cell. Antibody-mediated eosinophil cell adherence and parasite toxicity have been shown in *in vitro* studies of schistosomiasis (MacKenzie *et al.*, 1977), fascioliasis (Duffus *et al.*, 1980) and onchocerciasis (Brattig *et al.*, 1991). Following the binding of antibody onto the eosinophil receptors, peroxidase, platelet-activating factor, MBP and the eosinophil-derived neurotoxin are released (Capron *et al.*, 1985; Capron and Jouault, 1986) onto the parasite surface. This causes permeability changes in the membrane resulting in the death of the parasite by intoxication (Butterworth, 1984).

In conclusion, the results of the studies in this chapter indicate that the eosinophil response of Scottish Blackface sheep to *H.contortus* infection is associated with the ability of the host to resist the infection and to control the effects of infection (resilience) and as such should be further investigated as a possible phenotypic marker for genetic resistance of sheep to helminth parasites.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Haemonchus contortus and *Ostertagia circumcincta* are the most pathogenic and economically important gastric nematodes of sheep in the tropical and temperate countries, respectively (Chapter 1). There are no commercial vaccines against these two parasites, and control methods rely mainly on the use of grazing management and anthelmintic treatment. However, there are increasing constraints on the current methods of controlling gastric nematodes of sheep (reviewed in Chapter 1).

Evidence for genetic variability in resistance of sheep to gastric nematode infections has existed for some time (e.g., Stoll, 1929; Whitlock, 1958; Whitlock and Madsen, 1958; Madsen and Whitlock, 1958). Some of the differences reported have been very dramatic. For example, Altaif *et al.*, (1978b) reported that almost 50% more *H.contortus* larvae matured to adults in Finn Dorset sheep of haemoglobin type BB compared to Scottish-Blackface sheep of the same haemoglobin type. Thus, a number of breeding programmes for sheep have been started in Australia (Piper, 1987; Albers *et al.*, 1987; Windon, 1990); New Zealand (Baker *et al.*, 1990); United States of America: West Indies (Gamble and Zalac, 1992); Scotland (current study) and in Kenya (Onyango-Abuje *et al.*, 1993). The overall objective of these breeding programmes is to investigate the amount of genetic variation and the mechanisms of resistance to the economically important gastrointestinal parasites of sheep.

Apart from the reduced cost of deworming, the use of sheep that are resistant to parasites would reduce the degree of pasture contamination with parasite eggs and infective larvae (Windon, 1990). Sheep that are genetically resistant to parasites are also likely to respond more effectively to helminth vaccines which will probably become available in the future.

The current emphasis on identification of sheep that are genetically resistant to gastric nematodes is not aimed at totally eliminating the conventional methods of controlling gastric nematodes. Instead, an integrated approach using conventional methods such as grazing management and anthelmintic treatment and genetically-resistant sheep would help limit production losses and minimise the costs of production. In such a system, the effective life of anthelmintics would be prolonged due to reduced selection pressure on the parasite to develop anthelmintic resistance.

At the same time, the existence of animals with extremes of degree of resistance or susceptibility provides a powerful research for studying the mechanisms of resistance to nematodes. Understanding these mechanisms (the basis of the selected trait in breeding programmes) is essential for identifying predictive (genetic) markers of resistance. In addition, the recognition of the limiting mechanisms responsible for susceptibility will allow the opportunity for immunological manipulations to induce resistance. Considerable efforts are being devoted to the production of recombinant vaccines (Emery and Wagland, 1991). Cellular and antibody probes from animals having extremes of resistance can be used to identify protective antigens upon which these vaccines can be based. However, it is important that a vaccine must be effective in genetically susceptible animals and in animals where the immune response is compromised. Genetic constitution may therefore be a major constraint on any success of any vaccination strategy.

In order to be acceptable to farmers and commercial stud breeders, the traits used to measure genetic variation in resistance to gastric parasites in sheep should fulfil certain criteria and heritability

levels must be high enough to ensure an efficient response to selection.

The most commonly used phenotypic traits for assessing resistance to gastric nematodes of sheep so far, have been faecal egg counts and packed red cell volume (Chapter 1). These have their own merit but they are limited to certain species of parasites, and also, to the adult stage of the parasite (Chapter 1).

The role of the eosinophil and the factors that lead to the selective accumulation of the cell have largely remained unexplained (Salman and Duncan, 1985; Douch *et al.*, 1986; Gill, 1991). Recent evidence has indicated that T cell factors (Fukuda and Gleich, 1989), parasite factors (Klesius *et al.*, 1986) and certain products of inflammatory cells are chemotactic to eosinophils. In Chapter 3, the degree of eosinophilia was moderately repeatable at 0.25, indicating that peripheral blood eosinophilia, at least for natural infections of *O.circumcincta* is moderately repeatable. Given that there are many variables that affect the degree of infection under natural conditions, including stocking density, age of the animal, other types of acquired immune responses such as maternal antibodies, plus the inhibitory effect of anthelmintic treatment on the development of immune response, it is necessary to design controlled experiments to measure the heritability of eosinophilia in pedigree sheep in order to estimate the amount of genetic gain possible if selection was based on the trait. At the same time, an ideal trait should be associated with a genetic marker. The advantage of using genetic markers for the identification of resistant sheep is that the sheep do not require prior infection with the parasite. Genetic markers are also not influenced by physiological or environmental factors, which may produce transient effects.

Some of the genetic markers most commonly examined for association with resistance are haemoglobin type (e.g., Cuperlovic *et al.*, 1978), lymphocyte blastogenic responses (Rifkin and Dobson, 1979), Ovine lymphocyte antigens (OLA) (Outerridge *et al.*, 1984, 1985, 1986) and restriction fragment length polymorphisms. In this study, there was no association between haemoglobin type with resistance to natural infections predominantly *O.circumcincta*, as measured by faecal egg counts, in spite of the relatively large number of animals tested. This is in contrast with the findings of Altaif and Dargie (1978b) who reported an association between resistance and haemoglobin types following *H.contortus* infections in sheep. It is possible that haemoglobin type is important as a genetic marker for resistance only in blood sucking parasites (Jain, 1986).

In mice, the degree of eosinophilia is controlled by genes of the MHC (Wakelin, 1985). Preliminary analysis of OLA typing revealed moderate polymorphism of MHC Class I antigens (unpublished observations). There is a need, therefore, to investigate for association between eosinophilia with the presence or absence of certain lymphocyte antigens.

An ideal trait should also be cost-effective and easy to measure. Peripheral blood is a convenient method for sampling eosinophils. In most field laboratories differential white cell counts from a blood smear is one of the routine procedures (Dacie and Lewis, 1984). However, this is not the case with tissue eosinophilia during infection with gastric nematodes of sheep. As with worm burdens, abomasal tissue eosinophilia is measurable only at necropsy. This study has shown that peripheral blood eosinophilia and tissue eosinophilia are highly correlated during the tissue stage of the parasite. Antigens from migrating or inhibited parasites, presumably stimulate the

lymphocytes to produce such cytokines as IL-3 and IL-5 which then stimulate eosinophilopoiesis. Subsequently, peripheral blood eosinophilia results from the transit of eosinophils from the bone marrow through blood into the infected tissue. Because of the close association between peripheral blood eosinophilia and faecal egg counts, especially during secondary and subsequent infections (Chapters 3 and 5), the use of the two traits should help increase the efficiency of selection for resistance to gastric nematode infections of sheep.

This study has also shown that there is a diurnal variation in peripheral blood eosinophilia which accounts for up to 20% of the total natural variation in degree of eosinophilia (Chapter 4). However, the timing of the peak of the diurnal variation seemed to be influenced by certain factors such as level of infection and possibly the time and level of feeding. Thus, further investigations should help to clarify the extent to whcih these factors influence levels of eosinophils in peripheral blood and, in the abomasal tissue. Among such factors are cortisol levels, catecholamines, insulin and glucose levels, for example, after feeding and other stress factors possibly due to parasite burdens.

Most of the studies in the functions of eosinophils have been done on patients with some degree of eosinophilia, and therefore, the cells are in active, functional state (Rothwell, 1989; McEwen, 1992; Fukuda and Gleich, 1989). Secondly, the cell preparation techniques have not yielded sufficiently pure populations of eosinophil cells, and therefore, most functional assays have used mixed preparation of cells which makes the interpretation of results difficult. Therefore, there is urgent need to develop appropriate techniques for isolating pure eosinophil populations from tissues, including blood, in order to

permit meaningful comparative functional assays, such as chemotaxis and adherence, to be carried out.

An ideal trait for selecting sheep that are genetically resistant to gastric nematodes should be non-specific in nature, thereby promoting competence against other nematode parasites encountered in the field. Previous reports have suggested that sheep which are more resistant to one nematode species are also more resistant to other nematodes (e.g., Woolaston *et al.*, 1990). One possible reason for this is non-specific mechanisms of resistance. Unlike antibody responses which are for specific parasite antigens, it is highly unlikely that there are subtypes of eosinophils which are produced as a result of stimulation by specific parasite antigens. However, there is need for investigations on the existence of eosinophils subpopulations in peripheral blood and in the abomasal tissue. For instance, preliminary results on yields and purity of eosinophils isolated from peripheral blood have revealed differences in the densities of eosinophils harvested (unpublished data). Fukuda and Gleich (1989) have reported similar findings, but it is not clear whether the different densities are due to different eosinophil subpopulations. Investigations along these lines should include differences in functions and activities of the different eosinophil subpopulations in resistance to parasitic infections.

Finally, it is not clear the extent to which parasites are able to adapt to the genotypic changes that are occurring in the host through selection for resistance (Gruner, 1991). An ideal trait should demonstrate that parasite adaptation to withstand host resistance mechanisms will remain within manageable levels. Preliminary investigations have not shown that such adaptation occurs but it is early to speculate (Windon, 1990). This is another area that requires

investigation. Animals with extremes of resistance and susceptibility could provide useful information.

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