FIELD STUDIES ON THE CONTROL OF A BENZIMIDAZOLE RESISTANT ISOLATE OF *TELADORSAGIA* (*OSTERTAGIA*) *CIRCUMCINCTA*

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Abstract

A three year field study was conducted at Moredun Research Institute's Firth Mains farm on five 0.9 hectare paddocks carrying *Nematodirus battus*, *Trichostrongylus vitrinus* and an isolate of *Teladorsagia* (*Ostertagia*) spp which was known, since 1983, to be resistant to drugs within the benzimidazole (Bz) class of anthelmintics. Ewes were randomly assigned to each paddock every year and their twin lambs treated at the manufacturer's recommended dose (MRD) for their allocated anthelmintic. A non-suppressive control regime was adopted consisting of an initial anthelmintic treatment in mid to late May to control *Nematodirus* and when necessary a summer drench to control other gastrointestinal nematodes.

There were very few differences between the dams of the treated and control lambs with no evidence of reduced body weight, condition score or fleece weight over the three year study. There were no clinical signs of Teladorsagiosis in any of the groups of lambs throughout the study and plasma pepsinogen concentrations remained relatively low each season. There was little evidence of marked differences in performance in the fenbendazole (Fbz) treated lambs despite the presence of a Bz resistant isolate of *T. circumcincta* on the paddocks at Firth Mains. The maximum difference in weight gain between the Fbz and best performing group at the end of the 1993, 1994 and 1995 seasons were 3.1, 3.6 and 2.6 kgs respectively. The immunogenicity and pathogenicity of the Firth Mains Bz resistant isolate was no different to that of a Bz-susceptible or a multiple resistant isolate of *T. circumcincta* in a subsequent investigation.

Limitations in faecal egg count reduction tests (FECRTs) and controlled efficacy tests (CETs) were apparent when dealing with naturally infected animals with differences in the acquisition and expression of immunity. Results of the FECRTs and CETs conclusively demonstrated the presence of Bz resistant *T.circumcincta* on the paddocks at Firth Mains and suggested that resistance to levamisole (Lev) may have developed since the start of the study. A Fbz/Lev combination remained effective with worm reductions >95 % at the end of each season. Ivermectin (Ivm) treatments were fully effective with worm reductions

>99.8 % each year. Whether the expression of drug resistance that results from continued use of Bz drugs had increased over the study was very difficult to ascertain since the apparent extent of resistance, measured in terms of efficacy, was influenced by seasonally variable factors such as climate, nutrition, pasture contamination and timing of treatment.

Increases in both the bioavailability and treatment efficacy of Fbz were seen as a result of feed withdrawal, divided dosing and the co-administration of piperonyl butoxide. If suitable penning is available, withholding feed offers a simple approach to maximizing the bioavailability and efficacy of Bz drugs against resistant parasites.

A naturally infected lamb from each of the treatment paddocks at Firth Mains was housed towards the end of the study in 1995 and its faeces collected to provide infective larvae for artificial infection of parasite naive animals. Both the Fbz/Lev combination and the Ivm treatments were highly effective against abomasal and small intestinal species with faecal egg and worm reductions of over 99 %. The calculated efficacy for Fbz treatment using the FECRT was 77.8% whereas arithmetic and geometric mean *T. circumcincta* reductions were 69.9 and 70.7 % respectively. The calculated efficacy for Lev using the FECRT was 89.2% with arithmetic and geometric mean *T. vitrinus* reductions of 86.4 and 98.9 % respectively, the arithmetic data clearly suggesting Lev resistance in the *T.vitrinus* population on the paddock were Lev was employed throughout the study.

A study into the use of an arbitrarily primed polymerase chain reaction (AP-PCR) in the detection of drug resistant nematode parasites sucessfully amplified DNA from individual larvae. It was unlikely that this approach could be used routinely to differentiate between susceptible and resistant parasites since there was variation in the banding patterns of single larvae from within a population. Its application as an epidemiological tool in the assessment of anthelmintic resistance however merits further study.

The results of these studies are promising as regards the use of anthelmintics against which resistance has already been selected in the management of parasitic gastroenteritis, at least when it involves less pathogenic species with a low biotic potential such as *Teladorsagia.*

Declaration

The work described in this thesis was conducted at the Parasitology Division of Moredun Research Institute, Edinburgh. The pharmacokinetic analyses were carried out in the Department of Veterinary Pharmacology at Glasgow Veterinary School. The mast cell data reported was obtained in collaboration with colleagues from the immunopathology department at Moredun. Meteorological data was kindly supplied by the Scottish Institute of Agricultural Engineering Department at the Bush Estate, Midlothian. The work presented in this thesis was carried out by myself and a full role was played in the design of the experiments and interpretation of the results.

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ABBREVIATIONS

AChE ..... Acetylcholinesterase
ANOVA ..... Analysis of variance
APS ..... Ammonium persulphate
AP-PCR ..... Arbitrarily primed polymerase chain reaction
AUC ..... Area under the plasma concentration-time curve
Bz ..... Benzimidazole
BSA ..... Bovine serum albumin
Cmax ..... Maximum plasma concentration
CET ..... Controlled efficacy test
CSF ..... Colony stimulating factor
CTMC ..... Connective tissue mast cell
DNA ..... Deoxyribonucleic acid
dNTPs ..... Deoxyribonucleoside triphosphates
ECF-A ..... Eosinophil chemotactic factor of anaphylaxis
EDTA ..... Ethylenediaminetetraacetate
ECP ..... Eosinophil cationic protein
EPG ..... Eggs per gramme
ES ..... Excretory/Secretory
Et ..... Ethidium bromide
Fbz ..... Fenbendazole
FbzSO ..... Fenbendazole sulphoxide
FbzSO2 ..... Fenbendazole sulphone
FEC ..... Faecal egg count
FECRT ..... Faecal egg count reduction test
g ..... gravity
GABA ..... γ-aminobutyric acid
GL ..... Globule leucocyte
GLP ..... Good laboratory practice
HPLC ..... High performance liquid chromatography
IFN ..... Interferon
IGF-1 ..... Insulin-like growth factor-1
IL ..... Interleukin
IMF ..... Immunomodulatory factor
Ivm ..... Ivermectin
kg ..... Kilogramme
L ..... Litre
L_1 ..... First-stage larva

L_2 ..... Second-stage larva
L_3 ..... Third-stage infective larva
L_4 ..... Fourth-stage larva
LL_4 ..... Late fourth-stage larva
L_5 ..... Fifth-stage larva
Lev ..... Levamisole
LMI ..... Larval migration inhibition
MBP ..... Major basic protein
MIF ..... Migration inhibitory factor
MMC ..... Mucosal mast cell
ml ..... Millilitre
mm ..... Millimetre
µg ..... Microgramme
µl ..... Microlitre
µm ..... Micrometre
ng ..... Nanogramme
nm ..... Nanometre
°C ..... Degrees Celsius
OD ..... Optical density
Ofz ..... Oxfendazole
PAGE ..... Polyacrylamide gel electrophoresis
PB ..... Piperonyl butoxide
PCR ..... Polymerase chain reaction
PGE ..... Parasitic gastroenteritis
PI ..... Post-infection
PPRI ..... Periparturient relaxation in immunity
RAPD ..... Random amplified polymorphic DNA
RE ..... Rapid expulsion
RFLP ..... Restriction fragment length polymorphism
RMCPII ..... Rat mast cell proteinase II
rpm ..... Revolutions per minute
RSD ..... Resistance selected drug
SD ..... Standard deviation
SEM ..... Standard error of the mean
SMCP ..... Sheep mast cell protease
Tmax ..... Time to maximum concentration
TEMED ..... Tetramethylethylenediamine
TNF ..... Tumour necrosis factor

CHAPTER 1

General Introduction

1.1 Introduction

Anthelmintics are used in all domestic species and man, however the ruminant market is the largest with approximately $ 1.7 billion spent annually throughout the world in an effort to reduce the deleterious effects of helminth parasites (Lanusse & Prichard, 1993). The currently available anthelmintics are scarce resources and there is little likelihood that the number will be substantially increased in the near future. Few companies are in the business of research and development for the veterinary anthelmintic market. The escalating costs of drug development are largely due to the growing sophistication of analytical methods for detecting drug residues, mandatory long-term trials to determine likely teratogenic or mutagenic effects and wide ranging studies on non-target invertebrate and vertebrate species (Waller, 1993b).

Parasitic gastroenteritis (PGE) attributable to nematodes, is a major source of lost production in sheep, particularly in lambs during their first grazing season and is commonly associated with high stocking densities and intensive animal husbandry systems. Though there are a number of species of nematodes which parasitize the ruminant gastrointestinal tract the economic impact of many of these are limited by their climatic and host range. In temperate areas such as northern Britain the most prevalent gastrointestinal nematodes of small ruminants are *Teladorsagia (Ostertagia) circumcincta*, *Trichostrongylus vitrinus* and *Nematodirus battus* (Parnell, Rayski, Dunn & MacKintosh, 1954; Boag & Thomas, 1971, 1977; Taylor & Cawthorne, 1972; Thomas & Boag, 1972, 1973; Reid & Armour, 1975a; Waller & Thomas, 1978). Other species such as *Trichostrongylus colubriformis*, *Trichostrongylus axei*, *Haemonchus contortus*, *Chabertia ovina*, *Oesophagostomum venulosum*, *Nematodirus fillicollis*, *Nematodirus spathiger*, *Trichuris ovis*, *Bunostomum trigonocephalum* and *Strongyloides papillosus* tend to occur less frequently and are generally not associated with outbreaks of disease. *Ostertagia circumcincta* (Stadelmann, 1894) has been re-classified and is synonymous with *Teladorsagia circumcincta* (Ransom, 1907) and will be referred to in accordance with the reference source.

1.2 Life-cycles

1.2.1 Teladorsagia (Ostertagia) circumcincta

In Northern Britain, *Teladorsagia* is the major species implicated in cases of ovine PGE, particularly during the summer months. In ovine faecal samples examined at Moredun on behalf of the Scottish Veterinary Investigation Service, *Teladorsagia* eggs predominated (73 %) followed by *T. vitrinus* (18 %), although the extent to which it occurs throughout Britain is unknown since most cases of PGE are not diagnosed specifically and are treated locally (F. Jackson, personal communication). *Teladorsagia* spp have simple direct life-cycles typical of the trichostrongylids (Figure 1.1). Eggs are passed out of the host and hatch within the faecal pat to produce the free-living first larval stages (L_1). The L_1 undergo two moults separated by periods of lethargus to produce the infective third larval stages (L_3). The L_3 retain the cuticle of the L_2, which serves a protective role, and thus considerable numbers are able to survive the winter on pasture and in soil (Urquhart, Armour, Duncan, Dunn & Jennings, 1991). The time taken to hatch and develop to the L_3 stage is dependent upon the prevailing microclimatological conditions. The optimal temperature range for development is between 18-26 °C and takes approximately 2 weeks. Development is faster at higher temperatures but the larvae become hyperactive and mortality increases as a result of depleted lipid reserves. At lower temperatures (<10°C) development from egg to L_3 does not usually take place and the movement and metabolism of developed L_3 is minimal (Urquhart *et al.*, 1991).

When moist conditions prevail, the free-living L_3 migrate from the faeces on to herbage where they can be ingested by sheep. Following ingestion, the L_3 exsheath in the rumen before reaching their predilection site, the gastric glands of the abomasum. Two further moults occur before the immature adult (L_5) emerges from the gland, which may be as early as 8 days post infection (PI), and matures on the mucosa living closely associated with the surface (Armour, Jarrett & Jennings, 1966). Sexually mature worms may then copulate and eggs laid by the female are deposited on to the pasture in the host's faeces; the complete parasitic life-cycle taking around 3 weeks.

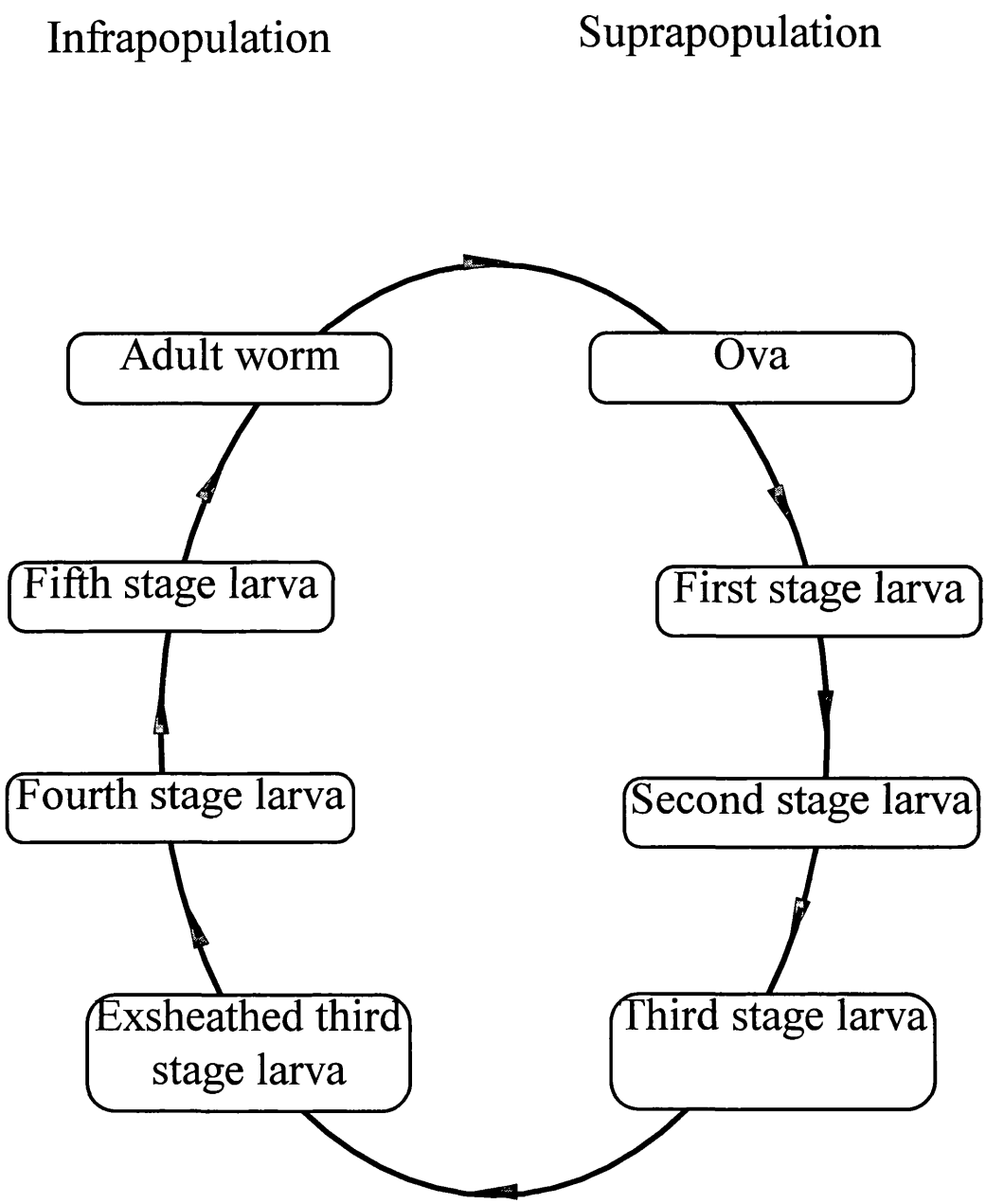

Figure 1.1 *Typical trichostrongylid life-cycle*

1.2.2 Trichostrongylus vitrinus

Trichostrongylus spp also have a simple direct life-cycle, similar to that of *Teladorsagia* spp, the time taken from hatch to infective L_3 varying with microclimatological conditions. For example, the minimum time for development of *T. vitrinus* in the field may vary from 4 days in western Victoria, Australia (Callinan, 1979) and the south of England (Rose & Small, 1984) to 24 days in south-east Scotland (Jackson, 1982). As with *Teladorsagia* spp, development from egg to L_3 does not usually take place at lower temperatures (<10°C) and the movement and metabolism of developed L_3 is minimal. Infective L_3 of *T. vitrinus* have also been shown to survive over winter on pastures in Scotland (Jackson, 1982) as well as in the south-east of England (Rose & Small, 1984).

Following ingestion the L_3 exsheath in the alimentary tract before reaching their predilection site, the anterior small intestine (Taylor & Kilpatrick, 1980). Within two days PI, larvae penetrate the base of the villi and undergo the third moult to L_4. Development takes place in sub-epithelial tunnels where larvae increase in size before entering lethargus around 8-10 days PI prior to the moult to L_5. There is a marked increase in growth between days 10-14 and the majority of worms attain maturity within 17 days PI, with eggs appearing in host faeces within 3 weeks of infection (Taylor, 1977; Taylor & Pearson, 1979a; Jackson, 1989).

1.2.3 Nematodirus battus

The direct life-cycle of *N. battus* differs from that of other strongyles in that eggs voided in the host faeces do not hatch to release first-stage free-living larvae (L_1) but successively moult within the egg to the L_3 stage. The L_3 then hatch from the egg following an increase in temperature (spring and summer months) which has been preceded by a period of cold exposure (autumn and winter months). Consequently, eggs passed by infected lambs can overwinter on pasture and hatch the following spring, thereby infecting the succeeding year's lamb crop (Boag & Thomas, 1975).

Infective L_3, when ingested, exsheath before penetrating the mucosa between the villi of the anterior small intestine where they undergo their third moult to the L_4 stage by day 4 PI. Between days 4 and 6 PI many L_4 return to the mucosal surface

before the final moult, the majority of worms reaching the L_5 stage by day 10 PI. Adult worms can be distinguished as early as day 10 and eggs may appear in host faeces by day 15 PI (Thomas, 1959a; Mapes & Coop, 1972).

1.3 Epidemiology

Gordon (1948) recognised that the epidemiology of gastrointestinal nematode infections differed markedly from that of bacterial, viral and protozoal diseases and preferred to consider nematode epidemiology in terms of the population dynamics of the parasite. Armour (1980) identified three key factors which precipitate production loss due to helminth infection in endemic areas. The first was an increase in infecting mass which is usually the result of a change in contamination and translation of infective stages. The second was due to an alteration in the susceptibility of existing stock and the third was the introduction of susceptible stock into an infected area. Helminthoses may also occur in non-endemic areas due to the movement of infected stock. Other key factors which affect the population dynamics of nematode infection and hence the occurrence of nematodoses are acquired immunity, the peri-parturient relaxation in immunity (PPRI) and the ability of the different species to enter arrested development (hypobiosis).

1.3.1 Periparturient relaxation in immunity (PPRI)

A periparturient relaxation in immunity during pregnancy and lactation has been observed in a number of species including rabbits (Dunsmore, 1966), sheep (Connan, 1968), rats (Connan, 1970), mice (Ngwenya, 1977) and more recently goats (Rahman & Collins, 1992). In considering the epidemiology of infection it is important to take into account the rise in faecal egg counts seen as a result of this PPRI. Host immunity, acquired during previous exposure, is invariably lost in the periparturient period in sheep (Brunsdon, 1970; Reid & Armour, 1975b). This phenomenon seems to result from a temporary relaxation in host immunity and it has been suggested that it may be due to the suppression of T-cell dependent mechanisms (Lloyd, 1983). This relaxation is thought to affect a wide variety of effector

mechanisms including those responsible for controlling larval establishment, development and hypobiosis, as well as adult fecundity and mortality (Gibbs & Barger, 1986). Consequently, eggs passed in the faeces of ewes, from about 2 weeks pre- until 6 weeks post-lambing, serve to provide large numbers of larvae at a time when highly susceptible lambs are present.

Although circulating levels of prolactin, which functions to promote mammary development and milk production, may play a role in maintaining the PPRI, it may not be responsible for initiating the response and the exact cause is still unknown (Barger, 1993a). By inducing high prolactin levels Coop, Mellor, Jackson, Jackson, Flint & Vernon (1990) showed that susceptibility to infection with *T. circumcincta* was not increased in inbred ewes. Similarly, by administering the prolactin antagonist bromocryptine, Jeffcoate, Fishwick, Bairden, Armour & Holmes (1990) found little influence on the periparturient rise in faecal egg counts (FECs) of lactating ewes.

In the case of *Teladorsagia* spp, considerable numbers of larvae may overwinter on the pasture and in soil, but the most important source of contamination is derived from the PPRI. The development of eggs deposited in late spring, from both ewe and lamb, is slow but becomes more rapid towards mid-summer, giving rise to potentially dangerous populations of L_3 on pasture from July to October.

The epidemiology of *Trichostrongylus* spp is also influenced by the PPRI, although it is not as significant since pre-parturient ewes retain the ability to regulate new *T. vitrinus* infections (Jackson, Jackson & Williams, 1988). The presence of *O.circumcincta* has also been shown to reduce the establishment of *T. vitrinus* in young lambs, at least over the first 8 weeks of exposure to both species (Jackson, 1989). As a result this species often becomes apparent in lambs later in the season (Boag & Thomas, 1977). Parnell *et al.*(1954) concluded that *T. vitrinus* was one of the main causes of helminthoses in the late winter and spring in Scottish hill sheep aged between 6 and 12 months.

Since immunity towards *N. battus* infections is acquired rapidly the PPRI does not play a major role in the epidemiology of this species. Essentially transmission is lamb-to-lamb, with eggs passed from the current year's lambs overwintering to infect the following lamb generation. Generally, the disease is prevalent during late spring

affecting young lambs of about 4 to 8 weeks of age (Coop, 1989), rarely affecting sheep aged over 4 months (Boag & Thomas, 1975). The possibility of an alteration in epidemiology of *Nematodirus* spp has been proposed since increased levels of pasture contamination during the autumn months and incidences of patent infections have been noted (McKellar, Bairden, Duncan & Armour, 1983; Hollands, 1984) and have been correlated to changes in the climate (Thomas, 1991).

1.3.2 Arrested Larval Development (Hypobiosis)

The temporary cessation in the development of nematodes, known as hypobiosis, serves to synchronize events within the host and is thought to coincide with the onset of environmental conditions adverse to the survival of the free-living stages, or as a consequence of host immunity (Gibbs, 1986). The proportion of challenge which undergo inhibition is also governed by the age, acquired immunity and reproductive status of the host (Michel, Lancaster & Hong, 1979). Resumption of development appears to be timed to occur when environmental conditions are suitable for the survival of the free-living stages, the mechanisms of which are not fully understood, involving host reproductive status, immune status, nutrition and stress (Armour, 1980).

In a temperate climate the primary stimulus for the induction of inhibited development of *Ostertagia* spp appears to be declining temperatures in the autumn (Armour & Bruce, 1974). Following ingestion of 'conditioned' free-living stages, development is arrested at the L_4 stage and the larvae remain in the gastric glands. Significant numbers of established adult worms may also survive within the host (Waller & Thomas, 1978). Type II ostertagiosis disease may occur in yearling calves following their first grazing season and results from the synchronous maturation of hypobiotic larvae ingested during the previous Autumn.

Ovine *Trichostrongylus* spp also have the capacity to undergo inhibited development within the host but differ from other trichostrongylids in that they inhibit at the L_3 stage rather than L_4 (Eysker, 1978; Waller, Donald & Dobson, 1981), but in other geographical locations they may survive adverse environmental conditions primarily as adult worms (Ogunsusi & Eysker, 1979). Eysker (1978) concluded that under European conditions the main cause of inhibition was

developing host immunity, rather than the effect of environmental stimuli upon the free-living stages.

Larval arrest in *Nematodirus* spp has been demonstrated under natural grazing conditions in sheep (Waller & Thomas, 1983). The authors suggested that seasonal factors and age of the host played an important role in inhibition, and it was not associated with host immunity or density-dependent effects, although Taylor & Thomas (1986) attributed the increased numbers of L_4 stages in their study to acquired immunity.

1.3.3 General

As the grazing season progresses there appears to be a definite succession of parasite species on the pasture and within the host. The epidemiological studies of Boag & Thomas (1977) demonstrated a succession of nematode species throughout the season in north-east England (with *Ostertagia* spp and *Nematodirus* spp recorded in June, *H. contortus* in July, *T. vitrinus* in August and *T. axei*, *T. colubriformis* and *Cooperia curticei* in September) and was attributed to the overwintering capabilities and rate of larval development of individual species. The relative abilities of different species to be transmitted in the PPRI, their biotic potential (fecundity) and host susceptibility are also associated with these changes. Generally, *H. contortus* is not as prevalent in Scotland, being seen only occasionally in ewes and lambs (Reid & Armour, 1975a).

In particular, the epidemiology and pattern of larval contamination is governed by climatic conditions which affect larval development and translation. For example, a dry summer would delay the translation of larvae on to the herbage and limit the size of larval challenge. Providing the moisture content of faecal matter is sufficient to support infective larvae, the onset of autumn rainfall would result in the release of large numbers of larvae 'preconditioned' to arrest upon ingestion. If these animals remain on the farm the magnitude of the PPRI would be increased, resulting in heavier levels of pasture contamination the following year. Thus, the epidemiology of nematode species is often complex and has a propensity for modification by a number of factors.

1.4 Pathogenicity and Productivity

Although infestations with *Teladorsagia* spp, *Trichostrongylus* spp and *Nematodirus* spp cause specific diseases, infections are commonly multispecific having similar effects, the clinical signs of which are weight loss, poor body condition, inappetence and often diarrhoea. The pathophysiological changes induced have been extensively studied, revealing significant effects upon appetite, gut function, protein and energy metabolism, all of which are dependent on the age, nutritional and immunological status of the host as well as the level of infection (Holmes, 1985). The loss of protein into the gastrointestinal tract is a distinctive feature of gastrointestinal parasitism and is associated with plasma leakage, exfoliated epithelial cells and increased mucus secretion (Holmes, 1985). Reduction in voluntary food intake is thought to be the most important factor accounting for decreased rates of liveweight gain, although little is known about the underlying mechanisms. Symons (1985) has reviewed the situation and identified some of the possible causes including pain, changes in pH (affecting protein digestion and /or absorption), gastrointestinal motility, circulating hormonal concentrations and direct neural effects.

1.4.1 Teladorsagia (Ostertagia) spp

The response to *Ostertagia* spp infection involves complex biochemical, hormonal, nutritional and immunological interactions between the parasite and host and has recently been reviewed by McKellar (1993). The presence of *O.circumcincta* in the abomasum in sufficient numbers gives rise to extensive pathological and biochemical changes which are largely due to the emergence of immature adults from the gastric glands as early as 8 days post infection (Armour *et al.*, 1966). Cells occupied by parasites lose their specialized secretory function such that parietal (HCl secreting) and zymogenic (pepsinogen secreting) cells are replaced by irregular cuboidal epithelium (McKellar, 1993). Emerging parasites spread the damage to surrounding un-parasitized cells which also lose their differentiation; the end result being a thickened hyperplastic gastric mucosa with impaired cell junctional integrity. Abomasal pH is elevated due to the loss of functional parietal

cells, this leads to increased circulating pepsinogen levels since pepsinogen conversion to the proteolytic enzyme pepsin is most effective at low pH (Jennings, Armour, Lawson & Roberts, 1966). The exact mechanism for elevated blood pepsinogen is not yet completely defined and it may be that it is multifactorial involving direct stimulation of zymogenic cells by the parasite, indirect stimulation via elevated circulating concentrations of hormones and leakage from abomasal fluid between poorly differentiated epithelial cells (McKellar, 1993). In the study of Armour *et al.* (1966) a single infection with 100,000 *O. circumcincta* L_3 failed to produce clinical signs of ovine ostertagiosis, although it should be noted that the sheep were aged 6 months at the start of the investigation. Holmes & MacLean (1971) also commented on the mildness of clinical signs following infection with 300,000 or 900,000 infective larvae, but these animals were also older (9 to 13 months) and, as the authors noted, pathogenicity may be dependent on the breeds of sheep employed.

1.4.2 Trichostrongylus spp

Taylor & Pearson (1979a and b) described the pathological changes which occurred in the mucosa of the small intestine in lambs infected with 50,000 or 250,000 *T. vitrinus* L_3 which were found to be similar to those seen with *T.colubriformis.* The major effects evident within the first five weeks of infection include villous atrophy, flattening of the mucosa and erosion of the epithelium and an associated inflammatory infiltration of lymphocytes and neutrophils into the damaged areas.

1.4.3 Nematodirus spp

The general feature of nematodirosis is local villous atrophy which is believed to be a sequel of pressure/damage upon the epithelial cells which are then lost into the lumen, although the mechanism of damage is unclear (Coop, Angus & Mapes, 1973). Mucus hypersecretion is another common pathological change seen with *Nematodirus* spp infection and is thought to be involved with the rejection mechanism of immunity (Martin & Lee, 1980). Irreparable tissue damage is unlikely since the tissue phase is transient, superficial and limited to the larval stages which

do not penetrate deep into the mucosa (Rowlands & Probert, 1972). The actual number of worms associated with mortality is extremely variable; deaths have been reported with burdens of 10,000 (Thomas & Stevens, 1956) whereas lambs with over 30,000 have appeared healthy (R. L. Coop, personal communication).

1.4.4 Continuous infections

Initial work on the pathogenicity of nematode species employed large single doses, which is not comparable with the continuous daily ingestion of small numbers of infective larvae by the majority of lambs at pasture. In an attempt to mimic the field situation by continuous larval dosing Coop, Sykes & Angus (1976) demonstrated that *T. colubriformis* can significantly reduce animal performance without obvious clinical signs of parasitism. Using the same techniques and experimental design, *O. circumcincta* was also shown to adversely affect the performance of sheep without clinical signs (Coop, Sykes & Angus, 1977; Sykes & Coop, 1977). Symons, Steel & Jones (1981), in a comparison with another study using *T. colubriformis* (Steel, Symons & Jones, 1980) concluded that, on a larval intake basis, *O. circumcincta* is considerably less pathogenic. Based on impairment of liveweight gain, wool growth and food consumption, the effects of 3,000 *T.colubriformis* infective larvae per week were generally more severe and lasted longer than those observed with a weekly dose of 37,500 *O. circumcincta* larvae.

Coop, Sykes & Angus (1977 and 1982) concluded that the effect of parasitism was dose related and seemed to have a threshold level since only larval intakes above 1,000 *O. circumcincta* per day affected food intake and growth rate. This reduction in growth rate was due not only to a depression of appetite but also to a reduction in the efficiency of food utilisation since infected sheep grew at only half the rate of pair-fed controls. In a later study Coop, Sykes, Spence & Aitchison (1981) demonstrated that intakes above 1,000 also affected skeletal growth. These results are consistent with the suggestion made by Dineen (1963) that the initiation of immunological control of helminth infections requires the presence in the host of a threshold level of parasitic material. From a practical point of view the existence of some threshold level of infection, below which there are no obvious effects on performance is important since it is clearly more feasible, both practically and

economically, to reduce a parasite population on pasture below a given level than to eliminate it altogether. Symons *et al.* (1981) found a threshold level of between 12,000 and 37,500 larvae per week in their study with lambs continuously exposed to *O. circumcincta.* Differences in the age and breed of lambs and the parasite isolate used between these studies may account for the different findings. The lambs in the study conducted by Coop *et al.* (1982) were aged 16 weeks and dosed on a daily basis whereas those in the study of Symons *et al.* (1981) were aged between 18-24 weeks by the time dosing, which was divided into three equal aliquots throughout the week, had started. Breed and genotype differences may be particularly important when assessing threshold levels. Abbott, Parkins & Holmes (1984) found that as well as having higher faecal egg counts, the effects of chronic haemonchosis were more severe in Merino lambs compared to Scottish Blackface lambs. Steel *et al.* (1980) concluded that the threshold level, under their experimental conditions, for *T.colubriformis* infections was between 950 and 3,000 larvae per week, but pointed out that under poor dietary conditions this level may well be lowered.

Since most studies concerning the consequences of infection with *Trichostrongylus* species have used *T. colubriformis* rather than *T. vitrinus*, investigations have been conducted to determine the relative pathogenicities of the two species. Coop, Angus & Sykes (1979) reported the pathological changes in the small intestine whilst Sykes, Coop & Angus (1979) looked at the effects on food utilisation and animal performance in sheep chronically infected with *T. vitrinus.* The authors compared the results to an earlier study using sheep of a similar age and breed, fed the same diet and identical intakes of *T. colubriformis* larvae (Coop *et al.*, 1976; Sykes & Coop, 1976). Assuming that the viability of larvae was similar the authors concluded that there was a lower fecundity and earlier expulsion of worms with *T. vitrinus* infections. Furthermore, although the intestinal lesions caused by both species were similar in most respects they were not as extensive and there appeared to be less of an effect upon food intake with *T. vitrinus* infection.

1.4.5 Mixed infections

Most studies on the effects of sub-clinical gastro-intestinal parasitism in sheep have involved single species nematode infections. In the field situation ruminants

are generally exposed to both abomasal and small intestinal parasites concurrently. Attempts to determine whether the effects of multiple infections are additive or otherwise have provided conflicting results. Steel, Jones & Symons (1982) investigated the threshold levels of single *T. colubriformis* (900 larvae per week) or *O. circumcincta* (38,000 larvae per week) as well as concurrent infections. These authors found respective reductions in liveweight gains of approximately 20% and 30% with single infections whereas concurrent infection resulted in a reduction of approximately 75 %. In a similar experiment using *T. vitrinus* as the small-intestinal species, Coop, Field, Graham, Angus & Jackson (1986) saw 17 % and 20 % reductions in liveweight gain with monospecific *T. vitrinus* (5,000 larvae per week) and *O. circumcincta* (12,500 larvae per week) infections respectively. Concurrent infection however resulted in a reduction of 30 % with no evidence of additive or multiplicative effects.

Dobson, Barnes & Windon (1992a) investigated the population dynamics and acquisition of host immunity to *T. colubriformis* and *O. circumcincta* in both single (10,000 and 5,000 larvae per week respectively) and concurrent infections using lambs aged 21 weeks. Under their experimental conditions, the establishment of infective larvae, cumulative worm burdens, faecal egg counts and lamb bodyweights were not affected by concurrent or pre-existing infection with the other species. Weight gains of the single *O. circumcincta* infected sheep were similar to those of the uninfected controls confirming the threshold level of approximately 1,000 larvae per day noted by Coop *et al.* (1982) and agreeing with the findings of Coop *et al.* (1986), using *T. vitrinus*, that no additive or multiplicative effects on performance are seen with the abomasal and small-intestinal species. An interaction between these species as regards establishment rates has been demonstrated by Jackson, Jackson, Coop & Huntley (1992b). These workers noted that the initial establishment rate of *T. vitrinus* was reduced as a result of concurrent *T.circumcincta* infection and may be an important factor for differences in their seasonal prevalence.

The major limitation of previously mentioned studies is the confidence with which the results from housed animals consuming conserved feeds can be extrapolated to grazing animals consuming fresh herbage. Both the plane of nutrition and the increased energy expenditure associated with grazing may influence the

responsiveness of sheep to infection under field conditions, such that threshold levels of larval intake which affect productivity may well be considerably lower (Symons *et al.*, 1981). Another problem associated with pelleted feed is the palatability of the diet. For example, in the study of Steel *et al.* (1980) the feed intake of several sheep including uninfected controls was temporarily depressed, but their appetite recovered rapidly when a freshly prepared batch of feed was offered to them.

In an attempt to overcome this problem Sykes, Poppi & Elliot (1988) investigated the effect of a concurrent infection on growing lambs consuming fresh herbage. The 4 month old lambs were fed freshly cut rygrass-white clover pasture which had been managed during the previous eighteen months to achieve minimal larval contamination. Bodyweight gain was reduced by 13 and 43 grammes per day following infection with 4,000 *O. circumcincta* and 3,000 *T. colubriformis* larvae per day respectively. Concurrent infection on the otherhand resulted in a reduction of 92 grammes per day. The multiplicative effect of the mixed infection that resulted on animal performance was in agreement with Steel *et al.* (1982). The contrary findings of Coop *et al.* (1986) may be explained by the different trichostrongyle species employed and also by the considerable differences between the experimental conditions in terms of larval dosages, animal breed, age, dosing schedule and diet. The possibility of differing pathogenicities, not only between the trichostrongyle species but also between different isolates, should also be taken into account. It should also be noted that although pasture counts revealed no larval contamination the uninfected control animals in the study by Sykes *et al.* (1988) had positive faecal egg counts and harboured small numbers of worms indicating that the freshly cut herbage carried some infective larvae.

A study by Coop, Graham, Jackson, Wright & Angus (1985) went one step further by monitoring the effects of experimental infection on grazing lambs. Minimally contaminated grazing for the ten 0.9 hectare paddocks used in the study was achieved by taking a hay crop each year and grazing the aftermath with adult cattle or ewes given anthelmintic every two weeks. Three and a half month old lambs were infected daily, five days a week, with either zero, 500, 1,500, 3,000 or 5,000 infective *O. circumcincta* larvae for twelve weeks. The procedures used to produce 'clean' pasture and the practice of moving the lambs to a fresh paddock

every 7 - 10 days were very successful, as judged by the low level of parasitism in the control lambs. Faecal egg counts followed a stereotypic pattern in all the infected groups, peaking at week four and declining to low levels by week six. Reductions in liveweight gain compared to the uninfected controls for the groups dosed with 1,500, 3,000 and 5,000 larvae per day were 24 %, 31 % and 37 % respectively. No differences in liveweight gain were apparent in the group dosed with 500 larvae per day. These figures are similar to those recorded in the same breed of lambs receiving a complete ruminant ration and on a comparable infection regimen with *O. circumcincta* when housed, and suggest that a threshold level of exposure for a significant effect on productivity of between 1000 and 1500 larvae per day for this age of lamb (Sykes & Coop, 1977; Coop *et al.*, 1982). This figure is lower than that suggested by Symons *et al.* (1981) for housed Merino-cross lambs of between 12,000 and 37,500 *O.circumcincta* larvae per week. However, as mentioned earlier, differences in animal age, breed, larval isolate, dosing schedule and diet may account for these discrepancies.

1.5 Development of immunity to gastrointestinal parasites

The effects of acquired resistance on the course of gastro-intestinal nematode infections in sheep under both natural conditions (Gordon, 1948; Morgan, Parnell & Rayski, 1951) and in experimental infections (Dineen, Donald, Wagland & Offner, 1965) have long been noted. These and similar studies have identified some of the means by which the host regulates its nematode burden during prolonged and continuous exposure to infection. These include the periodic spontaneous expulsion of the established adult parasite by resistant individuals (Gordon, 1948; Stewart, 1953), a reversible inhibition of larval development which may form part of a process for the continual replacement of adult worms, the inhibition of ovulation of female worms and the development of resistance to reinfection (Michel, 1963).

In natural infections the frequency of gastrointestinal nematode infection follows a negative binomial distribution describing a state of overdispersion (Barger, 1985). The result of this is that a relatively small proportion of the host population harbours a large proportion of the parasite population. Riffkin (1988) has estimated

that the most resistant 50% of grazing animals may produce less than 10% of the worm eggs counted, whereas the most susceptible 15% of the flock may be responsible for over 50% of the egg output. The existence of such overdispersed parasite populations illustrates the importance of host immunity to infection. Host immunity to infection can be expressed in one of two ways; innate and acquired. Innate immunity is a non-specific and pre-existing phenomenon which provides a measure of the host's ability to regulate parasite establishment, development, persistence and fecundity. In contrast, acquired immunity is highly specific for a particular pathogen and is an active, adaptive and aggressive response which improves with repeated exposure to the same pathogen (Emery & Wagland, 1991).

Young animals are most susceptible to gastrointestinal nematode infection. In temperate regions, where grazing animals are exposed to continuous trichostrongylid infection, these animals are gradually able to acquire an immunity to subsequent infection. The development and expression of acquired immunity is a complex process which may be strongly influenced by a number of factors, most notably the age of the host, the plane of host nutrition, the level of exposure to infection and the animal management. The rate of development and degree of host immunity also varies with nematode species, such that resistance to *Nematodirus* spp. may be acquired within weeks, whereas that to *Teladorsagia* spp and *Trichostrongylus* spp may take between 3 to 5 months (Sykes, 1994).

1.5.1 Age

Growing lambs acquire immunity to gastrointestinal nematode infections at a slower rate than older sheep (Manton, Peacock, Poynter, Silverman & Terry, 1962; Dineen, Gregg & Lascelles, 1978; Douch & Morum, 1993). Generally, immunity to gastrointestinal nematode infection in sheep gradually improves with age over the first 12 months (Watson & Gill, 1991). This has been reported for sheep infected with *H. contortus* (Manton *et al.*, 1962; Benitez-Usher, Armour, Duncan, Urquhart & Gettinby, 1977), *T. colubriformis* (Gibson & Parfitt, 1972 and 1973; Chiejina & Sewell, 1974a and 1974b; Dineen *et al.*, 1978) and *T. circumcincta* (Smith, Jackson, Jackson & Williams, 1985). The relative immunological unresponsiveness of immature lambs has been described by Watson, Colditz, Andrew, Gill & Altmann

(1994) and supports other studies which suggest that lambs are unable to mount an effective immunity to nematode infection until they are at least 6 months of age (Waller & Thomas, 1981; Soulsby, 1985). It has been suggested that the unresponsiveness of young lambs may be partly due to the immaturity of gut effector mechanisms rather than the failure to produce parasite-specific antibodies (Gregg, Dineen, Rothwell & Kelly, 1978). The factors involved in the development of age-related immunity are as yet unknown, but have been linked to factors such as puberty, bodyweight and condition rather than chronological age (Abbott & Holmes, 1990). It is also possible that in parasitised ruminant animals there are competing demands for the available nutrients between growth, the repair of gastrointestinal pathology and the immune response with the first two processes taking priority in the young lamb (Coop, Huntley & Smith, 1995).

1.5.2 Weaning

Spedding, Brown & Large (1963) investigated the effects of milk intake upon lamb growth rate, food consumption and worm infestation. These workers concluded that milk intake and growth rate were highly correlated and that a negative relationship existed between milk intake and intake of solid foods. As a result, lambs receiving more milk consumed less grass in the field and had fewer worms *post mortem*. A direct effect of ewe's milk was not excluded and it was also noted that since milk supply influences growth rate, lambs require less time for fattening on pasture and are exposed to fewer larvae.

The effect of weaning on antibody responses and nematode parasitism in Merino lambs was investigated by Watson & Gill (1991). As expected, weaning seriously compromised growth rate and was attributed to the cessation of milk intake, but it was noted that the anxiety and stress of weaning may also have lowered pasture intake by increasing the amount of time that weaned lambs spent calling and searching for their mothers. When the lambs were experimentally infected with *H.contortus* and *T. colubriformis* larvae at eight weeks old, the mean faecal egg count for weaned lambs was twice that for controls and there was also a significant decline in haematocrit values. Antibody responses following immunisation with either proteinaceous (ovalbumin) or bacterial (*Brucella abortus*) antigens did not

differ significantly between control and weaned lambs at 4 and 8 weeks of age. However, serum antibody responses to *H. contortus* and *T. colubriformis* differed significantly with controls responding earlier and more strongly than weaned lambs. From these findings it was concluded that up to the age of three months suckled lambs, when faced with substantial parasite challenge, have much better prospects than weaned lambs.

Chapple & Lynch (1986) suggested that lambs' diet selection after weaning is strongly influenced by learning experiences while still with their dam. Weaned lambs therefore may not learn to select as nutritious a diet as those grazing close to their mother and there may also be dominance effects at sites of competition.

Shaw, Nolan, Lynch, Coverdale & Gill (1995) investigated whether delaying the age of weaning or feeding a protein-rich supplement altered the rate at which lambs developed immunity to *H. contortus*. There were no differences evident in the response to a trickle infection between suckling lambs and those weaned at four months but an earlier immune response was mounted in groups given protein supplementation. Gender differences were also evident with castrated males, whether supplemented or not, having lower faecal egg counts than females. Unweaned females were also more susceptible to parasitism than weaned females. Whether these differences were due to the effect of maternal hormone, differences between the maternal relationship between male and female offspring or due to other reasons was not clear.

1.5.3 Nutrition

The nutritional status of the host and the consequences of infection have long been established with malnourished animals showing increased susceptibility to parasitism (Gibson, 1963). Baird, Vegors, Sell & Stewart (1954) showed that the actual protein content of the diet, rather than the total consumption of forage, was associated with increased susceptibility in ruminants. Dobson & Bawden (1974) studied the effects of a low-protein diet on the resistance of sheep to infection with the large intestinal nematode *Oesophagostomum columbianum* and concluded that increased susceptibility was associated with malfunctions of the innate immunity of the gut. This involved decreased peristalsis, failure of mucin cell hyperplasia,

reduced lymphocyte and plasma cell reactions and possibly poor functional coordination between lymphocytes, antibodies and mast cell-granulocyte effector mechanisms.

The importance of protein intake for the development and expression of immunity towards *T. colubriformis* was demonstrated by Wagland, Steel, Windon & Dineen (1984). Dietary protein does not appear to influence parasite establishment and pathogenesis in lambs of varying ages and breed when given a single challenge of infective *H. contortus* larvae (Abbott, Parkins & Holmes, 1985 and 1986). However, when lambs aged four months were continuously infected with small numbers of larvae and fed a high protein diet many animals developed resistance to further infection (Abbott, Parkins & Holmes, 1988). In a vaccination trial using irradiated *H. contortus* larvae Abbott & Holmes (1990) demonstrated that dietary protein was not as important in older lambs, aged eight months, since a strong resistance to a challenge infection developed in these animals regardless of dietary intake.

Bown, Poppi & Sykes (1991) described the effects of an abomasal infusion of casein and glucose on protein and energy deposition in lambs chronically infected with *T. colubriformis*. The pathological damage to the small intestine caused by infection was a result of induced protein rather than energy deficiency and the authors concluded that this was probably a consequence of increased endogenous protein loss into the gastrointestinal tract rather than a failure of protein absorption. The authors demonstrated that this deficiency may be overcome by a post-ruminal infusion of extra protein with a consequent reduction in the effects of parasitism.

Kambara, McFarlane, Abell, McAnulty & Sykes (1993) investigated the effect of age, 8-26 or 33-51 weeks old, and dietary protein, 11 % or 20 %, on the development of immunity and resistance in lambs infected with *T. colubriformis*. Immunity was measured by an *in vitro* lymphocyte blastogenesis test to T and B cell mitogens and to a larval antigen. Resistance to challenge infection was ascertained by worm burden, FEC and eggs *in utero* in the nematode. The young lambs on the high protein diet developed a better resistance and greater live-weight gain than those fed a low protein diet but there were no differences between the older groups. Lymphocyte responsiveness to T cell mitogens was higher in lambs on the high

protein diet, being greater in the older animals. Increased sensitization to larval antigen was only observed in the older lambs. It was concluded that the response to larval antigen was affected by age and that this mechanism of resistance is not evident in young lambs regardless of protein intake.

Using a protected protein (resistant to microbial degradation in the rumen) van Houtert, Barger, Steel, Windon & Emery (1995) demonstrated an increased resistance to the effects of parasitism by *T. colubriformis* in Merino lambs aged three months. As well as significantly higher liveweight gains, the rate of worm expulsion was higher in supplemented animals and was correlated with eosinophil numbers and sheep mast cell protease (SMCP) levels. Although there was no difference between circulating antibody levels, supplemented animals did show an enhanced responsiveness to *T. colubriformis* larval antigen *in vitro*. It was suggested that the reduction in production losses attributed to *T. colubriformis* infection were immunologically mediated but that the mechanism(s) through which nutrition enhances this response requires further investigation.

Coop *et al.* (1995) investigated the effect of protein supplementation on the rate of development of immunity in growing lambs infected with *O. circumcincta.* Protein supplement was administered directly to lambs aged four and a half months by means of an abomasal catheter so as to avoid degradation by the rumen microflora. As well as lower mean faecal egg counts, worm burdens were significantly lower in supplemented lambs suggesting that the provision of protein accelerated the development of immunity to *O. circumcincta* in these lambs. There was also a greater percentage of worms at the early fourth stage and higher levels of SMCP in the supplemented animals. Coop & Holmes (1996) recently reviewed the interaction between nutritional status and gastrointestinal parasitism in ruminants. The effect of host nutrition upon parasite establishment and survival, the development of immunity and the pathophysiology of infection was discussed as was the influence of the parasite upon host nutrition. In addition to dietary protein, the influence of both macro-minerals and trace elements was highlighted and it was concluded that further research is required into the complex parasite/nutrition relationship within the host.

1.5.4 Teladorsagia (Ostertagia) spp

Gibson & Whitehead (1981) examined the development of immunity and the consequent changes in worm populations in sheep continuously infected with *O.circumcincta.* Using lambs aged between 20-22 weeks and a daily dose of 2000 infective larvae, faecal egg counts declined after 7 weeks whereas worm burdens fell abruptly by week 12. Although the lambs in this study were older than those of a similar study with *T. colubriformis* (Gibson & Parfitt, 1973), the authors attributed the differences in results between the experiments to the different life cycles of the nematodes. They concluded that the close contact between the histotrophic phase of *O. circumcincta* and host tissues promotes a more rapid development of resistance to infection than with *T. colubriformis* where the contact between immature stages and host is not so intimate.

In their study on the population dynamics of *O. circumcincta*, Hong, Michel & Lancaster (1987) infected groups of lambs aged 18 weeks at three daily dosage levels. Although there were a few abnormal individuals, the number of established worms appeared to be related to the rate of larval intake. Faecal egg counts fell rapidly in all groups after about 6 weeks and worm burdens between weeks 9-12. A decrease in worm length was observed with time and the numbers of females with reduced vulval flaps was more prevalent indicating that there was a rapid turnover of worms, a phenomenon originally noted by Michel (1963) with *O. ostertagi* in calves.

1.5.5 Trichostrongylus spp

Jackson, Angus & Coop (1983) monitored the development of morphological changes in the small-intestine of lambs aged 12 weeks infected daily with 2,500 *T.vitrinus* larvae five days a week. These authors noted that worm burdens were declining and animals were immune to larval infection by week 14. Gibson & Parfitt (1973) found that worm burdens increased up to the 20th week of infection using similar aged lambs and a dosing regime of 2,000 infective *T. colubriformis* per day. Comparison of the worm burdens between these two studies and that of a similar study by Coop *et al.* (1976) show that much larger burdens may become established with *T. colubriformis.* These results led Jackson *et al.* (1983) to conclude that there

is an apparent ability of lambs to mount a more rapid and effective response to *T.vitrinus* than to *T.colubriformis* species.

1.5.6 Nematodirus spp

Within 5-6 weeks experience of infection with *N. battus* lambs are capable of regulating faecal egg output and by 3 months are capable of regulating adult populations (Gibson & Everett, 1963). Using larger numbers of lambs Taylor & Thomas (1986) confirmed that immunity to *N. battus* may develop in the first 3 months of life but that it is a true acquired immunity rather than a manifestation of age immunity.

1.5.7 Use of radiolabelled larvae

While serial killing of sheep in continuous infection experiments has provided information on the total population of parasites at any given time, radiolabelling has made it possible to distinguish newly acquired, normally developing worms from earlier arrivals which may have become retarded, or arrested in their development. By following the fate of radiolabelled larvae given as a challenge to lambs previously exposed to a continuous infection, Seaton, Jackson, Smith & Angus (1989a and 1989b) have studied the development of immunity in lambs dosed with *T. vitrinus* and *O. circumcincta* respectively.

The development of immunity to incoming radiolabelled *T. vitrinus* larvae was studied in lambs aged 6 months (Seaton *et al.*, 1989a). The mean faecal egg count increased from day 17 of the experiment to reach a peak during week 7, afterwhich they declined. Nevertheless, partial immunity to freshly acquired larvae was present as early as 4 weeks in the form of reduced establishment and by 8 - 12 weeks there was almost complete immunity to further infection as well as retardation of established worms.

In the case of *O. circumcincta* the first indication of immunity in lambs aged 5 months was a retardation of developing worms after 4 weeks of dosing (Seaton *et al.*, 1989b). Faecal egg counts were variable, peaking after 7 weeks but remaining high until week 11. Immunity to the establishment of incoming larvae had developed between weeks 4 and 8 of continuous challenge and by week 12 the animals were

almost completely immune to further infection. The authors suggested that there was a brief period of population turnover with a loss of established worms between 4 and 8 weeks, agreeing with the findings of Hong *et al.* (1987).

This work has provided direct evidence that immunity to continuous infection begins earlier than suggested by indirect studies and despite rising faecal egg counts. It also demonstrated an apparent difference in the development of immunity towards the different species. With *O. circumcincta* the first indication of immunity was retardation of developing worms whereas resistance to establishment was the first sign with *T. vitrinus*. It would seem therefore, that the relative importance of each aspect in the initial development of immunity is different for these abomasal and small-intestinal species. Barger, Le Jambre, Georgi & Davies (1985) working with radiolabelled *H. contortus* concluded that resistance to establishment and inhibition of parasite development were separate aspects of the immune response to continuous challenge, dependent upon the host's previous experience of infection and the current rate of larval intake.

1.6 Patterns of worm exclusion/expulsion

1.6.1 *Expulsion of infective larvae*

The term 'rapid expulsion' was first introduced by Bell, McGregor & Despommier (1979) to describe the speed of larval expulsion in secondary *Trichinella spiralis* infections in rats and the premature rejection of larvae surviving the initial phase. Rapid expulsion (RE) is directed against infective larvae as they enter the gastrointestinal tract and is ultra-rapid, apparently occurring before establishment takes place, the majority of worms being expelled in the faeces within 24 hours (McClure, Emery, Wagland & Jones, 1992) and as quick as 4 hours following challenge (Jackson, Miller, Newlands, Wright & Hay, 1988). However, as pointed out by Rothwell (1989), the term RE should not necessarily be applied to all expulsion processes which are rapidly initiated and should be restricted to that of *T.spiralis* in rats until its relationship with other host-parasite systems is established. The establishment of *T. spiralis* larvae requires attachment to and penetration of

intestinal epithelial cells; processes that are not required by many other nematode species and may well be uniquely susceptible to host intervention.

There was no evidence of immune exclusion of a single challenge of 10,000 *H.contortus* L_3 in the study of Jackson *et al.* (1988) whereas higher levels did elicit a response. However, as the authors pointed out, there is no reason to suggest that this mechanism does not play a part in the regulation of worm populations in animals continuously exposed to low levels of infection since marked increases in initial lymphocyte traffic and raised antibody levels were maintained as a result of daily challenge with 2,000 *O. circumcincta* L_3 (Smith, Jackson, Jackson, Williams & Miller, 1984a; Smith, Jackson, Graham, Jackson & Williams, 1987). Several mechanisms have been proposed that may account for the immune expulsion of incoming larvae, reviewed by Miller (1984). These include the cooperation between mucus and antibodies and their interaction with inflammatory mediators released from a variety of effector cells, the components of which will be discussed later, all of which must be immediately available for activation.

1.6.2 Expulsion of established worms

Primary infections with gastrointestinal nematodes in their natural hosts invariably progress to patency and the eventual immune elimination of adult worms occurs over a variable period of time, the quantitative features of which depend upon the host genotype, immune status and number of infective larvae given (Rothwell, 1989). Immune responses may be directed against developing larvae or pre-adults which have already established but are expelled before they reach adulthood, or there is the more usually described spontaneous cure wherein the adult worms are expelled over a period of several days, weeks or months. Alternatively, the response may merely cause stunting of parasites, a reduction in worm fecundity or, under certain circumstances, inhibition of the infective larvae after they have reached their niche in the mucosa (Miller, 1984).

In experimental *O. ostertagi* trickle infections in cattle the worm burden undergoes continuous turnover with new worms being acquired as old worms are lost. Under field conditions adult worms have an average lifespan of 26 days (Michel, 1970) despite the potential to survive for more than 100 days in single

pulse, low-level infections (Michel, Lancaster & Hong, 1978). Thus, there is a continuous turnover of the worm population and, since immunity to infection increases with continued exposure, incoming larvae are gradually less successful in their rate of establishment and ultimately worm numbers decline (Michel, 1970).

A different pattern of events is observed in animals repeatedly infected with *T.vitrinus* or *T.colubriformis*. When sheep are exposed to daily infection with *Trichostrongylus* species, adult worm burdens accumulate until immunity develops to incoming larvae, after which adult worms may persist for varying periods of time before they are ultimately expelled (Jackson *et al.*, 1983).

The regulation of *H. contortus* populations in sheep grazing on endemic pastures is more complex. Adult worms may accumulate for the first 4 weeks of infection before immunity develops against incoming larvae, after which adult worms may persist for varying periods of time before they are ultimately expelled, the length of time depending upon the level of larval intake (Barger *et al.*, 1985).

Trichostrongylus and *Haemonchus* infections, by means of some immunomodulatory activity, are similar in that adult worms appear to survive in hosts which are immune to reinfection but eventually these modulatory factors are overturned and expulsion of adult worms takes place (Behnke, 1987). In explaining worm turnover in terms of host protection and immunomodulation it is possible that larvae are themselves immunomodulatory, keeping host responses at bay until a particularly high threshold of stimulation is exceeded and anti-larval immunity initiated. Presumably the modulatory factors of adult worm *Ostertagia* species are less effective than those of *Trichostrongylus* and *Haemonchus* species (Behnke, 1987).

Expulsion of established worms is probably brought about by a combination of immunologically specific (e.g. T cells and antibodies) and non-specific components (e.g. mucus and inflammatory mediators) interacting in concert, the mechanisms of which will be discussed later. As well as host genotype, immune status and number of infective larvae given, the immune response is influenced by the age of the sheep and its nutritional and hormonal status (Smith, 1988) the complexities of which may help to explain the reason for conflicting results from studies on immunity to gastrointestinal nematodes.

1.6.3 Cross-protection

Initial studies by Stewart (1953 and 1955) revealed the phenomenon of 'self-cure' in sheep that had been rendered hypersensitive as a result of previous infestations. Immunity to *H. contortus* resulted in the rejection of a combined challenge with *T. colubriformis*, however, *H. contortus* could establish in *T.colubriformis*-immune sheep indicating that expulsive mechanisms could work down-stream but not up-stream (i.e. antigenic material from larvae in the abomasum could pass readily to the small intestine, whereas similar material from the small intestine could not pass to the abomasum). The ability of *T. colubriformis*-immune sheep to regulate a combined challenge with *T. colubriformis*, *N. spathiger* and *T.vitrinus* L_3 led Dineen, Gregg, Windon, Donald & Kelly (1977) to suggest that expulsion of gastrointestinal nematodes from immune sheep is mediated by an effector mechanism that is non-specific in nature. Similar findings have also been recorded with *T. colubriformis*-immune sheep challenged with a mixed infection at pasture (Douch, 1989). Compared with naive controls, immune animals had reduced adult worm burdens of *T. colubriformis* (87%), *N. spathiger* (91%), *T. vitrinus* (44%), *T. axei* (67%), *O. circumcincta* (42%), but increased numbers of arrested larvae of *Nematodirus* and *Ostertagia* species, and similar numbers of *H. contortus* and *C.curticei*. Emery, Wagland & McClure (1993) have recently confirmed the findings of non-specific rejection of unrelated parasites living in the same or downstream niches of the gut and suggested that a similar mechanism(s) may operate as a result of antigens produced by L_4 and later stages following challenge.

In the study of Dineen *et al.* (1977) a single challenge infection with *T. vitrinus* administered to the *T. colubriformis*-immune sheep resulted in 34 % protection, and it was suggested that shared antigens may have evoked this cross-protection. This theory may help to explain the rejection of the abomasal parasite *T. axei* in the study of Douch (1989) or it may well be that the effector mechanism can also work up-stream. Douch (1989), suggested that the unexpected cross-protection seen with *O.circumcincta*, which was associated with increased numbers of globule leucocytes in the abomasal mucosa, may have resulted from the small numbers of *T.colubriformis* present in the abomasa of some sheep. Also evident from the study

of Douch (1989) was the failure of the effector mechanism to expel *C. curticei* populations down-steam and suggests that the non-specific effector mechanism(s) may work in either direction, but in close proximity, to the site of parasitism. Adams, Anderson & Windon (1989), on the otherhand, failed to demonstrate cross-protection between *H. contortus* and *T. colubriformis* when the vaccinating infections were removed by anthelmintic.

It should be noted that physiological changes induced by one species have also been shown to affect the establishment of a second species. For example, alterations in abomasal pH and sodium ion concentration induced by *H. contortus* infections have been shown to alter the intestinal environment down-stream thereby affecting the development of concurrent *N. battus* infections (Mapes & Coop, 1970 and 1971 and 1973). Similarly, it has been suggested that alterations in gut pH caused by prior exposure to *O. circumcincta* interferes with the establishment and fecundity of subsequent *H. contortus* infections (Blanchard & Wescott, 1985). It is also thought that physiological changes may account for the reduced establishment rate of *T.vitrinus* in concurrent *T.circumcincta* infections noted by Jackson *et al.* (1992b). Cross protection, therefore, may involve a combination of immunological, physiological, pathological and parasitological changes occurring simultaneously.

1.7 Mechanisms of immunity to gastrointestinal parasites

1.7.1 Humoral (antibody) response

Despite numerous observations of increased levels of parasite-specific antibodies in ovine gastrointestinal nematode infections their role in immunity remains unclear (Miller, 1984). Early studies on the development of immunity described anti-parasitic antibodies in the abomasa and intestines of sheep infected with *H. contortus* (Smith, 1977) and *T. colubriformis* (Adams & Cripps, 1977) respectively, with the IgA isotype predominating. Although IgE is the isotype most widely associated with parasitic infections and immediate type hypersensitivity reactions, the class of immunoglobulin responsible for anaphylactic activity in sheep has yet to be fully characterised (Miller, 1984). Elevated levels of serum IgG, IgM and mucosal/lymph IgA antibodies are typical responses to sheep nematode

infections, although local IgG has been observed in both *H. contortus* and *O.circumcincta* infected sheep (Smith, 1977; Smith, Jackson, Jackson & Williams, 1983a). Recent studies comparing the antibody responses of immune and randomly-bred sheep demonstrated the presence of local IgG_1 as well as IgA in immune animals, indicating that both isotypes may play an important role in mediating resistance (Gill, Husband, Watson & Gray, 1994).

Large temporary increases in the concentration of lymph antibodies, peaking around 6 days after challenge, have been consistently recorded in immune sheep (Smith, 1988). However, attempts to transfer protection against gastrointestinal nematodes in sheep by intravenous infusion of large quantities of IgA-rich immune lymph plasma have been unsuccessful (Adams, Merritt & Cripps, 1980; W. D. Smith, personal communication). The fact that resistance was not transferred does not rule out a role for antibody since the concentrations achieved in the mucosa of the recipients of such lymph are much lower than those in immune sheep (Smith, 1988).

Stunting, reduced fecundity and loss of worms are frequently associated with immunity and various mechanisms by which antibodies may exert their protective effects have been proposed. Bound antibody makes it easier for phagocytic cells to attach to parasites and can also activate a system of blood proteins (collectively known as complement) that attack the worm (Taverne, 1993). Other suggestions include the ability of antibodies to interfere with the worms' capacity to feed by blocking or neutralizing vital parasite enzymes (Smith *et al.*, 1985; Gill *et al.*, 1994) or inhibiting essential metabolic processes which are vital for parasite establishment and maintenance (Carlisle, McGregor & Appleton, 1990). Furthermore, the demonstration that IgA is capable of inducing eosinophil degranulation has led to the suggestion that the interaction of eosinophils and IgA, under cytokine control, may play an important role in mucosal immunity (Abu-Ghazaleh, Fujisawa, Mestecky, Kyle & Gleich, 1989).

1.7.2 Cell-mediated response

The cell-mediated response involves T lymphocytes and the production of specialized cells that react with foreign antigens on the surface of other host cells. Studies of gastrointestinal nematodoses in neonatally thymectomized or athymic

laboratory animals have demonstrated the fundamental involvement of T lymphocytes in the acquired immune response (Mitchell, 1980). T lymphocytes play a regulatory role in immunity, acting either to enhance or suppress the responses of other white blood cells (leucocytes), referred to as T helper and T suppressor cells respectively. Cytokines, which include interleukins (IL), interferons (IFN), colony stimulating factors (CSF), tumour necrosis factors (TNF) and migration inhibitory factors (MIF), are soluble low molecular weight proteins which regulate the amplitude and duration of the immune-inflammatory responses. Principally, cytokines are produced by T lymphocytes, macrophages and mast cells but may also derive from B lymphocytes, fibroblasts and endothelial cells. Other T lymphocytes, called cytotoxic T cells, along with B lymphocytes and cells such as macrophages, eosinophils, mast cells and neutrophils are involved directly in defence against infection and are collectively referred to as effector cells. Many of the anti-parasitic activities of effector cells are enhanced by interaction with inflammatory mediators released by other types of cell in response to infection (Taverne, 1993).

Typically the cellular response reaches a peak 3 days after challenge, comprising a large transient increase in the output of lymphoblastic and IgA containing cells in the lymph (Smith, 1988). The infusion of lymphocytes from the gastric lymph of sheep immune to *H. contortus* (Smith, Jackson, Jackson, Williams, Willadsen & Fehilly, 1984b) and *O. circumcincta* (Smith, Jackson, Jackson, Graham, Williams, Willadsen & Fehilly, 1986) resulted in the transfer of partial immunity to parasite naive histocompatible recipients. Miller (1984) emphasised that the transfer of lymphocytes is unlikely to affect gastrointestinal nematodes directly and that an indirect mechanism triggered by the donor cells seems a much more plausible scenario. The immunity adopted by Smith and his co-workers (1984b and 1986) was associated with a local IgA response and mastocytosis but, as the authors pointed out, these were only two of the many potential effector cell populations that were monitored and the extent to which they influenced worm retardation and/or expulsion remains speculative.

Recently, helper T-cell lines ($CD4^+$) have been shown to play a pivotal role in acquired immunity. In genetically resistant sheep treated with a monoclonal antibody against $CD4^+$ subsets, cellular and humoral responses were abolished, with

consequent increases in faecal egg counts and worm burdens (Gill, Watson & Brandon , 1993). However, the type of T cell response involved in protection may vary depending upon the parasite species and different phenotypes may be required at different stages of infection (Taverne, 1993).

1.7.3 Gastrointestinal mucus

Invariably infective stages come into contact with superficial mucus at the epithelial surface and hyperplasia of mucus secreting goblet cells has been described in a variety of infections (Rothwell, 1989). Christie, Hart, Angus, Devoy & Patterson (1978) reported hyperplasia of the abomasal mucosa in sheep repeatedly infected with *H. contortus*, whilst Jackson *et al.* (1983) recorded similar findings in the intestinal mucosa of sheep infected with *T. vitrinus*; both sets of authors associated these changes with the development of immunity. Mucus is not thought to act as a physical barrier since it is penetrated in some systems (Miller, Jackson, Newlands & Appleyard, 1983a), but rather it may have a protective role, dislodging established worms or acting as a medium for trapping larvae in which they are exposed to parasite specific antibodies and/or inflammatory mediators (Miller, 1987). Bell, Adams & Ogden (1984) demonstrated expulsion without significant mucus trapping in rats exposed to an abbreviated primary infection regime in which *T.spiralis* did not reach patency, although Rothwell (1989) proposed that adequate mediator levels may well be achieved in the absence of mucus. Antibody and complement are known to be present in mucus and are thought to enhance retention of larvae in the superficial mucus by interacting with the nematode cuticle (Miller, 1984). Gill *et al.* (1994) demonstrated significantly higher numbers of parasite specific antibody containing cells in the abomasal mucosa of sheep bred for resistance to *H. contortus* compared to randomly bred animals. The IgA isotype predominated followed by IgG_1 and IgM. Similarly, elevated IgA concentrations were seen in sheep previously infected with *O. circumcincta*, as judged by levels in gastric lymph (Smith *et al.*, 1987).

Douch, Harrison, Buchanan & Greer (1983) investigated the antiparasitic property of mucus from immune sheep using an *in vitro* larval migration inhibition (LMI) bioassay. Inhibition of *T. colubriformis* larval migration was attributed to

mucus components having similar properties to slow reacting substance of anaphylaxis (SRS-A) and was shown to be non-specific since abomasal species were also unable to migrate. The major constituents of SRS-A are the leukotrienes B4, C4, D4 and E4, two of which were assessed, along with histamine, in lambs genetically defined as high or low responders to vaccination and challenge with *T.colubriformis* (Jones, Windon, Steel & Outteridge, 1990). The authors found increased concentrations of these mediators in mucus of responsive animals compared to intestinal tissue and suggested that they may play a role in preventing establishment of incoming larvae. As well as measuring inflammatory mediators Jones, Emery, McClure & Wagland (1994) used a LMI assay to examine possible causal relationships between mediator release and kinetics of parasite rejection. Challenge of immune sheep evoked a release of mediators and the intestinal contents inhibited migration of larvae significantly more than those of worm-free sheep given a single infection of *T. colubriformis*. Mediator concentrations in gut contents were considerably lower than those found in mucus indicating that the adjacent mucosa was the likely source of these mediators. Increased concentrations of mucosal mediators were evident at the time when egg counts declined in primary infected animals suggesting a role for these mediators and/or their cellular source in the expression of immunity.

1.7.4 Mast cells

Mast cells are often indistinguishable from circulatory basophils in a number of properties although their relationship is not clear. There are two different kinds of mast cell; one is associated with the mucosa (MMC) whilst the other is found in connective tissue (CTMC). Like basophils, mast cells contain numerous lysosomes and secretory granules and can be activated by cytokines. They also contain specific high affinity receptors for the Fc portion of IgE which causes them to degranulate upon antibody binding. The two types of mast cell have common precursors and seem to be interconvertable depending upon the level of cytokines in the local environment (Rothwell, 1989). Histopathological changes which develop in the alimentary tract following infection by nematode parasites are characterized by mast

cell hyperplasia (mastocytosis) and their accumulation, especially in the mucosa (Rothwell, 1989).

The appearance in the mucosa of cells with large acidophilic granules or globules, designated globule leucocytes (GL), has been recorded by many workers. The relationship of a GL response to nematode infestation was demonstrated by Whur (1966) in a comparison of worm-free and parasitized sheep; reviewed by Gregory (1979). Investigation into the ultrastructural, morphological and histochemical properties of GLs have confirmed that in ruminants, GLs are derived from MMCs as a consequence of prolonged antigenic challenge (Huntley, Newlands & Miller, 1984).

Appropriately stimulated MMCs release a variety of mediators including biogenic amines (histamine, serotonin), lipid mediators (prostaglandin, leukotrienes, platelet-activating factor), granule proteins (serine proteases, aryl sulphatase) and proteoglycans (heparin, chondroitin sulphate) as well as cytokines (Taverne, 1993). Studies in rats have shown that the immune expulsion of nematodes is associated with local immediate hypersensitivity reactions involving increased mucosal permeability and concomitant systemic release of a mucosal mast cell proteinase, termed rat mast cell proteinase II (RMCPII) (Miller, Woodbury, Huntley & Newlands, 1983b). A similar although fundamentally different proteinase, sheep mast cell proteinase (SMCP), has been isolated from ovine MMCs (Huntley, Gibson, Knox & Miller, 1986) and the subsequent development of an ELISA has provided a means of monitoring the functional activities of sheep MMCs *in vivo* (Huntley, Gibson, Brown, Smith, Jackson & Miller, 1987). Although the presence of proteinase inhibitor(s) interfered with this assay the authors detected high concentrations of SMCP in the sera and gastric lymph of immune sheep challenged with *H. contortus* and *O. circumcincta* respectively. Release of SMCP from intestinal MMCs has also been demonstrated *in vitro* following the addition of specific parasite antigens (Jones, Huntley & Emery, 1992) providing further evidence of the involvement of mast cells in protective immunity.

Nevertheless, the question of an effector role for mast cells and their products in worm expulsion has not been resolved and technical problems such as inadequate fixing of cells, unreliable counts in tissue sections and the possible involvement of

basophils (which may be functionally similar) has been overlooked in many studies. Other sources of mediators include enterochromaffin cells and platelets; the absence of MMCs therefore does not preclude the involvement of these mediators in the expulsion process (Rothwell, 1989).

1.7.5 Granulocytes

Peripheral blood contains three types of granulocytes; neutrophils (polymorphonuclear leukocytes), eosinophils and basophils all of which contain numerous lysosomes and secretory granules and are activated by cytokines. Granulocytes are short-lived (2-3 days) relative to monocyte/macrophages which may live for months or years. Neutrophils possess receptors for the Fc portions of antibodies and for complement proteins, and they migrate to and accumulate at sites of complement activation. Eosinophils express specific receptors for the Fc portion of IgE antibodies and are important effector cells in immune reactions to antigens that induce high levels of IgE, such as parasites. *In vitro* experiments suggest that IgE antibody-dependent cytotoxicity mediated by eosinophils may be particularly effective at restricting helminth infections. This is because the major basic protein of eosinophil granules may be more toxic for helminths than the proteolytic enzymes and reactive oxygen species produced by neutrophils and macrophages (Taverne, 1993).

Eosinophils are a major and well known component of the cellular infiltrate in parasitized tissues and some hypersensitivity reactions (Rothwell, 1989). Increases in the numbers of eosinophils (eosinophilia) in blood and tissues is a characteristic of parasitic infection in most species although their role against helminths is still uncertain (Butterworth, 1984). Nevertheless, studies conducted with sheep have shown a correlation between eosinophilia and the ability of lambs to respond to *T.colubriformis* (Dawkins, Windon & Eagleson, 1989; Buddle, Jowett, Green, Douch & Risdon, 1992; Rothwell, Windon, Horsburgh & Anderson, 1993) and *H. contortus* (Gill, 1991). The association of eosinophils with hypersensitivity reactions led to suggestions that they were attracted by chemotactic factors, such as eosinophil chemotactic factor of anaphylaxis (ECF-A), in order to regulate the effects of mast cells. However, as well as containing a major basic protein (MBP) which can

activate MMCs and basophils (Rothwell, 1989) eosinophils contain other proteins including a cationic protein (ECP) which is a potent helminthotoxin for a range of parasites including schistosomula of *Schistosoma mansoni* (Ackerman, Gleich, Loegering, Richardson & Butterworth, 1985). Eosinophils are also known to generate leukotrienes, platelet activating factor and reactive oxygen metabolites and their categorisation as an end-stage effector cell appears to be an oversimplification (Rothwell, 1989). As well as having receptors for IgG, IgE and complement it has recently been demonstrated that IgA is capable of inducing eosinophil degranulation (Abu-Ghazaleh *et al.*, 1989). Since local levels of IgA have been shown to be significantly elevated in ovine gastrointestinal infections (Smith *et al.*, 1987; Gill *et al.*, 1994) it has been suggested that the interaction of eosinophils and IgA, under cytokine control, may play an important role in mucosal immunity (Abu-Ghazaleh *et al.* 1989).

Basophils are the circulating counterparts of tissue mast cells possessing high affinity receptors and their possible involvement has been overlooked in many studies. Peripheral basophilia and infiltration of the site of infection with basophils accompanies mast cell proliferation and the expulsion of *T. colubriformis* by guinea pigs (Rothwell, 1975). However, as pointed out by Rothwell (1989), there are no published quantitative studies in other species from which conclusions can be drawn. Nevertheless, basophils are an important source of mediators and may well be involved in the expulsion process.

1.7.6 Monocytes/Macrophages

Monocytes originate in the bone marrow and enter the bloodstream as incompletely differentiated cells containing granular lysosomes, phagocytic vacuoles and cytoskeletal filaments. Once they settle in tissues, monocytes mature and become macrophages, both cell types being members of the mononuclear phagocyte system. As well as phagocytosis and the production of cytokines involved with innate immunity, mononuclear phagocytes play an important role in specific immune responses. Macrophages can act as antigen presenting cells, displaying foreign antigens on their surface in a form that can be recognized by antigen specific T lymphocytes resulting in a more efficient phagocytic, degradative and cytocidal

function. Macrophages also express surface receptors for antibodies and certain complement proteins and as a result can participate in the elimination of antibody coated (opsonized) antigens (Taverne, 1993). Very little is known about macrophages, monocytes and other bone marrow-derived cells, all of which, presumably, are of considerable importance in the immune response to parasitic nematodes (Miller, 1984).

1.7.7 Complement

Complement, so called because it complements and amplifies the action of antibodies, is the name given to a series of some 20 proteins forming an enzyme system (cascade) found in plasma. It can be activated either by the classical pathway, triggered by IgG or IgM binding to antigen, or by the alternative pathway which is triggered by the cell coat of some organisms. The most important and pivotal component is the C3 protein which when activated binds covalently to nearby membranes and is referred to as C3b. Each catalytic site thereby leads to the deposition of a large number of C3b molecules on the organism which initiate the assembly of further complement components, the end result being an ampipathic molecule that forms a transmembrane channel that can cause organisms to lyse. Neutrophils, macrophages and eosinophils have receptors for C3b which also facilitates their adherence to the organism. The activation of complement also releases a variety of small soluble peptides (C3a and C5a) that attract and activate neutrophils and stimulate mast cell degranulation (Taverne, 1993).

1.7.8 General

Phagocytes, complement, mast cells, goblet cells, cytokines and the leukocytes that mediate inflammation are all components of innate immunity, because they do not specifically recognize or distinguish between different foreign antigens. Thus, specific immunity serves to amplify and focus foreign antigens to this variety of effector mechanisms. In order to determine the mechanism of immune protection it is necessary to examine not only isolated parameters but also their interaction. McClure *et al.* (1992) attempted such an approach by examining a range of parasitological, morphological, biochemical and immunological parameters in

immune and susceptible sheep at intervals after challenge with *T. colubriformis*. The majority of challenge larvae were rejected within the first day and was attributed to local mast cell degranulation and increased concentrations of parasite specific IgG_1 and IgG_2 in the intestinal mucus. Rejection of the remaining worms occurred between days 3 and 14 following challenge and, as well as continued mast cell degranulation, was associated with increased parasite specific IgA and IgG_2 in the intestinal mucus, local T cell infiltration and epithelial necrosis and sloughing.

Despite increasingly sophisticated immunoparasitological research it is still not clear which components of the humoral and cellular immune responses are protective and how they interact to achieve effective immunoregulation. There may be a number of alternate pathways which vary for incoming larvae, established worms, different nematode species and different host species or they may all lead to a common final effector process (Rothwell, 1989). In immune sheep, expulsion is probably brought about by a combination of immunologically specific (e.g. T cells and antibodies) and non-specific components (e.g. mucus and inflammatory mediators) interacting in concert. This response is influenced by the age and genotype of the sheep as well as by its nutritional and hormonal status (Smith, 1988). Nematodes inhabit different niches in the gastrointestinal tract, including the lumen, the surface of the mucosa, the mucosal epithelium and the lamina propria, and different effector mechanisms are thought to act at each of these sites. In the gut lumen, antibodies, mucus and inflammatory mediators may exclude, damage and paralyze the worms. The mechanisms operating within the epithelium are not understood although inflammatory mediators may have some effect on nematode survival. Those worms penetrating into the tissues would be susceptible to direct helminthotoxic activity of inflammatory cells as well as the action of mediators (Miller, 1984).

1.8 Evasion of Immunity by Gastrointestinal Parasites

The ability of gastrointestinal nematodes to survive in their hosts reflects evolutionary adaptations that permit these organisms to evade and resist immune effector mechanisms (reviewed by Behnke, 1987). Immune responses in the intestine

are particularly complex and poorly understood, the complexity of these interactions reflecting the importance of distinguishing between pathogens and irrelevant immunogenic molecules in the intestinal contents. It is conceivable therefore that parasites have evolved mechanisms which interfere with the various immunoregulatory pathways present in the intestine (Behnke, 1987).

If the immune responses in the intestine operate in a very local manner, worms in low-level infections may simply avoid expulsion by moving on to a fresh location (Miller, 1984). Gastrointestinal nematodes, including *Ostertagia*, *Trichostrongylus* and *Nematodirus* species, seldom distribute themselves evenly along the intestine (Behnke, 1987). For example, *T. vitrinus*, which can survive in lambs for some 12 weeks post infection, accumulate in "finger print" lesions (Coop *et al.*, 1979). These are patches of the intestine which have undergone villous atrophy, below which the mucosa is deficient in globule leucocytes and mast cells, even though both cell types abound in the surrounding tissue (Jackson *et al.*, 1983; Angus & Coop, 1984). Survival of *Trichostrongylus* species therefore may be mediated through immunomodulatory factors (IMF) having a local effect which create foci of relative safety from the host responses. Characterization of IMFs should therefore enable ways of neutralizing their effects to be devised. For example, the synthesis of analogues with no immunodepressive activity but sufficient similarity to parasite IMF, could elicit cross reactive neutralizing antibody (Behnke, 1987).

1.9 Control of gastrointestinal nematodes

The main aim of all control measures is simply to limit host-parasite contact to levels which do not impair performance (Brunsdon, 1980). Presently, there are two key strategies for the control of gastrointestinal nematodes; namely grazing management and chemotherapy, the effectiveness of which are dependent upon detailed epidemiological knowledge (Pandey, 1995). Research into alternative means of control such as vaccine development, selective breeding and biological control will be discussed in section 1.10.

1.9.1 Grazing management

Grazing management is aimed at reducing the rate of infection from pastures by means of several practices which have been discussed by Morley & Donald (1980). These include changes in stocking rate, timing of reproductive events (parturition and weaning), pasture resting (including rotational grazing), alternate grazing using different host species (or older animals of the same species with acquired immunity) and the use of fodder crops, new sowings and aftermaths. Variations in the numbers of young and adult livestock and the choice of pasture species are also discussed in relation to the size of larval challenge met by grazing animals. The availability of land is one of the major constraints involved with grazing management and as a result many of the strategies are not applicable to intensive livestock production systems such as those practiced in Britain.

1.9.2 Chemotherapy

The earliest attempts to control the economic impact of gastrointestinal nematodes was the use of a variety of narrow spectrum drugs which were relatively inefficient and often dangerous. The discovery of the anthelmintic properties of phenothiazine was a major advance in helminth chemotherapy, but the first highly efficacious broad spectrum anthelmintic produced was thiabendazole (Brown, Matzuk, Ilves, Peterson, Harris, Sarett, Egerton, Yakstis, Campbell & Cuckler, 1961). Since its introduction 35 years ago, benzimidazoles and pro-benzimidazoles with improved efficacy and extended spectra of activity have been developed (McKellar & Scott, 1990). The success of thiabendazole stimulated considerable research into anthelmintic discovery which has led to the availability of three classes of broad spectrum drugs. These are the benzimidazoles, the imidazothiazoles /tetrahydropyrimidines and the avermectins/milbemycins, each having activity against most gastrointestinal nematodes, lung worms and some tissue nematodes. Other compounds in the organophosphorus, salicylanilide and substituted nitrophenols have only a limited application in the control of ovine nematodes in the UK.

Table 1.1 *Currently licensed anthelmintics for sheep in the UK*

Anthelmintic class	Chemical	Number of registered products
Benzimidazoles and probenzimidazoles	Netobimin	1
	Febantel	5
	Mebendazole	2
	Ricobendazole	3
	Oxfendazole	3
	Albendazole	4
	Fenbendazole	12
Imidazothiazoles / Tetrahydropyrimidines	Morantel	1
	Levamisole	24
Avermectins	Ivermectin	2
	Moxidectin	1

(Adapted from Compendium of Data Sheets for Veterinary Products (1995-96))

Benzimidazole anthelmintics are generally believed to work primarily by interfering with the cytoskeletal protein, tubulin, thereby blocking its polymerization into microtubules which are essential for the normal functioning of all eukaryotic cells (Lacey, 1988 and 1990). The selectivity of these drugs is explained by their higher affinity for parasite tubulin compared to mammalian tubulin and is also reflected in a considerably slower rate of benzimidazole dissociation. The metabolic processes within parasites may also be disrupted by the activity of benzimidazole anthelmintics (McKellar & Scott, 1990).

The imidazothiazole/tetrahydropyrimidine class of anthelmintics is represented by levamisole, morantel and pyrantel, all of which act as cholinergic agonists, causing an outflow of Na^+ ions from depolarised nematode muscle bag membranes, resulting in spastic paralysis (Coles, East & Jenkins, 1975; Harrow & Gration, 1985).

Early reports suggested that the mechanism of action of the avermectin class of anthelmintics, which includes ivermectin, work by associating with γ-aminobutyric acid (GABA) receptors, thereby irreversibly opening chloride channels of nematode neuromuscular membranes, resulting in worm paralysis (Barragry, 1984). However, recent evidence (reviewed by Geary, Klein, Vanover, Bowman & Thompson, 1992a) indicates that the chloride channels are glutamate-dependent and are not under the control of GABA. It has also been speculated that altered pharyngeal function may be the key effect of this anthelmintic since the pumping of this organ was inhibited more than muscular motility by ivermectin in *H. contortus* (Geary, Sims, Thomas, Vanover, Davis, Winterrowd, Klein, Ho & Thompson, 1993).

1.10 Alternative means of control

The problem of anthelmintic resistance is increasing so much to the extent that livestock producers in some regions of South Africa are being forced out of business due to the failure of available anthelmintics to control nematode disease in their animals (van Wyk, Malan, Gerber & Alves, 1989). The interest in alternative control strategies for helminth diseases has not only been spurred on by the widespread emergence of anthelmintic resistance but also due to public demands in relation to human health and attitudes from an environmental standpoint (Waller, 1993b).

1.10.1 Vaccines

In comparison with viral and bacterial infections the success in developing vaccines against helminths has been limited. Only one vaccine, against the lung-worm *Dictyocaulus viviparus* in cattle is commercially available (Clegg & Smith, 1978). Most lambs aged 6 months or less which have been immunized with either infective *H. contortus* or irradiated *H. contortus* or *T. colubriformis* are not immune to homologous challenge with normal larvae, whereas the same immunizing procedure produces a consistently high degree of protection in older sheep (Manton *et al.*, 1962; Urquhart, Jarrett, Jennings, MacIntyre & Mulligan, 1966; Dineen *et al.*, 1978). The reason for this age-related unresponsiveness is unknown and difficult to understand since lambs are capable of mounting protective immune responses against

a wide range of bacterial and viral infections (Soulsby, 1985). Strategies for vaccine development fall into two broad categories; excretory/secretory antigens and 'hidden' antigens (Newton, 1995).

1.10.1.1 Excretory/secretory antigens (ES products)

Besides oral and anal openings, nematodes have prominent secretory glands, excretory pores and genital openings from which a wide range of substances emanate, including enzymes and metabolites, commonly known as excretory/secretory antigens (ES products). The function of many ES products in terms of parasite survival are unknown but are thought to play a role in the maintenance between parasite and host (Miller, 1984; Knox, 1994). Rothwell & Love (1974) found that vaccination of guinea pigs with ES products from *T.colubriformis* L_4 was highly effective against further infection and consequently ES products are of particular interest as reagents for vaccination. One group of ES products which have received particular attention are the acetylcholinesterases (AChE) which are secreted in comparatively large amounts *in vitro* by many nematode species (Ogilvie, Rothwell, Bremner, Schnitzerling, Nolan & Keith, 1973; Douch, Harrison, Buchanan & Greer, 1988). AChE is thought to act as a biochemical holdfast for worms by slowing intestinal movements in the vicinity (Ogilvie & Jones, 1971) or inhibiting mucus production by goblet cells (Philipp, 1984).

Many other ES proteins and glycoproteins are produced by nematodes besides AChE, but to date these are poorly characterized. Savin, Dopheide, Frenkel, Wagland, Grant & Ward (1990) isolated and characterized a 30-kilodalton glycoprotein secreted from *T. colubriformis* L_4 and adult worms whilst Dopheide, Tachedjian, Phillips, Frenkel, Wagland & Ward (1991) have isolated an 11-kilodalton protein from the ES fluid, both of which afforded good protection in guinea pigs towards subsequent infection. The biological importance of these ES products has yet to be determined but sequence homology of the 30-kilodalton product to vasolin, a porcine intestinal peptide, implicates possible alteration of gut physiology whilst the 11-kilodalton product displays similarity to a γ-interferon-induced protein and may play a role in immune modulation. Recent findings suggest

that AChE does not act as a biochemical holdfast, but rather a vasoactive intestinal polypeptide exists, similar to that isolated by Savin *et al.* (1990), and is responsible for the reduction in motility of the alimentary tract (Lee & Foster, 1995). The potentially crucial functions at the host/parasite interface of ES products therefore makes them attractive targets for chemotherapeutic or immunological intervention, however, this requires detailed characterization *in vivo* at the molecular level (Knox, 1994). A recent immunization trial, using lambs aged 5 months given surface antigens from infective larvae that were recognized by bile antibodies from immune sheep, resulted in 72 % reduction of *O.circumcincta* worm burdens compared to challenge controls (Wedrychowicz, Bairden, Dunlop, Holmes & Tait, 1995).

1.10.1.2 "Hidden" antigens

It is now apparent that vaccination does not have to concentrate exclusively on surface proteins or ES products. Antigens in the parasite gut can also elicit an immune response and vaccination to these 'hidden' antigens could see the animal mount a truly novel assault on the parasite. The potential value of vaccination with preparations containing proteins associated with the luminal surface of the parasite's gut was first demonstrated by Munn, Greenwood & Coadwell (1987) against experimental haemonchosis in sheep. Search for other potential protective antigens from this site, particularly those associated closely with the microvillar membrane, have established integral membrane glycoproteins, including H11 (Munn, Smith, Graham, Greenwood, Tavernor & Coetzee, 1993) and H-gal-GP (Smith, Smith & Murray, 1994) which have been shown to be 88 % and 72 % effective, respectively, in reducing challenge *H. contortus* worm burdens in sheep.

1.10.1.3 General

Recent advances in genetic engineering mean that it is now possible to isolate genes coding for antigenic proteins which can then be produced in quantity by bacterial or viral vectors. Tertiary structure may be important however and expression systems may therefore be crucial. Similarly, once a potential vaccine has been developed the route of administration, the timing and the adjuvant to be employed must be determined. Individual results of vaccination trials are very

variable, both in test and control animals (Dopheide *et al.*, 1991) and are typical of the genetic variability in outbred populations (Rothwell, Le Jambre, Adams & Love, 1978). Other problems that have to be tackled include the possibility that there may be immunologically non-responding sheep, and in the case of 'hidden' antigens absence of continued stimulation by the parasite may only result in short-term protection. Considerable research is still required therefore before the availability of vaccines becomes a commercial reality. Furthermore, multi-species vaccines are required since non-targeted species may then pose a threat to livestock.

1.10.2 Breeding for immunoresponsiveness

The heritable nature of 'resistance' to gastrointestinal nematodes was first demonstrated in studies with *H. contortus* (Warwick, Berry, Turk & Morgan, 1949) and subsequently with *Ostertagia* spp (Scrivner, 1967). Using a programme of assortative mating, Windon, Dineen & Kelly (1980) identified lambs, previously vaccinated with irradiated *T. colubriformis* larvae, with highly heritable immunity (responders) or susceptibility (non-responders) traits following challenge. Selective breeding for genetically resistant hosts is therefore seen as a means of reducing the reliance upon chemotherapy (reviewed by Gray, 1991; Windon, 1991). Research into selected lines of sheep is particularly advanced in Australia and New Zealand, with flocks selected for resistance to *H. contortus*, *T. colubriformis*, *O. circumcincta* or mixed infections (Barger, 1993b). Heritability of immunity, indicating the proportion of variation between individuals which is under genetic control, has been estimated to be in the order of 0.23-0.44. This is of a similar magnitude to production traits such as fleece weight or liveweight gain and some commercial breeders in Australia and New Zealand have now incorporated immunoresponsiveness into their stud operations (Barger, 1993b). Worries as to whether breeding programmes based on host immunity may also select for parasite genotypes capable of withstanding immunological attack seem unfounded. Woolaston, Elwin & Barger (1992) examined this possibility by serial passage of *H.contortus* and *T. colubriformis* in resistant or susceptible sheep and found that after 14 generations there were no indications that parasites passaged in one genotype were any more successful than those from another. Stear, Bairden, Bishop,

Buitkamp, Epplen, Gostomski, McKellar, Schwaiger & Wallace (1996) have recently identified an allele in Scottish Blackface sheep which is associated with reduced faecal egg counts. Breeding for immunoresponsiveness is inevitably a lengthy process but the identification of genetic markers provides a means of enhancing the rate of progress. Since immunity does not appear to be acquired by young lambs, selective breeding is unlikely to provide the sole means of controlling nematodoses but nonetheless can provide a useful adjunct to existing methods of control and reduce reliance upon chemotherapy.

1.10.3 Biological control

Potential biological control agents include bacteria, viruses and protozoa but the most promising group of all are fungi (Waller, 1991). The predacious fungus *Duddingtonia flagrans* has been shown to survive gut passage in calves and to grow and entrap trichostrongyle larvae in dung pats (Larsen, Wolstrup, Henriksen, Grønvold & Nansen, 1992). A subsequent field study in which fungal spores were strategically fed to calves resulted in significant reductions on pasture of *Ostertagia* and *Cooperia* larval populations and the prevention of clinical disease (Nansen, Larsen, Grønvold, Wolstrup, Zorn & Henriksen, 1995). Even more promising is the recently reported effects of the fungus *Harposporium anguillulae* (Charles, Roque & Santos, 1996). Sheep faecal cultures treated with spores from this species reduced the number of *H. contortus* infective larvae by over 99 % compared to control samples, making this an excellent candidate in the development of an alternative control strategy. Although this type of control requires knowledge about the complex natural biological systems and antagonists that may be involved, industry may become more interested, not only because of anthelmintic resistance and the increasing cost of drug development, but in particular the increasing public concern about chemical residues in animal products and the environment. Simply cleaning faeces from pasture may also be adopted as a method of biological control and has been shown to be effective in reducing the use of anthelmintics in horses (Herd, 1990).

1.10.4 Alternative compounds

There is a paucity of novel anthelmintic chemicals under investigation, however paraherquamide, an oxindole alkaloid metabolite of *Penicillium paraherquei*, with potential broad-spectrum use is currently under evaluation (Shoop, Haines, Eary & Michael, 1992). Interest in compounds other than conventional anthelmintics includes nematode growth regulators such as triflumuron which was shown to exert potent larvacidal effects upon free-living stages of *T. colubriformis* (Waller & Lacey, 1986). Unfortunately the effects were not as marked on closely related nematodes and further research into this class of compounds is required. There is also the possibility of developing novel anthelmintic compounds from bioactive natural products. For example, a variety of plants containing condensed tannins (Waghorn, Charleston, Niezen & Robertson, 1995), naturally occurring compounds from Eucalyptus trees (Bryant & Bennett-Jenkins, 1995) and herbal preparations (Sharma, 1994) have been shown to have detrimental effects on gastrointestinal nematodes. The use of herbage species such as chicory has also been examined as a means of reducing the effects of parasites (Scales, Knight & Saville, 1995). Conditions for larval development and migration were thought to be less suitable with chicory swards, although the possibility of a metabolic effect was not discounted. The implementation of mechanism-based screening for new anthelmintics rather than whole organism toxicity offers promise for antiparasitic drug discovery. Such screening must be based on a thorough understanding of the proteins or processes that offer the best chance for selective chemotherapeutic intervention, of which nematode neuropeptides look promising targets (Geary, Bowman, Friedman, Maule, Davis, Winterrowd, Klein & Thompson, 1995). Further research is required however, in order to increase our knowledge of the physiology of nematodes so that potential targets for novel anthelmintics may be identified.

1.11 Anthelmintic resistance

The term 'anthelmintic drug resistance' was defined by Prichard, Hall, Kelly, Martin & Donald (1980) as a greater frequency of individuals within a population able to tolerate doses of a compound than in a normal population of the same species

and is heritable. More recently Taylor & Hunt (1989) described resistance as the heritable ability of some nematode parasites to survive treatment with anthelmintic drugs at the recommended therapeutic dose levels. Resistance to different compounds within a class of anthelmintic is termed side resistance whereas resistance to compounds from more than one class is defined as multiple resistance (Prichard *et al.*, 1980). The first reports of anthelmintic resistance in nematodes of sheep were made in the USA by Drudge, Leland & Wyant (1957) who identified *H.contortus* resistant to phenothiazine. Workers from the same group also identified the first case of benzimidazole (Bz) resistance within three years of thiabendazole being released onto the market in the same species of nematode (Drudge, Szanto, Wyant & Elam, 1964). Further reports of resistance to Bz's in *H. contortus* followed in Australia (Smeal, Gough, Jackson & Hotson, 1968) and it soon became apparent, from increasing reports and more comprehensive surveys, that resistance to Bz's was becoming widespread in the *H. contortus* endemic region of Australia (Waller, 1986). Resistance to this class of broad spectrum anthelmintic was subsequently reported in field isolates of *T. colubriformis* (Hotson, Campell & Smeal, 1970) and *O. circumcincta* (Hall, Campbell & Carroll, 1979) and has since become a worldwide problem mainly involving *H. contortus*, *Ostertagia* spp and *Trichostrongylus* spp, with resistance in other genera having a more limited distribution.

Resistance to Bz's in populations of small strongyles in horses has been widely reported throughout the world (Prichard, 1990). Resistance to pyrantel is also believed to be present for small strongyles in the United States (Herd, 1992). Parasitic nematodes of cattle however, have evolved resistance much more slowly than related species of the same genera in sheep. The reason for this is unclear but it is possible that there are differences in the population dynamics of sheep and cattle parasites, or in the intensity of anthelmintic treatment between the two species (Barger, 1993b). Cattle are known to metabolise Bz anthelmintics more quickly than sheep (Prichard, Hennessy, Steel & Lacey, 1985) and it has been suggested that this may have reduced selection for resistant parasites in this species (Coles & Taylor, 1990). It is also possible that bovine dung-pats may provide a relatively larger refugia of susceptible infective larvae, thereby reducing the proportion of the population exposed to anthelmintic selection (Martin, 1990). Nevertheless, there

have been reports of Bz and levamisole/morantel resistance in *O. ostertagi* of cattle. Both of these drugs provide less than optimal protection against inhibited larval stages and it is possible that resistance has developed in what amounts to subtherapeutic dosing (Conder & Campbell, 1995). Populations of *T. axei* (Eagleson & Bowie, 1986) and *C. oncophora* (Jackson, Townsend, Pyke & Lance, 1987) resistant to the Bz's have also been identified in cattle. Although reports in other hosts are rare, resistance to levamisole (Lev) has been reported in *Oesophagostomum quadrispinulatum* and *O.dentatum* of pigs in Denmark (Bjørn, Roepstorff, Waller & Nansen, 1990).

Although Bz resistance predominates in small ruminants (Prichard *et al.*, 1980), there are also field reports of resistance against Lev and ivermectin (Ivm) classes. Levamisole resistant strains of *H. contortus*, *Trichostrongylus* spp and *Ostertagia* spp have been identified in Australia, New Zealand and South America (reviewed by Prichard, 1990). Ivermectin resistant *H. contortus* has been described in sheep in South Africa (van Wyk & Malan, 1988), South America (Echevaria & Trindade, 1989), Australia (Le Jambre, 1993) and goats in the USA (Craig & Miller, 1990). Ivermectin resistant *Ostertagia* spp have been described in goats in New Zealand (Badger & McKenna, 1990) and in Scotland (Jackson, Jackson & Coop, 1992a) and recently a field case has been described in sheep in Australia (Swan, Gardner, Besier & Wroth, 1994). Reports of multiple resistance to Bz's and Lev have been well documented, being particularly frequent in Australia (reviewed by Overend, Phillips, Poulton & Foster, 1994) but there are also increasing reports of multiple resistance involving Ivm in sheep (van Wyk & Malan, 1988) and goats (Watson & Hosking, 1990; Jackson *et al.*, 1992a). In South Africa (van Wyk, Malan, Gerber & Alves, 1987) and Australia (Rolfe, Boray, Fitzgibbon, Parsons, Kemsley & Sangster, 1990) it is also evident that the narrow-spectrum salicylanilides, used almost exclusively to control strains of *H. contortus* resistant to the broad-spectrum anthelmintics, are rapidly becoming less effective because of resistance development. Drudge, Lyons, Tolliver & Fallon (1990) suggested that phenothiazine may have a similar mode of action as the Bz's and its use may have preselected some small strongyle populations in horses for Bz resistance. However, it is unlikely that the mode of action of these two anthelmintic classes are directly related and it possible

that some degree of cross-resistance was present in the study of Drudge *et al.* (1990) (R. J. Martin, personal communication).

Comparison with previous surveys has provided evidence that the problem of resistance is on the increase, in terms of both the level of resistance and the number of cases reported: UK (Coles, Hong & Hunt, 1991), Australia (Love, Johns & Coverdale, 1992), New Zealand (McKenna, Badger, McKinley & Taylor, 1990), South Africa (van Wyk *et al.*, 1989) and the Netherlands (Borgsteede, Schavemaker, Van der Burg, Gaasenbeek & Pekelder, 1991). Anthelmintic resistance has been the subject of many reviews, the latest of which include Prichard (1990 and 1994), Waller (1994) and Conder & Cambell (1995). A recent survey of South American sheep flocks indicates that the problem of anthelmintic resistance is particularly widespread in this area (Waller, Echevarria, Eddi, Maciel, Nari & Hansen, 1996).

1.11.1 Anthelmintic resistance in Britain

Bz resistance involving *O. circumcincta* was first reported in Britain by Britt (1982) and subsequently by Cawthorne & Whitehead (1983). Evidence of Bz resistance involving *H. contortus* species was reported on farms in southern England by Cawthorne & Cheong (1984). The incidence of anthelmintic resistance in Scotland was reviewed by Scott, McKellar, Armour, Coop, Jackson & Mitchell (1990) and the need for large scale surveys was stressed. There is evidence in the UK of an increasing prevalence of anthelmintic resistant nematode species in both sheep (Mitchell, Jackson & Coop, 1991; Hong, Hunt, Harris, Coles, Grimshaw & McMullin, 1992) and goats (Jackson, Jackson, Little, Coop & Russel, 1992c; Hunt, Hong, Coles & Jones, 1994), reviewed by Hazelby, Probert & Rowlands (1994). Anthelmintic resistance has been reported in the UK against all three chemical classes of anthelmintics: Bz's (Cawthorne & Whitehead, 1983), Ivm (Jackson *et al.*, 1992a) and Lev (Hong, Hunt & Coles, 1994; Coles & Simkins, 1996).

1.11.2 Emergence of resistance

The rapid emergence of anthelmintic resistance in sheep and/or goats is primarily due to two important aspects of management; frequent dosing and the practice of running these animals together (Conder & Campbell, 1995). Suppressive

treatment regimes, in which animals are treated within or close to the prepatent period of the parasite population, inevitably favour the selection of a parasitic population (infrapopulation) which contains only resistant phenotypes (Jackson, 1993). The population dynamics of the free-living stages (suprapopulation) are another important factor that can influence the rate of development of resistance. For species such as *Teladorsagia*, the size of the suprapopulation on permanent pasture in the UK, with its prevailing moist and mild climate and the relatively long survival times for infective larvae, tends to follow a stable annual pattern. This stability may initially provide a reservoir of susceptibility but unfortunately the converse is also true and highly selected resistant populations may survive for some time on pasture (Jackson, 1993).

Differences in the metabolism of anthelmintics in sheep and goats are known to exist. Bogan, Benoit & Delatour (1987) compared the pharmacokinetics of oxfendazole metabolism and noted that the bioavailability of drug was lower in goats compared to sheep. In a comparative *in vitro* study of fenbendazole (Fbz) metabolism Short, Flory, Hsieh & Barker (1988) noted variation in the relative quantities of metabolites produced in hepatic microsomal fractions between goats and sheep. Hennessy, Sangster, Steel & Collins (1993a) monitored the kinetic disposition of oxfendazole and its metabolites in plasma and abomasal fluid and suggested that goats possessed a faster hepatic metabolism than sheep which results in a more rapid elimination of drug. Other studies have provided evidence that anthelmintics have not been applied at adequate dose rates in goats (McKenna 1984; Charles, Pompeu & Miranda, 1989) and it has been suggested that the correct dose rate for the Bz's in goats may be twice that of sheep (Sangster, Rickard, Hennessy, Steel & Collins, 1991b). Similarly, studies with levamisole (McKenna & Watson, 1987; Coles, Giordano & Tritschler, 1989a) have suggested that higher dosages are required for drug efficacy in goats than in sheep. Sub-optimal dosing may increase the frequency of resistant genes since it allows the survival of heterozygous resistant individuals and has been shown to rapidly select for anthelmintic resistance in *Ostertagia* spp (Martin, 1989). Since goats share the same genera of nematode infections with sheep, to which they appear more susceptible in terms of faecal egg counts and worm burdens, the consequences of grazing these animals together and

the spread of resistant parasites is clear (McKenna, 1984). However, it should be noted that simply increasing dosage rates does not offer a viable solution in delaying resistance. Not only does overdosing have obvious drawbacks in terms of increased tissue residues, cost and toxicity, but it has also shown to offer little benefit in terms of systemic availability and efficacy in goats (Sangster *et al.*, 1991b).

1.11.3 Mechanisms of anthelmintic resistance

It appears that resistance to the Bz's is due to a change in the β-tubulin isotype pattern, resulting in the loss of high affinity receptor binding sites, the biochemical mechanisms of which are discussed by Lacey & Gill (1994). Geary, Nulf, Favreau, Tang, Prichard, Hatzenbuhler, Shea, Alexander & Klein (1992b) cloned and sequenced β-tubulin genes from *H. contortus* and showed that there are at least two different isotypes of the gene. Kwa, Kooyman, Boersema & Roos (1993) have shown that the restriction fragment length polymorphism (RFLP) patterns for both isotype 1 and isotype 2 β-tubulin genes are reduced with Bz resistance, and in very resistant strains of *H. contortus* isotype 2 may be absent. A subsequent study, using an allele-specific polymerase chain reaction (PCR), has shown that all Bz resistant isolates of *H. contortus* and *T. colubriformis* examined have a change from phenylalanine to tyrosine at amino acid position 200 in isotype 1 β-tubulin (Kwa, Veenstra & Roos, 1994). The same mechanism for resistance to Bz drugs has also been shown in *T. circumcincta* (Elard, Comes & Humbert, 1996). Whether this amino acid substitution is the actual cause of resistance or simply a genetically linked factor is unclear. If the tyrosine amino acid is critical then it is unlikely that Bz resistance could be overcome by a change in drug chemistry since mammalian β-tubulins also have tyrosine at this position (Prichard, 1994). It is possible that there are at least two mechanisms that select for Bz resistance; selection of a pre-existing 9kb isotype1 β-tubulin with reduced affinity for Bz's and the elimination of isotype 2 genes from highly resistant individuals (Conder & Campbell, 1995). Recent work by Grant & Mascord (1996) supports the hypothesis that a single allele is selected for in the evolution of Bz resistance. Furthermore, these workers proposed that the loci involved with *T. colubriformis* and *H. contortus* can be regarded as homologues with closely related sequences.

Knowledge of the site of action and the molecular mechanisms of Lev/morantel and Ivm resistance are still sparse although, as with Bz's, it seems that multiple mechanisms may operate. Backcross studies conducted with a Lev resistant strain of *T. colubriformis* suggest that resistance may be due to a major sex-linked gene, single recessive gene or linked gene complex (Martin & McKenzie, 1990). Resistance appears to be the result of a reduction in the number of Lev receptors or in their affinity (Sangster, Davis & Collins, 1991a) which is consistent with studies in *Caenorhabditis elegans* (Lewis, Fleming, McLafferty, Murphy & Wu, 1987). Less is known about the genetics of Ivm resistance in parasitic nematodes, but if the mechanisms are similar to those in the free-living nematode *C. elegans* then resistance may also be under polygenic control (Le Jambre, 1990).

1.11.4 Delaying anthelmintic resistance

The first step in the control of resistance is to avoid incorrectly attributing treatment failure as a case of resistance. A variety of other infections (protozoal, bacterial and viral), disease syndromes (mineral deficiency) or even changes in diet can mimic clinical signs of nematode infections, unrelated to resistance. Underdosing animals, either as a result of under estimating liveweight or as a consequence of pharmacokinetic differences such as those that exist between sheep and goats, sets the stage for rapid development of resistance. Similarly, inadequate attention to calibration or condition of dosing equipment can also result in underdosing. Quarantining, monitoring and treating all replacement stock is also a critical management practice in preventing the introduction of resistant parasites (Conder & Campbell, 1995).

Control measures outlining the principles of delaying the spread of resistance have been published (Coles & Roush, 1992; Waller, 1993a; Barger, 1993b; Coles, Borgsteede & Geerts, 1994). Although reducing the number of anthelmintic treatments reduces the selection pressure for resistance it requires local epidemiological knowledge in order that strategic treatments can be given to optimize production. It is also generally supposed that the alternation of drug classes on an annual basis can slow down the development of resistance (Prichard *et al.*, 1980). It was initially hoped that a 'slow' rotation of drugs would result in reversion

to susceptibility during the intervening years but evidence to date suggests that this is not the case (Jackson, 1993).

Maximising treatment efficacy to ensure that heterozygous resistant individuals are removed is another method to delay the onset of resistance. Extending the period of drug administration by dividing the dose (Sangster *et al.*, 1991b) or reducing feed intake before treatment (Ali & Hennessy, 1993) have resulted in increased efficacies of Bz treatment against resistant species. Combinations of anthelmintics offer another means of maximizing efficacy and have been shown to be effective by Martin, Anderson & McKenzie (1990). These authors reported that resistance was not evident following six generations of parasite exposure to a Bz/Lev combination but it was apparent to the single drugs within three to four generations of application. Sustained- or pulse-release anthelmintics may also provide a useful tool in controlling resistant nematodes or preventing their selection if used in a judicious manner (Sangster, Rickard, Collins, Hennessy & Steel, 1992).

The need for integrated systems of control, incorporating anthelmintic treatment and grazing management has long been recognized (Thomas & Boag, 1973) but requires sound epidemiological knowledge. In Australia, Donald & Waller (1982) confirmed the benefits of an integrated system, in terms of liveweight gain and fleece production, when weaner sheep were grazed alternatively on pastures with cattle and treated three times per season. Similarly, Armour (1983) demonstrated that where clean grazing is available in Britain, dosing and moving at weaning can provide adequate control in lambs destined for market in autumn. However, dose and move strategies involving minimally contaminated pasture may increase the rate at which resistance is selected. Depending upon the efficacy and epidemiological timing of treatment, it is possible that the infrapopulation being transferred within the host to clean pasture may be composed entirely of resistant parasites which will then be transmitted to the aftermath (Taylor & Hunt, 1989). Consequently, the benefits gained by reducing the suprapopulation have to be weighed against the potential risk of increased selection for resistance (Jackson, 1993).

Mathematical models have been developed in order to evaluate factors contributing to resistance and/or strategies to limit its development. These include models for *O. circumcincta* (Gettinby, Soutar, Armour & Evans, 1989),

T.colubriformis (Barnes & Dobson, 1990) and *H. contortus* (Echevarria, Gettinby & Hazelwood, 1993). Optimal strategies to minimize production losses whilst prolonging the useful life of anthelmintics, by a combination of their judicious use with other management practices, can be predicted. However, although useful tools, these models should also be treated with caution (Conder & Campbell, 1995). They are dependent on accurate information regarding nematode biology and epidemiology, genetics of resistance, acquired immunity, management practices and environmental factors, all of which vary locally and over time and therefore require field confirmation.

Employing a narrow-spectrum drug along with a broad-spectrum anthelmintic has been shown to be effective in the control of some resistant species. For example, programs such as 'Wormkill' that were introduced in Australia to control resistant *H.contortus* with the strategic use of closantel has led to the eradication of this species in some areas (Barger, Hall & Dash, 1991). However, as mentioned earlier, resistance to closantel and other narrow-spectrum anthelmintics is emerging, jeopardizing this and similar control programs (van Wyk *et al.*, 1987; Rolfe *et al.*, 1990). An interesting strategy proposed by van Wyk & van Schalkwyk (1990) is the reintroduction of susceptible strains using donor sheep into areas/herds where resistance is a problem. However, this approach has only a limited application and is unlikely to be successful in temperate climates due to the low fecundity of the more important species such as *Teladorsagia* (Jackson, 1993). Selectively deworming animals from a population has also been proposed as a means of limiting selection for anthelmintic resistance (Duncan & Love, 1991).

1.12 Detection of anthelmintic resistance

As well as the introduction of guidelines for evaluating the efficacy of anthelmintics in ruminants (Powers, Wood, Eckert, Gibson & Smith, 1982) a range of *in vivo* and *in vitro* techniques have been developed for the detection of anthelmintic resistance (reviewed by Taylor & Hunt, 1989; Coles, 1990; Conder & Campbell, 1995). An updated second edition of the guidelines for evaluating the efficacy of anthelmintics in ruminants has recently been published (Wood, Amaral,

Bairden, Duncan, Kassai, Malone, Pankavich, Reinecke, Slocombe, Taylor & Vercruysse, 1995). The procedure of choice for field survey investigation is the faecal egg count reduction test (FECRT) which allows all anthelmintics to be tested at the same time and does not require sophisticated equipment or highly trained personnel (Presidente, 1985; Johansen, 1989). This test provides an estimate of anthelmintic efficacy by comparing worm egg counts from a group of animals before and after treatment but has several shortcomings. In an effort to overcome these limitations, WAAVP guidelines have been produced to standardize the procedures and encourage uniformity of data analysis (Coles, Bauer, Borgsteede, Geerts, Klei, Taylor & Waller, 1992). However, it should be noted that such tests may be host and drug specific since longer periods are required before post-treatment samples are taken in goats treated with Ivm (Jackson, 1993). Furthermore, resistance to minor species in a mixed population may be missed when vastly outnumbered (Martin, Anderson & Jarrett, 1989), and although larval culture can be used for identification, highly fecund species may mask species with low fecundity (West, Pomroy, Probert & Charleston, 1989). Species with a high mortality in culture may also be underestimated (Dobson, Barnes, Birclijin & Gill, 1992b). Caution must be taken when assessing efficacies with Lev since there is a potential for misinterpretation of FECRT results. Grimshaw, Hong & Hunt (1996) noted that faecal egg counts taken 11 or more days after treatment with Lev may allow time for development of immature stages to egg producing adults.

The controlled efficacy test (CET) is the most reliable test for assessing anthelmintic efficacy against mixed nematode infections and despite being costly in terms of animals and labour, is the definitive test for resistance (Johansen, 1989). Animals are slaughtered following treatment and their worm burdens collected and drug efficacies calculated by comparison to untreated controls. However, due to the economic considerations this assay is generally used for research purposes only. Small animal models offer a more cost-effective approach and the opportunity to monitor dose-responses by systematic slaughter would allow the precise identification of changes in susceptibility, thereby measuring resistance rather than efficacy (Conder & Campbell, 1995). This approach has been used to assess resistance with an isolate of *H. contortus* in jirds (Conder, Thompson & Johnson,

1993) the results of which were substantiated in the host species (Shoop, Haines, Michael & Eary, 1993). Guinea pigs (Kelly, Sangster, Porter, Martin & Gunawan, 1981) and more recently immunosuppressed rats (Gration, Bishop, Martin-Short & Herbert, 1992) have been used for efficacy trials with *T. colubriformis*. However, although these models offer attractive alternatives further research needs to be carried out before they can be adopted with confidence (Hazelby *et al.*, 1994).

A number of *in vitro* assays, based on anthelmintic effects on physiological processes, have been developed to support egg count reduction assays and are summarized in Table 1.2. These include the egg hatch assay as well as larval development, paralysis, motility and migration assays. In addition, a variety of biochemical based assays have been developed which include the colorimetric assay of acetylcholinesterases and tubulin binding/polymerisation assays. At present genetic assays are limited to the Bz's where resistant populations have been identified using cloned β-tubulin probes. With the exception of the egg hatch and larval development assays most other approaches are not suitable for field use because of technical requirements and are only applicable to certain anthelmintic classes.

Understanding the biochemistry and the molecular genetics of different types of anthelmintic resistance will lead to improvements in methods for the detection of resistance in parasites. Knowledge of the site of action and the mechanisms of ivermectin and levamisole/morantel is still sparse but DNA probes for Bz susceptible/resistant individual worms, larvae or eggs may soon be available. The ability to detect heterozygous resistant individuals would allow earlier identification of the problem and a more accurate assessment and likely effectiveness of different approaches to the prevention and reversion of anthelmintic resistance (Prichard, 1994).

Table 1.2 *In vitro assays available for the detection of resistance*

Assay	Spectrum	Author(s)
Physiological		
Egg hatch assay (1)	Bz	Le Jambre (1976)
Egg hatch assay (2)	Lev	Dobson *et al.* * (1986)
Larval paralysis	Ivm	Martin & Le Jambre (1979)
Larval motility (1)	Bz	Coles, Folz & Tritschler (1989)
Larval motility (2)	Ivm	Gill, Redwin, van Wyk & Lacey (1991)
Larval development (1)	Bz Lev Ivm	Taylor (1990)
Larval development (2)	Bz Lev Ivm	Lacey *et al.* † (1990)
Larval development (3)	Bz Lev Ivm	Hubert & Kerboeuf (1992)
Larval migration inhibition	Bz Lev Ivm Sal	Rothwell & Sangster (1993)
Biochemical		
Tubulin binding	Bz	Lacey & Snowden (1988)
Acetylcholinesterase	Bz	Sutherland, Lee & Lewis (1989)
Larval paralysis	Bz	Sutherland & Lee (1990)
Isoenzyme analysis	Ivm	Echevaria, Gennari & Tait (1992)
Genetic		
β-tubulin probe (1)	Bz	Roos *et al.* ‡ (1990)
β-tubulin probe (2)	Bz	Le Jambre (1990)

Bz-benzimidazoles; Lev-levamisole; Ivm-ivermectin; Sal-salicylanilides.

* Dobson, Donald, Waller & Snowdon (1986).

† Lacey, Redwin, Gill, Demargheriti & Waller (1990).

‡ Roos, Boersema, Borgsteede, Cornelissen, Taylor & Ruitenberg (1990).

1.13 Summary

Since it may be many years before alternative methods of controlling helminth infections become widely available, chemotherapy will necessarily remain as an important means of achieving control (Jackson, 1993). Consequently, the need to conserve currently available anthelmintics is of the utmost importance and even when vaccines become available, chemotherapy has the advantage of not only prophylaxis but also treatment, including that of immunocompromised animals

(Gutteridge, 1989). It is too late to prevent anthelmintic resistance for two of the three broad spectrum classes in most sheep and goat regions throughout the world where nematode parasitism is a problem. This essentially means that, for sheep and goat industries at least, strategies need to be devised and implemented to manage an existing resistance problem, rather than preventing its occurrence (Waller 1993a). The emergence of multiple resistant strains in Australia (Swan *et al.*, 1994) has focused attention upon the use of anthelmintics against which resistance has already been selected in the management of resistance. It has been suggested that effective control may be achieved on well-managed farms by treatment of animals with an anthelmintic of reduced efficacy (70-80 %) provided it is used infrequently (Barger, 1993b). The risks associated with the re-introduction of 'selected' drugs for therapeutic and prophylactic purposes are influenced largely by the pathogenicity and fecundity of the prevailing resistant species and the extent of any increase in resistance that results from further exposure. Given that the production and welfare of treated animals are not compromised then it may be possible, under carefully monitored circumstances, to re-introduce 'selected' drugs into slow chemoprophylactic rotations, particularly when resistance involves less pathogenic species with a low biotic potential such as *Teladorsagia* (Jackson, 1993). By doing so the life expectancy of the other drug classes may be extended and the risk of selecting multiple resistance, if it does not already exist, reduced. A change in attitude to nematode control is required, with the aim of ensuring that parasite populations do not exceed levels compatible with economic production rather than total eradication. However, if such control systems are to gain acceptance, large-scale field testing is essential to show that they work in practice (Waller, 1993b).

1.14 Aims of the study

Previous caprine studies at Moredun have demonstrated that adequate control may well be achieved with treatments which are not fully effective. No differences in performance were seen between groups of does and kids grazing on pastures contaminated with a benzimidazole resistant strain of *Teladorsagia*, that were treated

with fenbendazole, levamisole or ivermectin (F. Jackson, unpublished data). These findings formed the basis for a detailed field investigation to determine the extent to which drugs, against which resistance has been developed, can be used in the control of commonly occurring sheep parasites in the UK.

The main aims of the study were:

1. To determine what extent, if any, the use of a less effective drug compromised performance.
2. To measure any increase in the expression of resistance that results from continued use of an anthelmintic against which resistance has been selected.
3. To investigate ways of increasing the efficacy of resistance selected drugs.
4. To assess the use of arbitrarily primed polymerase chain reaction (AP-PCR) in the detection of resistant nematodes.

CHAPTER 2

General Materials and Methods

2.1 Animals

2.1.1 Ewes and lambs (grazing)

Greyface ewes (2 - 3 years old) grazing at Firth Mains farm were used with a Suffolk tup to provide lambs for the study. The ewes were brought indoors at the end of January each year and treated with anthelmintic immediately on housing (Oramec, MSD Agvet UK, 0.2 μg kg^{-1}). Terramycin antibiotic (oxytetracycline, Pfizer UK) was administered on day 100 of gestation as a prophylactic treatment against pneumonia and a clostridial vaccine (Covexin, Mallinckrodt Veterinary Ltd. UK) given three weeks before lambing. The ewes were not given a pre- or post-lambing treatment with anthelmintic since this has been shown to have little effect on lamb growth or ewe production when clean pastures cannot be provided (Donnelly, McKinney & Morley, 1972; Brunsdon, 1974). The ewes were drawn from a Greyface flock (n = 350) with different animals being used in each year of the study.

2.1.2 Worm free lambs

Greyface x Suffolk parasite naive lambs used in the study were born and reared indoors under conditions designed to preclude accidental infection.

2.2 Parasitological Techniques

2.2.1 Collection of faecal samples

Rectal faecal samples were taken into 230 x 300 mm, 30μm polythene bags (McKinnon and Hay, Edinburgh, UK) and transported to the laboratory. Samples were processed immediately or stored at 4 °C for no longer than 3 days before examination.

2.2.2 Scoring of sheep faeces samples

This procedure was carried out whilst weighing samples for faecal egg counts (2.2.3) in order to monitor consistency. Faecal scores were assigned ranging from 1 for a sample consisting almost wholly of blood and mucus to a score of 5 for a sample consisting of dry, hard pellets; the details of which are shown in Table 2.1.

Table 2.1 *Scoring system used to monitor faecal consistency*

Score	Condition
1	Sample consisting wholly of blood and mucus
2	Sample fluid consisting largely of faecal debris together with some mucus and or blood
2-3	Fluid sample containing no blood or mucus.
3	Sample soft, unformed consisting of faecal debris
3-4	Sample partially formed, obvious pellets within faecal mass.
4	Sample formed consisting of moist pellets.
5	Sample formed consisting of dry hard pellets.

2.2.3 Faecal Egg Count (Flotation Method)

Faecal egg counts were determined using a modification of the sensitive flotation technique described by Jackson (1974). The faecal samples were weighed and 10 ml of tap water added for every gramme of faeces. Each sample was then emulsified using a stomacher (Seward Medical Ltd., UK). A 10 ml sub-sample was then removed and passed through a tea strainer (1mm mesh) and washed through with an additional 5 ml of tap water. The retentate was squeezed and discarded. The filtrate obtained was poured into 15 ml polyallomer centrifuge tubes (16 x 102 mm, Beckman, USA) and centrifuged at 1000 rpm (228 g) for 2 minutes.

The supernatant was removed using a vacuum line and the remaining faecal pellet re-suspended with 12 ml saturated sodium chloride. The tubes were centrifuged again at the same speed and time as described earlier. Using artery - forceps, the tube was clamped just below the meniscus of the supernatant and the contents of the upper chamber was poured into a 4 ml disposable polystyrene cuvette (LIP Ltd., Shipley, UK). The upper chamber was rinsed with saturated NaCl and again poured into the cuvette which was filled totally and capped. The cuvette containing all the eggs in one gramme of faeces was placed under a compound microscope and examined under ×40 magnification.

The microscope contained a calibrated eyepiece graticule (Miller square, Graticules Ltd., UK) which was used to count eggs in samples with high egg concentrations. For samples with few eggs all the eggs in the cuvette were counted. Whenever samples contained high numbers of eggs the graticule was used. Either eggs that fell in the large square along two traverses of the cuvette were multiplied by 3 or those that fell in the small square multiplied by 9 in order to obtain the total number of eggs per gramme (EPG) of faeces.

Those eggs which could not be simply distinguished on their appearance or morphology (*Teladorsagia* spp, *Trichostrongylus* spp, *Haemonchus*, *Cooperia*, *Chabertia*, *Bunostomum* and *Oesophagostomum*) were referred to as trichostrongylid eggs. Species including *Nematodirus* spp, *Trichuris* and *Strongyloides* which are readily identifiable were noted separately. Other readily identifiable parasitic species such as *Moniezia*, lungworm and liverfluke were noted specifically. A score was also assigned for the density of coccidial oocysts present in each sample where:
1 = low, 2 = moderate and 3 = dense numbers of oocysts.

2.2.4 Specific Identification of Strongyle eggs

An egg dimension measurement technique (Christie & Jackson, 1982) was employed in order to improve the specificity of faecal egg counts. The method is a development of the approach made by Cunliffe and Crofton (1953) estimating proportions of species present using unique areas of size distribution plots. Eggs that had been counted using the flotation technique (2.2.3), either from an individual or a pooled sample from all of the animals within a group, were collected in a 38μm sieve and washed with water to remove the saturated salt. The eggs were then stored at 4°C in a test-tube with water and a couple of drops of iodine. Following at least one overnight sedimentation the supernatant was removed using a vacuum line and the eggs re-suspended in saturated salt and mounted in a cuvette and presented for measurement in the manner described in the egg count method. The dimensions (length and width) of 50 eggs were obtained using a strain gauge image shearing model (M14/2, Vickers Instruments, UK) which was fitted to a Vickers M14 microscope at a magnification of x60 and interfaced to a microcomputer. The file containing this data was saved and then analysed using an in-house programme

called 'Ovasort'. The programme works by identifying the numbers of eggs contained in specific unique areas of ellipses calculated for each species. These standard ellipses have been constructed from data from monospecifically infected sheep. From the numbers of eggs appearing in unique areas of the specific ellipses it is possible to estimate the overall prevalence of each species. The estimated percentage was noted for each genera/species and the data used in epidemiological studies or to increase the sensitivity of faecal egg count reduction tests. *O.circumcincta* and *T. colubriformis* cannot be separated on size alone and these two species form a composite group. Data for *C. oncophora* and *C. curticei* have recently been added and since the size distributions overlap with *T. axei* these three species also form a composite group. The distribution of 50 per cent ellipses for the common nematode species in Scotland are shown in Figure 2.1.

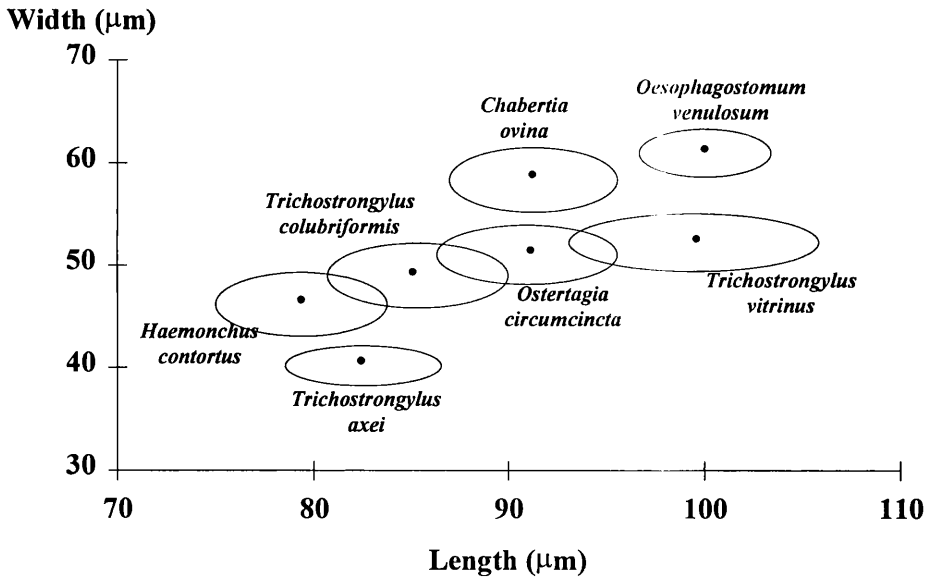

Figure 2.1 *The distribution of 50 per cent ellipses for the common nematode species in Scotland (adapted from Christie & Jackson, 1982)*

2.2.5 Larval Culture, Recovery, Storage and Infection Techniques

Faeces collected from either a donor animal or a pooled group sample were placed in culture trays (maximum depth 30 mm) and incubated inside a loosely sealed polythene bag (to enable some exchange of air) at 22 °C for ten days. Following incubation the sample was flooded with warm tap water (approx. 25 °C) and left to soak for 1-4 hours. Coarse faecal material was then separated from the fluid using a 1 mm sieve. Following a 2 hour sedimentation at 4 °C the fluid volume was reduced and the sample cleaned of fine faecal debris by Baermannization using high wet strength paper (Cleanaroll Ltd., UK). The sample was poured through the paper which was held on a filter holder (plastic tube 50 mm in diameter and 100 mm long) by a rubber band. The paper temporarily restrained the larvae which were sluggish following the cold incubation whilst releasing the water. The filter holder was then suspended in a jar containing warm tap water (approx. 25 °C) which allowed the larvae to migrate through the filter leaving the sediment behind. Baermannization was usually run overnight providing sufficient time for the larvae to migrate. The clean larval suspension (maximum density of 500,000 larvae per 250ml storage flask) was then labelled and stored at 4 °C until the larvae were required (within 4 weeks).

For infecting lambs, larval suspensions were made up to an appropriate volume in a volumetric flask and shaken to evenly disperse the L_3. From a 1 in 10 dilution (1ml + 9ml water) 5 samples of 100 µl were streaked on to a glass slide and the L_3 counted under a stereo microscope. The total numbers of larvae were calculated and the concentration of the suspension adjusted for the required larval dose. In most circumstances a 10 ml dose was conveniently delivered *per os* via a glass McCartney vial.

2.2.6 Parasite isolates

All of the parasites used in the study were isolated from field material and maintained at Moredun in worm-free donor lambs. The Bz-resistant *T. circumcincta* from Firth Mains was first isolated from lambs in 1983 and has been passaged twice per annum and is referred to as the Moredun ovine resistant isolate (MORI). A multiple resistant (Bz + Ivm) isolate of *T.circumcincta* was derived from goats

grazing a hill farm near the Scottish borders in 1991 and has been passaged twice per annum and is referred to as the Sourhope caprine resistant isolate (SCRI). The Bz-susceptible *T. circumcincta* was first isolated in 1979 from lambs grazing pastures at the Moredun Research Institute on which no anthelmintic treated animals had grazed previously. The Bz-susceptible *T. circumcincta* isolate has been passaged in worm-free donor lambs four times per annum and is referred to as the Moredun ovine susceptible isolate (MOSI). The Bz-susceptible isolate of *H. contortus* (HcS) has been maintained at Moredun in worm-free donor lambs since the late 1960's and has been passaged four times per annum. The multiple resistant (Bz + Ivm) white river isolate of *H.contortus* (HcR) from South Africa was provided by Glasgow University in 1992 and has been passaged twice per annum.

2.2.7 Pasture Larval Counts

Pastures were sampled using the method of Taylor (1939). Herbage samples were plucked from sites 10 metres apart along a series of 'V' shaped traverses up and down the pasture. A small sub-sample was removed and weighed, usually between 50-100 grammes, for dry matter estimation. The remaining sample was weighed, usually between 500 and 1000 grammes, and then soaked overnight in 40 litres of warm tap water (25°C) which contained a small amount of detergent (1 ml per 40L, Tween 20, Aldrich Chemical Co., UK).

Following soaking, the herbage was removed in small handfuls which were squeezed to remove as much water as possible. The sample was then sedimented in a cold room (4°C) overnight and its volume reduced to 2 litres. Following two further sedimentations to 500 ml initially the sample was reduced to 100 ml which was poured into 6 cellulose acetate tubes. The larvae were recovered from each tube by centrifugation using saturated NaCl in the manner described for the faecal egg count method. The recovered larvae were pooled, washed twice centrifugally using water and the sample volume reduced to 0.3 ml.

The numbers of infective larvae were determined at ×100 magnification in saturated potassium iodide in a cuvette. The counting of larvae was done in the same manner described for the faecal egg count method. The counts were converted to numbers of larvae per kg of wet herbage using the following formula:

No. L_3 kg^{-1} = No. L_3 counted x (1000 ÷ Wt. of sample in grammes)

The number of L_3 kg^{-1} wet herbage were then converted to numbers per kg dry herbage using the following formula:

No. L_3 kg^{-1} dry herbage = No. L_3 kg^{-1} wet herbage x $\frac{\text{Wt. of herbage sub-sample}}{\text{Dry Wt. of herbage sub-sample}}$

2.3 Worm Recovery Techniques

2.3.1 Removal of gastrointestinal tract for worm recovery.

Sheep were positioned on a cradle with all four limbs extended through the metal bars while an assistant restrained the sheep. A single captive bolt was delivered through the forehead; once stunned the sheep were exsanguinated and the spinal cord severed. Each sheep was restrained until any remaining nervous stimuli diminished and then turned onto its back and the abdomen opened along the ventral midline.

The entire gastrointestinal tract was removed following ligation of the omasal/abomasal, abomasal/ileal and jejunal/caecal junctions. If only the abomasum was required then only the first two ligatures were applied. The abomasum and small intestine were then detached into pre-labelled (sheep number, organ) 10 litre buckets prior to being opened to recover their contents.

The contents of the abomasum and first two thirds of the proximal small intestine were emptied into their respective buckets before each organ was split lengthwise using gut scissors and, along with the contents, soaked in approximately 4.5 litres of warm 0.85 % saline solution for 4-6 hours at 37 °C with regular agitation (Jackson, Jackson & Smith, 1984). Following incubation the superficial mucosa, together with any adhering worms, was removed by running abomasal folds or lengths of intestine through the thumb and index finger. The remaining tissue was then discarded before making the samples up to 5 litres with additional saline. Following thorough mixing of the bucket contents, two 250 ml aliquots were removed and pooled together to form a 10 % sub-sample which was fixed with 20 ml formalin.

2.3.2 Worm counts

2 % sub-samples (i.e. 100 ml) were stained with a few mls of helminthological iodine (Appendix). After a few minutes of staining the sample was rinsed over a 38μm sieve to remove excess iodine and fine debris and collected into a container. The stained sample was poured into 100 mm gridded contact plates (Sterilin, UK) and searched for worms at ×100 magnification using a stereo microscope. Worms were identified, staged and sexed according to the descriptions given by Denham (1969) *T.circumcincta*, Douvres (1957) *T. vitrinus*, and using keys supplied by the Ministry of Agriculture, Fisheries and Food (1986) and Thomas & Probert (1993). Any worms present were recovered and preserved in 2 % formalin in plastic bijou bottles.

2.4 Production Parameters

2.4.1 Weighing

Animals were weighed to the nearest half kilogram, at a similar time of day on each occasion, using a calibrated weigh crate. Following shearing in July of each year, individual ewe fleeces were rolled into a ball, placed into a bag and their weights recorded using a spring-suspension balance (Salter, UK).

2.4.2 Bleeding and Plasma samples

Animals were bled, at a similar time of day on each occasion, by jugular venepuncture into 10 ml vacutainer tubes (Becton Dickinson, UK) containing lithium heparin. Animals were bled at the relevant timed interval following treatment when pharmacokinetic analyses were required. The tubes were centrifuged at 3000 rpm (2060 g) for 20 minutes and the plasma carefully removed using a Pasteur pipette into two 2 ml aliquots which were frozen at $-20\ ^{\circ}C$ for subsequent analysis.

2.4.3 Condition scoring of ewes

The technique offers a means of subjectively assessing the degree of fatness of an animal based on a six point scale. The system was proposed by Russel, Doney &

Gunn (1969) and has since been adopted by the Meat and Livestock Commission. It relies on the principle that the loin is the last part of the body to put on fat and the first part to lose it. Readily identifiable anatomical characteristics were assessed by feeling on and around the backbone in the loin area immediately behind the last rib and above the kidneys. The first stage was an assessment of the degree of prominence (degree of sharpness or roundness) of the spinous processes of the lumbar vertebrae. The prominence and degree of fat cover over the transverse processes of the vertebrae were then assessed. Thirdly, the extent of the muscular and fatty tissues below the transverse processes was judged by the ease with which the fingers passed under the ends of these bones. Finally, the fullness of the eye muscle area, and its degree of fat cover, in the angle between the spinous and transverse processes, was estimated. Animals were then awarded a score according to the following scale in Table 2.2.

Table 2.2 *Condition scoring of ewes (Russel et al., 1969)*

Score*	Condition
0	Extremely emaciated and on the point of death. It is not possible to detect any muscular or fatty tissue between the skin and the bone
1	The spinous processes are felt to be prominent and sharp. The transverse processes are also sharp, the fingers pass easily under the ends and it is possible to feel between each process. The eye muscle areas are shallow with no fat cover.
2	The spinous processes still feel prominent, but smooth, and individual processes can be felt only as fine corrugations. The transverse processes are smooth and rounded, and it is possible to pass the fingers under the ends with a little pressure. The eye muscle areas are of moderate depth, but have little fat cover.
3	The spinous processes are detected only as small elevations; they are smooth and rounded, and individual bones can be felt only with pressure. The transverse processes are smooth and well covered, and firm pressure is required to feel over the ends. The eye muscle areas are full, and have a moderate degree of fat cover.
4	The spinous processes can just be detected, with pressure, as a hard line between the fat-covered muscle areas. The ends of the transverse processes cannot be felt. The eye muscle areas are full, and have a thick covering of fat.
5	The spinous processes cannot be detected even with firm pressure, and there is a depression between the layers of fat in the position where the spinous processes would normally be felt. The transverse processes cannot be detected. The eye muscle areas are very full with very thick fat cover. There may be large deposits of fat over the rump and tail.

* Intermediate scores were also assigned to animals, for example 2-3.

2.5 Biochemical Techniques

2.5.1 Plasma Pepsinogen Assay

(Refer to Appendix for reagent and buffer preparation)

Plasma pepsinogen concentrations were determined by a modification of the method of Mylrea & Hotson (1969) using bovine serum albumin as substrate and a glycine / hyrochloric acid buffer (pH 2.0). Pepsinogen activity was expressed as units (U) where 1 unit equals 1 μmole tyrosine released per minute per litre of plasma at 37°C. Stored plasma samples were removed from –20 °C and allowed to thaw out overnight in a fridge. Glycine buffer (0.1M pH 2) and 1% bovine serum albumin (BSA) were mixed together in a ratio of 4:1 before dispensing 0.5 ml of this mixture into each eppendorf, to which 0.1 ml of plasma was added (each sample was run in duplicate). These 'test' samples were mixed using a vortex mixer and incubated in a water bath at 37°C for 4 hours. To a further set of duplicate samples (unincubated controls) the 0.1 ml of plasma was immediately followed by the addition of 0.4 ml of 10 % Trichloroacetic acid (TCA), mixed and centrifuged for 3 minutes at 13,000 rpm (11,000 g). Following the incubation period 0.4 ml of 10 % TCA was added to the test samples, mixed and centrifuged as above.

A standard curve for tyrosine was constructed by making up 0μl, 100μl, 250μl, 500μl, 750μl and 1000μl volumes of working standard (1 in 10 dilution) to a total volume of 2 ml using 1 ml of 10 % TCA and distilled water. The appropriate number of rotors were loaded into a Monarch centrifugal semi-automated spectrophotometer (Instrumentation Laboratories, UK) with Folins and Ciocalteau's reagent (1 in 3 dilution) in one reagent boat and 1N NaOH in another. The standards and sample supernatants were then transferred into 0.2 ml sample cups (Instrumentation Laboratories, UK) and their absorbances read at 690 nm.

Plasma pepsinogen concentrations were then calculated using the mean absorbance of the standard curve having a tyrosine concentration of 0.0039 μM (for 75 μl volume used with the Monarch):

$$\frac{\text{Test - Control}}{\text{Mean Std - Blank}} \times \mu\text{M Tyrosine} \times \frac{1000}{\text{Vol plasma (ml)}} \times \frac{1}{\text{Time (mins)}}$$

$$\therefore \quad \frac{\text{Test - Control}}{\text{Mean Std - Blank}} \times 0.0039\ \mu\text{M} \times \frac{1000}{0.0075} \times \frac{1}{240}$$

$$\therefore \quad \frac{\text{Test - Control}}{\text{Mean Std - Blank}} \times 2.166 = \text{U (}\mu\text{M Tyrosine/min/litre at 37 °C)}$$

2.6 Molecular Techniques

2.6.1 Extraction of genomic DNA

(Refer to Appendix for reagent and buffer preparation)

2.6.1.1 Adult populations

Adult parasites were removed at necropsy from the abomasa of donor sheep and approximately 0.5 g (wet weight) of worms snap-frozen in liquid nitrogen, powdered and solubilized in 5 ml of Tris EDTA (TE) buffer using a pre-chilled (–70°C) pestle and mortar. The resulting solution was mixed and incubated at 55 °C for 30 minutes and then at 37 °C for one hour. DNase-free RNase (Pharmacia, UK) was added to a final concentration of 10 μg ml^{-1} and the solution incubated at 37 °C for a further 15 minutes. An equal volume of phenol/chloroform was added before the solution was mixed with a vortex. The aqueous phase was removed following centrifugation at 10,000 rpm (12,096 g) for 15 minutes at 4 °C and further extracted using an equal volume of chloroform/isoamyl alcohol (49 : 1). DNA was precipitated by the addition of a 0.1 volume of 3M sodium acetate (pH 5.2) and 2 volumes of ethanol before incubating at –20 °C for 2 hours. Following centrifugation at 10,000 rpm (12,096 g) for 15 minutes at 4 °C, the resulting DNA pellet was re-suspended in 1ml of TE buffer, sub-aliquoted and stored at –20 °C.

2.6.1.2 Individual larvae

Larvae were recovered from faecal cultures of donor lambs infected with different isolates of *T. circumcincta*; Bz-susceptible (MOSI), Bz-resistant (MORI) or

multiple (Bz + Ivm) resistant (SCRI) described in chapter 2.2.6. Larval suspensions were washed centrifugally in 1.5 ml eppendorfs by pelleting them three times and re-suspending them in 1 ml of sterile distilled water each time using a benchtop microfuge (13,000 rpm (11,000g) for 20 seconds). The larval suspension was poured out onto a petri dish and individuals were aliquoted into sterile 100 µl eppendorfs by taking up each larva into a 10 µl volume using an automatic pipette under the view of a stereo-microscope at x60 magnification. Individual larvae were then stored at –20°C until DNA extraction was required.

Individual larvae were thawed before adding 20 µl of lysis buffer (Appendix) to the eppendorf which was then incubated at 45 °C for 3 hours. An equal volume of TE saturated phenol was then added (30 µl) and mixed gently for 5 minutes. Following centrifugation at 13,000 rpm (11,000g) for 5 minutes the aqueous phase (approximately 20 µl) was removed and put into fresh sterile eppendorfs to which a 0.1 volume of 3M sodium acetate (pH 5.2) and 3 volumes of 100% ethanol were added before incubating at –20 °C for 60 minutes. Following centrifugation at 13,000 rpm (11,000g) for 15 minutes the ethanol was removed taking care not to disturb the invisible DNA pellet. The pellet was then washed with 70 % ethanol (60µl), re-centrifuged at 13,000 rpm (11,000g) for 15 minutes and the ethanol carefully removed once more before allowing the pellet to air dry. The extracted DNA was then re-suspended overnight at 4 °C in 20 µl of sterile distilled water, ready for AP-PCR.

2.6.2 Arbitrarily Primed Polymerase Chain Reaction (AP-PCR)

(Refer to Appendix for reagent and buffer preparation)

The following method acts as a general guideline and amendments to the protocol are indicated in the relevant chapter. All buffers, pipette tips, microfuge tubes and distilled water were autoclaved before use and gloves were always worn in order to prevent contamination. To a sterile 0.1 ml microfuge eppendorf tube, 2.5 µl PCR incubation buffer (Appendix), 2.5 µl Primer (10x, Oswell, Edinburgh, UK), 1.0µl target DNA (8 ng μl^{-1}), 1.0 µl $MgCl_2$ (0.1 M) and 12.0 µl of sterile water were added. A control was also set up containing all the components of the polymerase chain reaction except the template DNA (i.e. 13.0 µl of sterile water). The tubes

were then microfuged for a few seconds to deposit the reaction mixture in the base of the tube. The eppendorfs were then placed into a polystyrene stand and the reaction mixture heated in boiling water for 4 minutes to denature the DNA. The tubes were then cooled on ice for 5 minutes before the addition of 5 µl of a stock solution of deoxyribonucleoside triphosphates (dNTPs) and 1 µl of Taq DNA polymerase (0.5 Units μl^{-1}, Boehringer, UK). Total volume 25 µl. Finally, the tubes were microfuged for a few seconds before overlaying the reaction mixture with 25µl of mineral oil to prevent evaporation during the repeated cycles of heating and cooling. The PCR reaction was performed using a thermal cycler (Cetus, Perkin Elmer, USA) programmed with the following conditions: (30 cycles) denaturation (94 °C) for 1 minute, primer annealing (45 °C) for 1 minute, primer extension (72°C) for 3 minutes and a final extention (72 °C) for 8 minutes. The tubes were then stored at –20 °C for subsequent analysis.

2.6.3 Agarose Gel Electrophoresis.

(Refer to Appendix for reagent and buffer preparation)

The edges of a clean, dry plastic tray supplied with the electrophoresis apparatus (GN100, Pharmacia, UK) were sealed with autoclave tape so as to form a mould. Sufficient electrophoresis buffer (TAE 1x) from the same batch was used to fill the electrophoresis tank and to prepare the gel (approximately 600 ml per run). (It is important to use the same batch of buffer since small differences in ionic strength or pH can create fronts in the gel that can greatly affect the mobility of the DNA fragments). A 1.4 % gel was prepared using 1.4 g of agarose per 100 ml of buffer and heating the slurry in a microwave-oven until the agarose dissolved (approximately 2 minutes). The volume of the solution was then checked to ensure that it had not been decreased by evaporation and replenished with water if necessary. The solution was then cooled to about 60 °C before pouring it into the mould to which an appropriate comb had been inserted before leaving the gel to set.

If it was desired, 5 µl ethidium bromide (EtBr, 10 mg/ml) was added per 100ml agarose (final concentration of 0.5 µg/ml) and mixed thoroughly before pouring the gel. Although the electrophoretic mobility of linear double-stranded DNA is reduced by approximately 15 % in the presence of the dye, the ability to examine the gel

directly under UV illumination both during or at the end of a run is often an advantage. However, the gel may also be run in the absence of ethidium bromide and stained after electrophoresis is complete. After removing the comb 2µl of Bloo Joos was added to 5 µl of an AP-PCR sample before mixing and carefully pipetting into a well. A well containing 14 µl of 1 Kb DNA markers was also included in each run. Typical run conditions were 80V for 90 minutes. In cases were EtBr was not added, the gel was post-stained by immersing it in water containing the dye (0.5 µg/ml) and gently swirling for 30-45 minutes at room temperature. The gel was then photographed under UV illumination.

2.6.4 Polyacrylamide Gel Electrophoresis (PAGE)

(Refer to Appendix for reagent and buffer preparation)

The glass plates and spacers supplied with the electrophoresis apparatus (Mini PROTEAN II, Bio-Rad, UK) were prepared and placed into the clamp assembly stand supplied with the apparatus for pouring the gel. The gel was then prepared: A 7.5% gel was prepared using 6.5 ml distilled water, 1.0 ml Loening E buffer (10x), 2.5 ml acrylamide stock solution (30 %) and gently mixing the solution by swirling. The gel was then polymerised by the addition of: 50 µl N,N,N',N'-tetramethylethylenediamine (TEMED) and 80 µl of 10 % ammonium persulphate (APS) and poured immediately. The appropriate comb was added and the gel allowed to set.

After removing the comb 2 µl of Bloo Joos was added to 5 µl of an AP-PCR sample before mixing and carefully pipetting into a well. A well containing 7 µl of 1Kb DNA markers was also included in each run. Typical run conditions were 200V for 30 minutes. Gels were then silver-stained (see below).

2.6.5 Silver-staining of polyacrylamide gels

(Refer to Appendix for reagent and buffer preparation)

Following electrophoresis the glass plate sandwich was laid on a bench and one of the spacers gently twisted in order to lift off the upper plate smoothly. The gel and its attached plate was submerged in a plastic weighing boat containing enough FIX solution to cover the gel. Once in solution, the gel was removed by carefully teasing

it away from the glass plate and incubated in 100ml FIX for 10 minutes at room temperature with gentle shaking. The FIX was then carefully poured off and the gel incubated in 100ml STAIN for at least 15 minutes at room temperature with gentle shaking. The STAIN was then carefully poured off and the gel washed briefly with distilled water. The distilled water was carefully poured off and the gel incubated in 100ml DEVELOPER for about 10 minutes at room temperature with gentle shaking. When the desired contrast was obtained the DEVELOPER was carefully poured off and the gel washed twice in STOP (approximately 125ml STOP for each wash). Wearing gloves, the gel was carefully placed on a light box where it was examined and photographed if necessary.

2.7 High Performance Liquid Chromatography (HPLC)

(All HPLC analyses were carried out under good laboratory practice (GLP) in the department of veterinary pharmacology laboratory at Glasgow University Veterinary School. Refer to Appendix for preparation of standard compound solutions)

2.7.1 Preparation of samples

Stored plasma samples for a particular animal(s) were removed from –20°C along with blank ovine plasma (known to be free of drug) and allowed to thaw. Five spikes were set up in labelled ground glass tubes, each containing 0.5 ml blank plasma and 50 µl of either 0.5, 1.0, 2.5, 5.0, or 10.0 µg ml^{-1} fenbendazole mix (Appendix). A tube containing 0.5 ml blank plasma and 50 µl of methanol was also set up as a blank spike. 0.5 ml of plasma from each sample and 50 µl of methanol was pipetted into appropriately labelled ground glass tubes. Finally, 50 µl of albendazole (10 µg ml^{-1}) was added to every tube as an internal standard.

2.7.2 Extraction procedure

All manipulations involving chloroform were carried out in a fume cupboard (Holliday Fielding Hocking Ltd., UK). To each tube 100 µl of 0.1 M ammonium hydroxide solution and 0.4 g NaCl (using a calibrated scoop) was added and mixed

using a vortex mixer. Following the addition of 6 ml of chloroform (using a calibrated automatic dispenser) the tubes were sealed using ground glass stoppers and mixed for 10 minutes on a slow rotary mixer. The stoppers were then replaced by cling-film and the tubes centrifuged at 3500 rpm (2800 g) for 10 minutes. The plasma layer was carefully removed using a Pasteur pipette before transferring 4 ml of chloroform into an appropriately labelled glass drying tube using a bulb pipette. The tubes were then placed on a heated block and allowed to dry under high purity nitrogen at 50 °C. Once dry, the samples were reconstituted by the addition of 150µl of methanol, mixed using a vortex mixer and sonicated for 30 seconds. Following a second vortex 125 µl was transferred into appropriately labelled 300 µl glass vials which were then sealed with cling-film in preparation for analysis by HPLC.

2.7.3 HPLC run conditions

HPLC analyses were carried out on an integrated chromatographic system with automatic injection (Spectra Physics, UK). A C18 nucleoside column (15 cm x 4.6 mm id, Capital HPLC, Edinburgh, UK) was employed using the gradient solvent profile detailed in Table 2.3. The injection volume used was 20 µl, with a solvent flow rate of 1.5 ml min^{-1} and ultraviolet detector wavelength of 292 nm.

Table 2.3 *Solvent profile for HPLC analyses*

Time (mins)	% A	% B
0.0	35.0	65.0
5.0	55.0	45.0
10.0	55.0	45.0
10.2	35.0	65.0
13.0	35.0	65.0

A = Acetonitrile (0.5 % acetic acid)

B = H_2O (0.5 % acetic acid)

2.8 Tissue Histology

2.8.1 Enumeration of mast cells

Where mucosal mast cell counts were required a small section of an abomasal fold was taken and processed using the techniques described by Huntley *et al.* (1987). The processing of samples and enumeration of mast cells was kindly performed by John Huntley from the immunopathology department at Moredun. The tissue was fixed in 4 % paraformaldehyde in phosphate buffered saline pH 7.4 for 6 hours before being transferred into paraffin wax and 5μm sections cut. The sections were mounted, stained with Toluidine Blue pH 0.5 and the numbers of mucosal mast cells counted at ×250 magnification (Enerback, 1966). Five fields from each of three separate sections from each abomasal fold were counted and the results expressed as mast cells per 0.02 mm^2 of mucosa.

2.9 Statistical analyses

All statistical analyses were performed using Minitab statistical software. Arithmetic means are given in the text, tables and figures with ± 1 standard deviation (SD) or standard error of the mean (SEM) unless stated otherwise. Where data were skewed or had unequal variances they were transformed by $\log_{10}$ or $\log_{10}(x+1)$. The transformation for particular data sets and the tests used are explained in each chapter.

CHAPTER 3

Field study on the control of a fenbendazole resistant isolate of *Teladorsagia* (*Ostertagia*) - Effects on production in lambs and ewes

3.1 Introduction

It is important to recognize that few pharmaceutical companies direct resources towards research and development of anthelmintics for the veterinary market and that currently available anthelmintics are precious resources. Escalating costs are caused by the growing sophistication and sensitivity of analytical methods for detecting drug residues, the requirement for wide ranging studies on non-target invertebrate and vertebrate species and long-term trials to determine likely teratogenic or mutagenic effects (Waller, 1993b). It has been estimated that it costs up to $ 230 million to develop a novel anthelmintic from discovery to release onto the veterinary market (McKellar, 1994). Since biological diversity dictates that resistance is an inevitable occurrence it is not surprising therefore that very few chemicals warrant the investment to be taken from discovery through to marketing (D. R. Hennessy, personal communication). However since it may be many years before alternative methods of controlling helminth infections become widely available, chemotherapy will necessarily remain as an important means of achieving control (Jackson, 1993).

As discussed in the general introduction the emergence of multiple anthelmintic resistant nematode species in New Zealand (McKenna *et al.*, 1990) and Australia (Overend *et al.*, 1994) has focused attention upon the use of anthelmintics against which resistance has already been selected in the management of PGE. Given that the production and welfare of treated animals are not compromised then it may be possible, under carefully monitored circumstances, to re-introduce 'selected' drugs into slow chemoprophylactic rotations, particularly when resistance involves less pathogenic species with a low biotic potential such as *Teladorsagia* (Jackson, 1993). However, if such control systems are to gain acceptance, large-scale field testing is essential to show that they work in practice (Waller, 1993b).

Previous caprine studies at Moredun have demonstrated that adequate control of nematodes may well be achieved with treatments which are not 100 % effective. No differences in performance were seen between groups of does and kids grazing on pastures contaminated with a Bz resistant isolate of *Teladorsagia*, that were treated with fenbendazole (Fbz), levamisole (Lev) or ivermectin (Ivm) (F. Jackson, unpublished data). These findings formed the basis for this detailed field investigation to determine the extent to which drugs, against which resistance has

been selected, can be used in the control of commonly occurring sheep parasites in the UK.

Increasing attention has also focused on the use of combinations of Bz and Lev drugs in the control and prevention of anthelmintic resistance. The principal theory behind the use of combination drenches is largely based on the proposition that dual resistance is likely to be comparatively rare and that worms surviving one of these drugs will be removed by the other (McKenna, Allan & Taylor, 1996). Modelling studies have also suggested that the use of Bz-Lev combinations may well be a more effective means of delaying resistance than administering the same drugs in an annual alternation (Smith, 1990; Barnes, Dobson & Barger 1995). However, although Bz-Lev combinations offer greater potential as a means of slowing down the development of resistance they may, nevertheless, also provide satisfactory control in at least some circumstances where resistance already exists (Anderson, Martin & Jarrett, 1988a; McKenna, 1990b). Another objective of this study therefore was to assess the effects of such a combination in terms of lamb performance and treatment efficacy.

The three year field study was conducted on five 0.9 hectare paddocks on Moredun Research Institute's farm at Firth Mains, 14 kilometres south east of Edinburgh, elevation approximately 230 m (Plate 3.1). The pastures, originally sown with a mixture containing 60 % perennial rye grass, 10 % timothy and cocksfoot and 5 % red and white clover, had been grazed with sheep for several years prior to the study and were known to be carrying *N. battus*, *T. vitrinus* and an isolate of *Teladorsagia* (*Ostertagia*) spp which was known, since 1983, to be resistant to drugs within the Bz class of anthelmintics (Coop, Jackson, Coles & Hong, 1993). The Bz resistant isolate of *T. circumcincta* was selected by dosing non-lactating ewes that were used as 'mowers' on clean grazing. Drugs from within the Bz class of anthelmintics were withdrawn from use on the farm following the discovery and characterization of the resistant isolate of *Teladorsagia*. Sheep were treated with either Lev or Ivm on an annual basis and in 1991 a controlled efficacy test showed that there was no reversion to Bz-susceptibility in the intervening years (Jackson, Coop & Jackson, 1993).

Plate 3.1 *The paddocks at Firth Mains*

3.2 Materials and methods

3.2.1 Experimental Design

The paddocks were randomly assigned to their drug treatments at the start of the study in April 1993. Table 3.1 contains details of the stock on each paddock and their allocated drug treatments during 1993, 1994 and 1995. Details of the twin bearing Greyface × Suffolk ewes used in the study are described in chapter 2.1. Lambing took place indoors over a two week period during the middle of March, prior to the ewes being turned out to pasture in the second (1995) or third week (1993 and 1994) of April each year. Following shearing in July of each year, ewes were sprayed for blowfly/headfly (Vector, 1.25 % w/v high cis-cypermethrin, Young's Animal Health, UK). Ewes and their twin-lambs were randomly allocated to each paddock as lambing progressed to ensure each paddock contained lambs of a comparable age. The lambs were individually numbered with coloured ear tags (Allflex Ltd., UK) prior to turnout. Lambs were treated at the manufacturer's

recommended dose rate (MRD) for their allocated drug on the basis of liveweight outlined in Table 3.1. Lambs within the control group were treated only when required on welfare grounds using Ivm (Oramec, MSD Agvet UK). A non-suppressive control regime was adopted consisting of an initial anthelmintic treatment in mid to late May to control *Nematodirus* and a summer drench to control other gastrointestinal nematodes if necessary (Table 3.2). At weaning the ewes were removed from the paddocks and the lambs grazed until they were of marketable size. At the end of each season a number of lambs from each treatment group were used in a controlled efficacy test. During the course of the study some of the treatments were modified for groups of lambs in order to assess the benefits of altering bioavailability upon treatment efficacy (see chapter 4).

Table 3.1 *Treatment allocation to each paddock and stocking details*

Paddock No.	**1**	**2**	**3**	**4**	**5**
Anthelmintic	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
1993 Stock	12 Ewes 24 Lambs	12 Ewes 24 Lambs	12 Ewes 24 Lambs	12 Ewes 24 Lambs	12 Ewes 24 Lambs
1994 Stock	10 Ewes 20 Lambs	10 Ewes 20 Lambs	10 Ewes 20 Lambs	10 Ewes 20 Lambs	10 Ewes 20 Lambs
1995 Stock	10 Ewes 20 Lambs	10 Ewes 20 Lambs	10 Ewes 20 Lambs	10 Ewes 20 Lambs	10 Ewes 20 Lambs

Fbz (Fenbendazole) 5 mg kg^{-1} - Panacur 2.5 %, Hoechst UK.
Lev (Levamisole hydrochloride) 7.5 mg kg^{-1} - Levacide 7.5 %, Norbrook Animal Health UK.
Ivm (Ivermectin) 0.2 mg kg^{-1} - Oramec, MSD Agvet UK.

Table 3.2 *Treatment dates throughout the three year study*

Year	**Fbz/Lev, Lev, Fbz and Ivm Treatment Dates**	**Controls (Ivm)**
1993	May 19th	June 1st
1994	May 25th and August 17th	May 25th
1995	May 18th and July 12th	June 1st

Fbz (Fenbendazole) 5 mg kg^{-1} - Panacur 2.5 %, Hoechst UK.
Lev (Levamisole hydrochloride) 7.5 mg kg^{-1} - Levacide 7.5 %, Norbrook Animal Health UK.
Ivm (Ivermectin) 0.2 mg kg^{-1} - Oramec, MSD Agvet UK.

3.2.2 Meteorological data

Meteorological data was kindly supplied by Henry McGeechan from the Scottish Institute of Agricultural Engineering department of the Bush Estate, situated 3 miles West of Firth Mains, the nearest venue recording a full set of readings.

3.2.3 Parasitological Parameters

Ewe's were faecal sampled *per rectum* fortnightly and the lambs weekly in 1993. In 1994 and 1995 ewe's were sampled monthly and lambs fortnightly. Faecal consistencies were monitored as described in chapter 2.2.2. Faecal egg counts were determined using a flotation method described in chapter 2.2.3. Pasture larval counts were determined monthly throughout the grazing season using the methods described previously (chapter 2.2.7). The total trichostrongylid egg production for each group of lambs was calculated using the sum of the trichostrongylid faecal egg counts on each sampling date over the season.

3.2.4 Production Parameters

Ewe's were weighed and condition scored on a monthly basis and their individual fleece weights recorded following shearing in July of each year using the methods described in chapter 2.4. Lambs were weighed and bled fortnightly and plasma stored for subsequent pepsinogen analysis using the methods described in chapter 2.5.1.

3.2.5 Statistical analyses

Differences between treatment group faecal egg counts were determined by Log_{10} (x+1) transformation prior to analysis of variance (Minitab, version 10.0). Differences between faecal consistencies, ewe condition scores, ewe fleeceweights, ewe and lamb bodyweights and lamb pepsinogen values were determined by analysis of variance of untransformed data (Minitab, version 10.0).

3.3 Results

3.3.1 Meteorological data

Figures 3.1 to 3.5 show the respective monthly average rainfall, hours of sunshine, minimum grass temperature (measured 2 inches from the ground) and the minimum and maximum air temperatures over the three year study. A 36 year average (1955-90) for these parameters is also included in each of the graphs. 1994 and 1995 had particularly dry spells from April to June which is reflected in the amounts of daily rainfall and hours of sunshine. Differences between the average minimum daily grass temperatures were evident with 1994 having a cooler Spring and 1993 having a cooler late Summer/early Autumn than the other years. The average air temperatures were broadly similar over the three years.

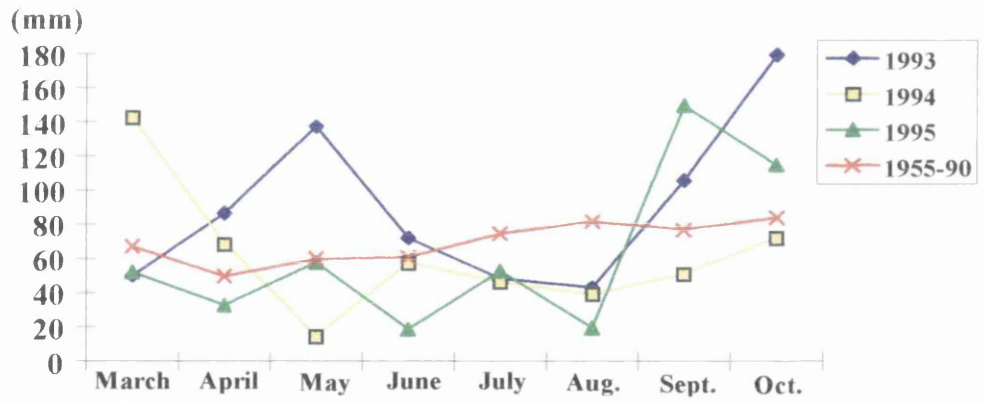

Figure 3.1 *Average monthly rainfall (mm) in each year of the study compared to a 36 year average (1955-90)*

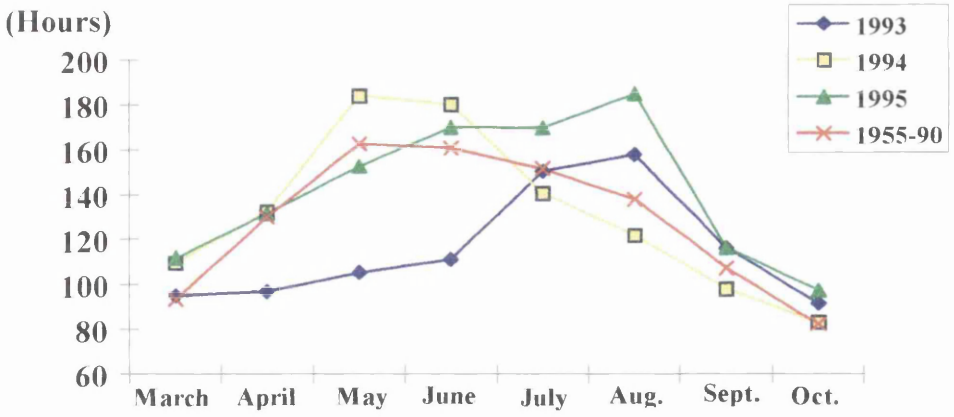

Figure 3.2 *Average monthly sunshine (hours) in each year of the study compared to a 36 year average (1955-90)*

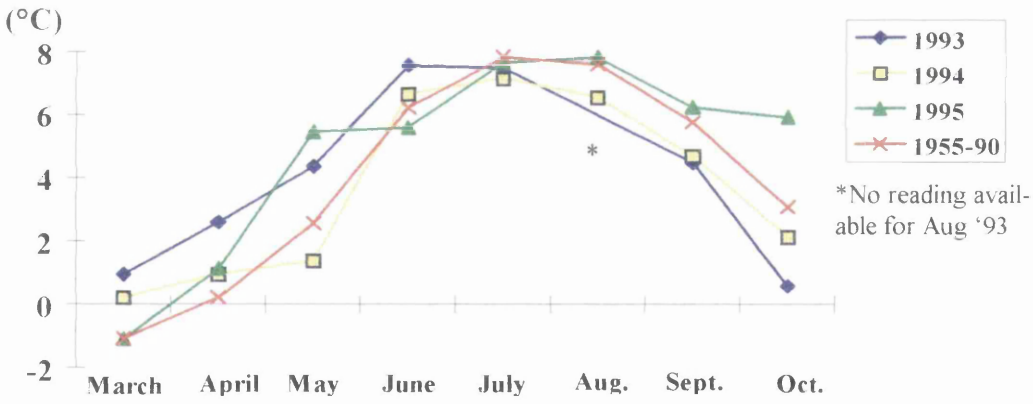

Figure 3.3 *Average minimum grass temperature (°C, measured 2 inches from the ground) in each year of the study compared to a 36 year average (1955-90)*

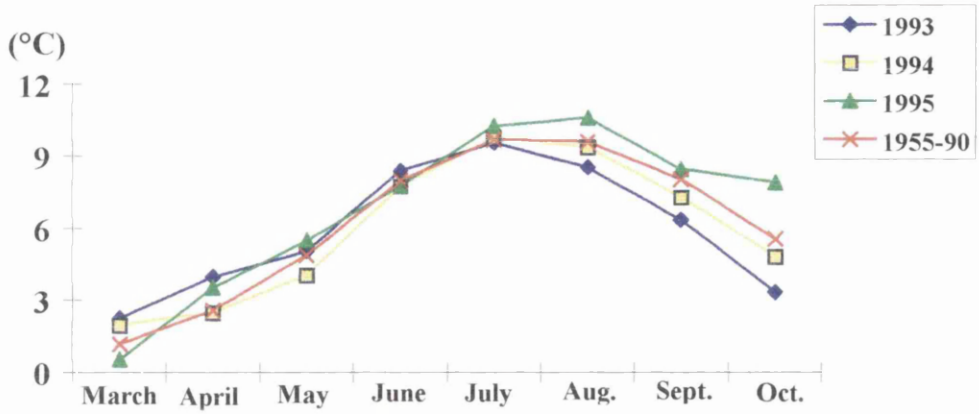

Figure 3.4 *Monthly average minimum daily air temperatures (°C) in each year of the study compared to a 36 year average (1955-90)*

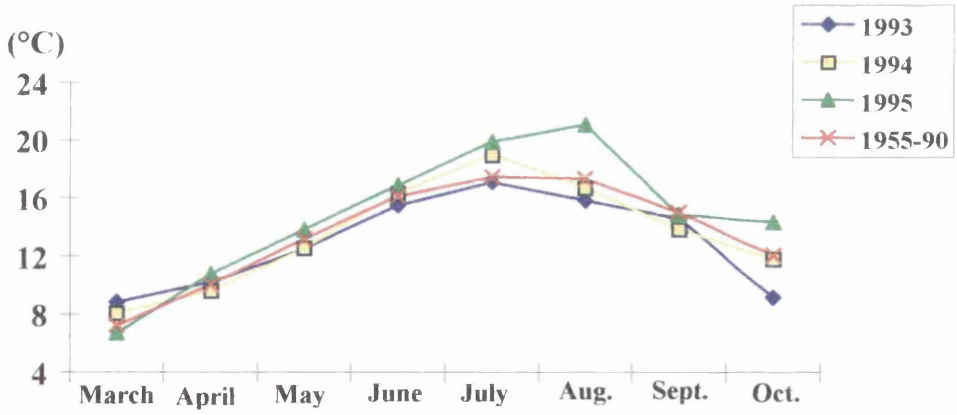

Figure 3.5 *Monthly average maximum daily air temperatures (°C) in each year of the study compared to a 36 year average (1955-90)*

3.3.2 Clinical Observations

1993 - Lambs were harbouring patent infections with both *N. battus* and trichostrongyles when first sampled on May 5th. Anthelmintic treatment was given on May 19th to control *N. battus* infection although trichostrongyle faecal egg counts were also reasonably high. The control group was treated with ivermectin 3 weeks later when the lambs became diarrhoeic. Following treatment there were no clinical signs of disease in any of the groups throughout the rest of the grazing season.

1994 - Lambs were again harbouring patent infections with both *N. battus* and trichostrongyles by the start of sampling on May 10th. Anthelmintic treatment was given to all groups, including the controls, on May 25th to treat both *N. battus* and trichostrongyle infection. Four lambs were removed from the study for various reasons: In the Ivm group one lamb (1161z) broke its hind leg; in the control group one lamb (1489z) died of pneumonia and in the Fbz/Lev group one lamb (1259z) gained access to another pasture and was withdrawn from the trial. In the Fbz group one lamb (1545z) was put down, histopathological examination of its kidneys showed lesions consistent with nephrosis. A second anthelmintic dose was given to each group, except the controls, on August 17th as a strategic treatment against trichostrongyles. Clinical signs of disease were not evident in any of the groups throughout the season.

1995 - Faecal egg counts for both *N. battus* and trichostrongyles were low at the start of sampling on May 5th indicating low levels of patent infection. The first anthelmintic treatment was administered on the 19th May when faecal scores were low and egg counts began to rise. The control group was treated with Ivm two weeks later to control *N. battus* and trichostrongyles. Three lambs were removed from the study for various reasons: In the Fbz/Lev group one lamb (1536a) died from chronic liver disease. In the control group one lamb (1426a) died of pneumonia and in the Fbz group one lamb (1421a) was diagnosed as having chronic liver disease and was withdrawn from the trial. A ewe from the Fbz group (1629x) was taken ill and removed from the study on the 28th June. Her lambs (1448a and 1449a) were weaned at this time and left on the paddock. A second anthelmintic dose was given to each group, except the controls, on the 12th July as a strategic treatment against

trichostrongyles. Clinical signs of disease were not evident in any of the groups throughout the season.

Faecal consistencies for the lambs over the three year study are shown in Figure 3.6. There were no statistical differences between any of the groups at any point throughout the study. Faecal consistencies were lowest soon after turnout in each year but increased following treatment and remained stable for the rest of the season. The density of coccidial oocysts present in the lamb faecal samples were also recorded and are summarised in Tables 3.3, 3.4 and 3.5. At the start of sampling in May 1993 and 1994 the numbers of oocysts remained low but in 1995 the density was notably higher. By the beginning of June in 1995 the density of oocysts had declined to a level similar to that seen in the previous two years.

Table 3.3 *Average density of coccidial oocysts during the early grazing season of 1993*

Group	5/5/93	11/5/93	19/5/93	26/5/93	1/6/93
Fbz/Lev	1.14	1.00	1.00	1.00	1.10
Lev	1.09	1.00	1.09	1.09	1.09
Fbz	1.00	1.14	1.00	1.00	1.00
Controls	1.00	1.25	1.04	1.00	1.00
Ivm	1.04	1.00	1.00	1.09	1.20
Average	**1.05**	**1.08**	**1.03**	**1.04**	**1.08**

1 = low, 2 = moderate, 3 = dense numbers of oocysts

Table 3.4 *Average density of coccidial oocysts during the early grazing season of 1994*

Group	10/5/94	25/5/94	3/6/94
Fbz/Lev	1.28	1.18	1.06
Lev	1.12	1.19	1.00
Fbz	1.00	1.05	1.16
Controls	1.26	1.00	1.00
Ivm	1.11	1.00	1.00
Average	**1.15**	**1.08**	**1.04**

1 = low, 2 = moderate, 3 = dense numbers of oocysts

Table 3.5 *Average density of coccidial oocysts during the early grazing season of 1995*

Group	4/5/95	18/5/95	1/6/95
Fbz/Lev	1.93	1.63	1.07
Lev	1.69	1.65	1.00
Fbz	1.60	1.29	1.05
Controls	1.50	1.72	1.00
Ivm	1.50	1.61	1.11
Average	**1.64**	**1.58**	**1.05**

1 = low, 2 = moderate, 3 = dense numbers of oocysts

3.3.3 Pasture larval counts

Figure 3.7 shows the *N. battus* pasture larval counts over the three year study. Pasture larval counts for this species were highest in May of each year but by June the numbers had rapidly declined. In September of 1994 there was a small increase in *N. battus* numbers on pasture which was even more evident in 1995. Figure 3.8 shows the trichostrongyle pasture larval counts over the three year study. The 1993 trichostrongyle pasture larval counts followed a characteristic biphasic pattern typically seen on 'dirty' pasture (Thomas & Boag, 1972). However, as a result of the particularly dry spells in May of 1994 and 1995, the size of the early season suprapopulation peak in late June of these years was limited thereby affecting the pattern of contamination throughout the rest of the season. Figures 3.9 and 3.10 show the average pasture larval counts for *N. battus* and trichostrongyles respectively and help to illustrate the epidemiological patterns seen in each year.

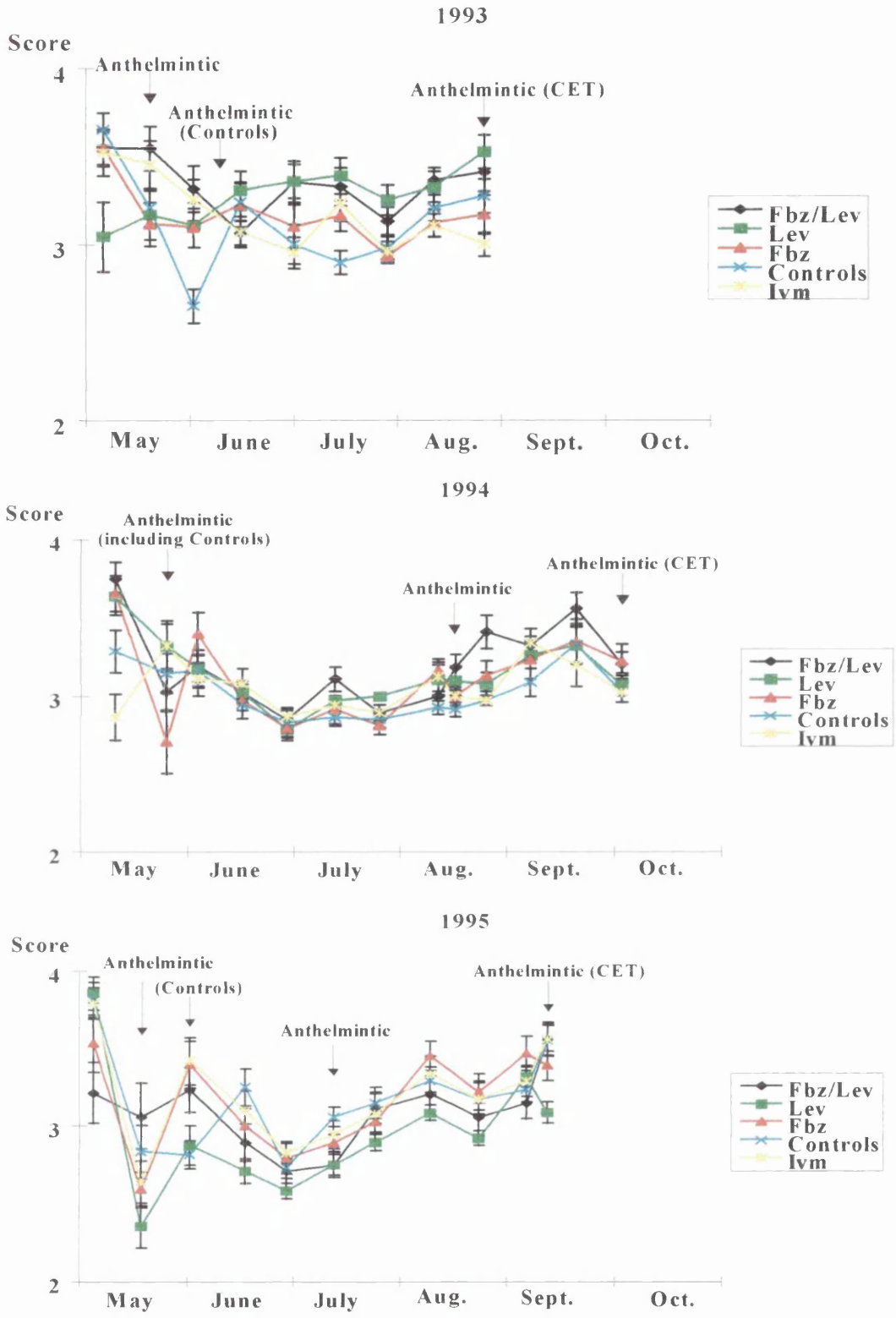

Figure 3.6 *Average faecal consistencies (±SEM) for the groups of lambs in each year of the study*

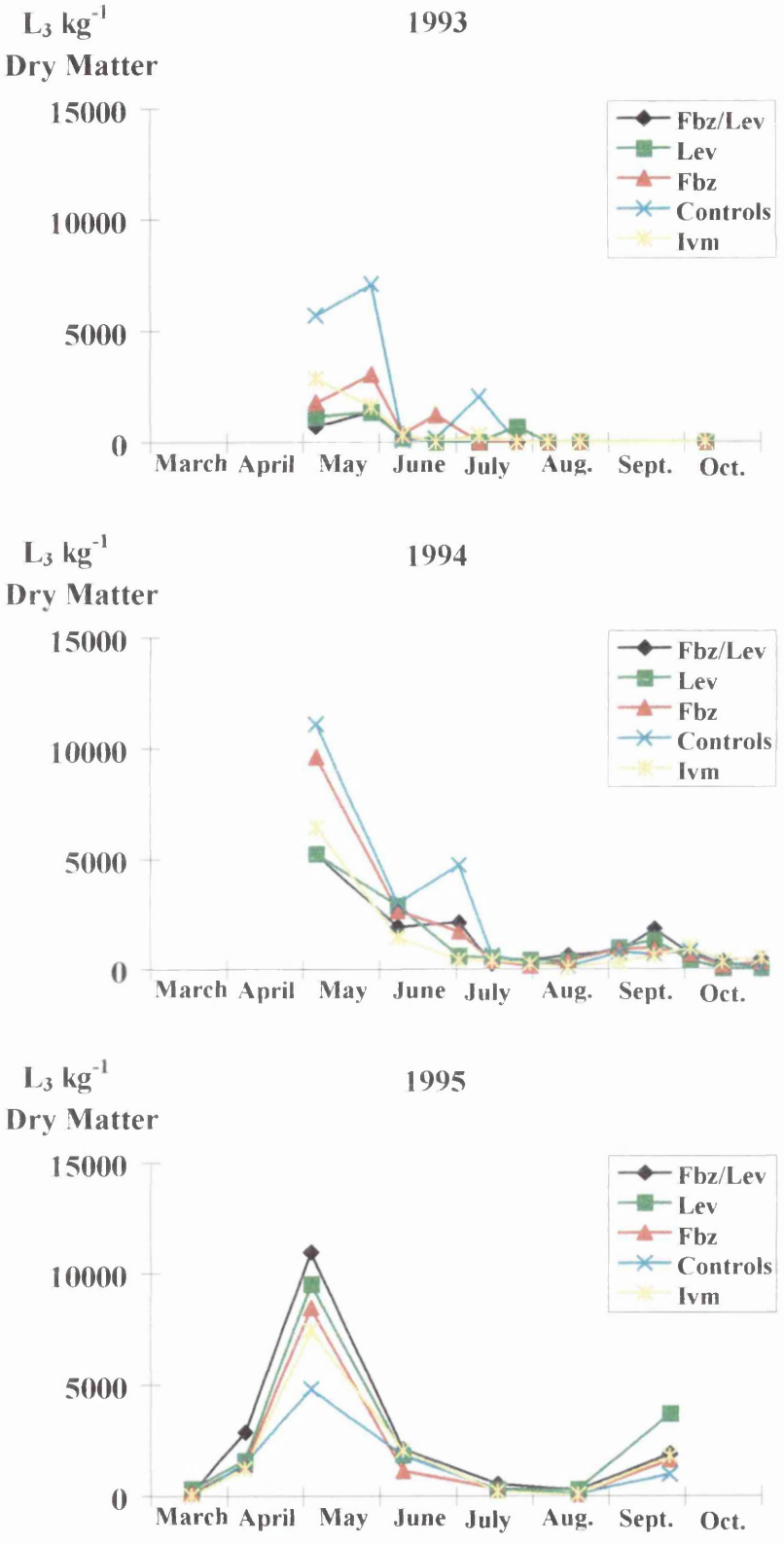

Figure 3.7 *Pasture larval counts for N. battus on paddocks 1 - 5 in each year of the study*

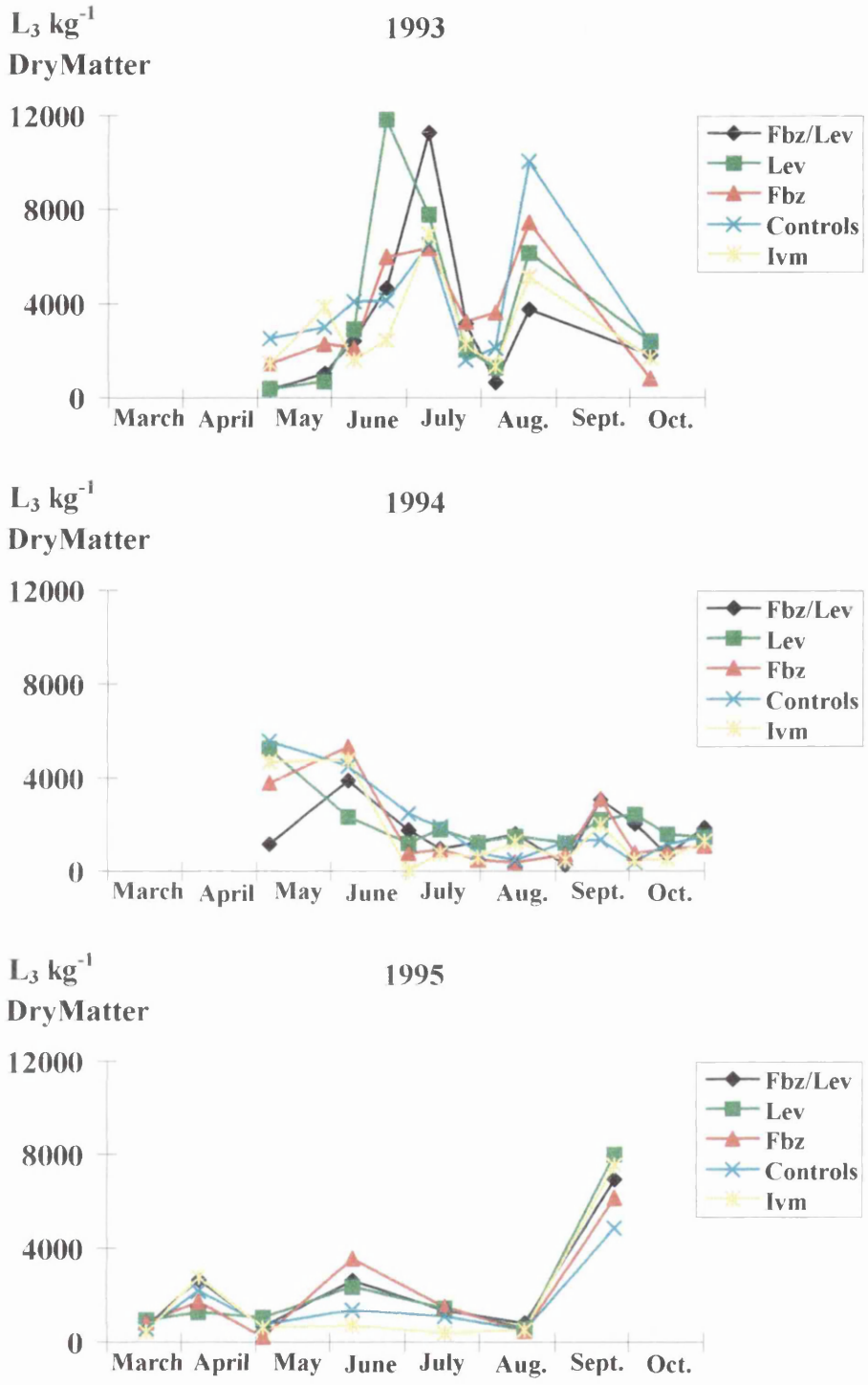

Figure 3.8 *Pasture larval counts for trichostrongylids on paddocks 1 - 5 in each year of the study*

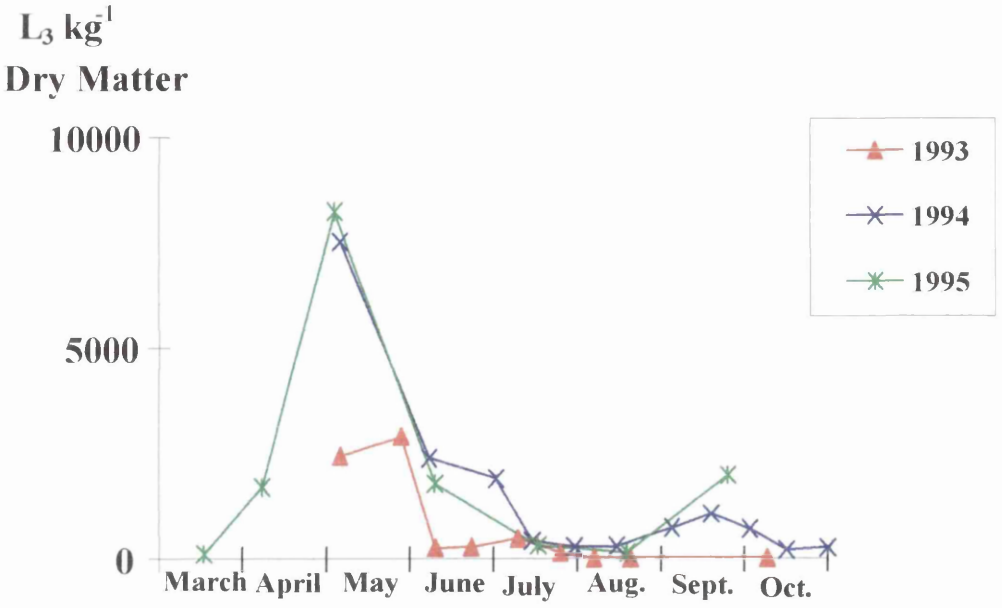

Figure 3.9 *Average pasture larval counts (paddocks 1-5) for N. battus in each year of the study*

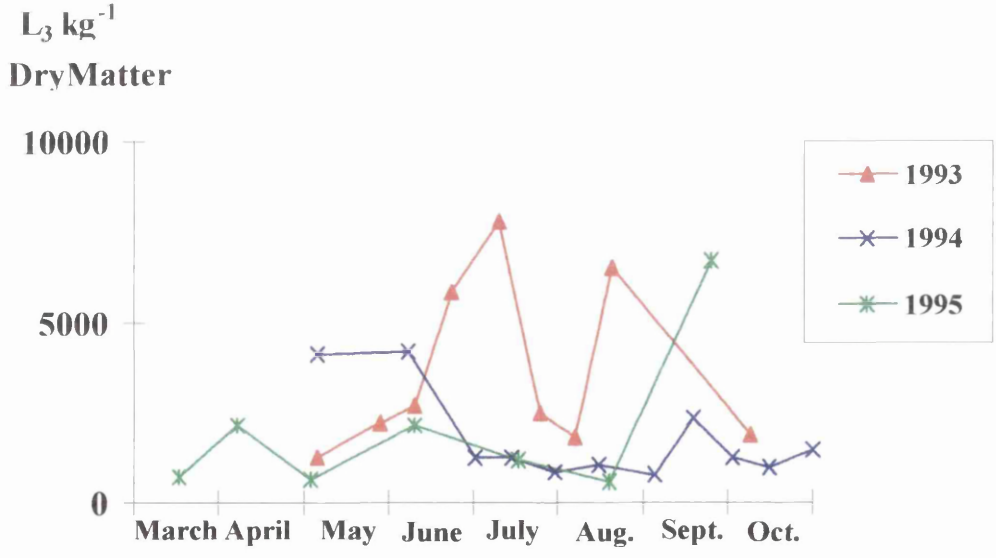

Figure 3.10 *Average pasture larval counts (paddocks 1-5) for trichostrongylids in each year of the study*

3.3.4 Ewe parasitological and production results

3.3.4.1 Ewe faecal egg counts

Figure 3.11 shows the trichostrongylid faecal egg counts (FECs) for the ewes over the three year study. The groups of ewes showed similar within-season patterns of egg count and no differences were observed in overall egg output. Throughout the study ewe *N. battus* faecal egg counts were either negative or very low with a maximum recorded individual egg count of 45 eggs per gramme (EPG). In the first two years of the study there was no evidence of a marked post-parturient rise in ewe trichostrongyle faecal egg count with an overall mean count of less than 100 EPG. In early May of 1995 there was a more pronounced post-parturient increase in trichostrongyle faecal egg count with overall mean of more than 230 EPG, although there was a rapid decline thereafter. Statistical comparison of faecal egg counts between groups over the three years are shown in Tables 3.6 - 3.8. It should be noted that on several occasions following herding a number of ewes were 'empty' and comparisons were made against different numbers of animals. There were very few differences in terms of faecal egg output between the groups over the three year study. Table 3.9 shows the % species of trichostrongylid eggs identified by dimension measurements. Since these animals had grazed the same pastures prior to lambing the egg counts for the groups of ewes were pooled which allowed positive identification of ewe faecal eggs when counts were declining. At the start of each season, egg output by the ewes was predominantly *Teladorsagia /T. colubriformis* with small numbers of *T. vitrinus*, the proportions of which remained remarkably constant over the three years. Although identification of ewe worm burdens was not performed, previous work at Firth Mains and results of the lamb CETs carried out at the end of each season identified only *T. circumcincta* and *T. vitrinus* worms. Though the dimension measurement technique cannot differentiate between *Teladorsagia / T. colubriformis* it seems unlikely that the latter species was present in this study.

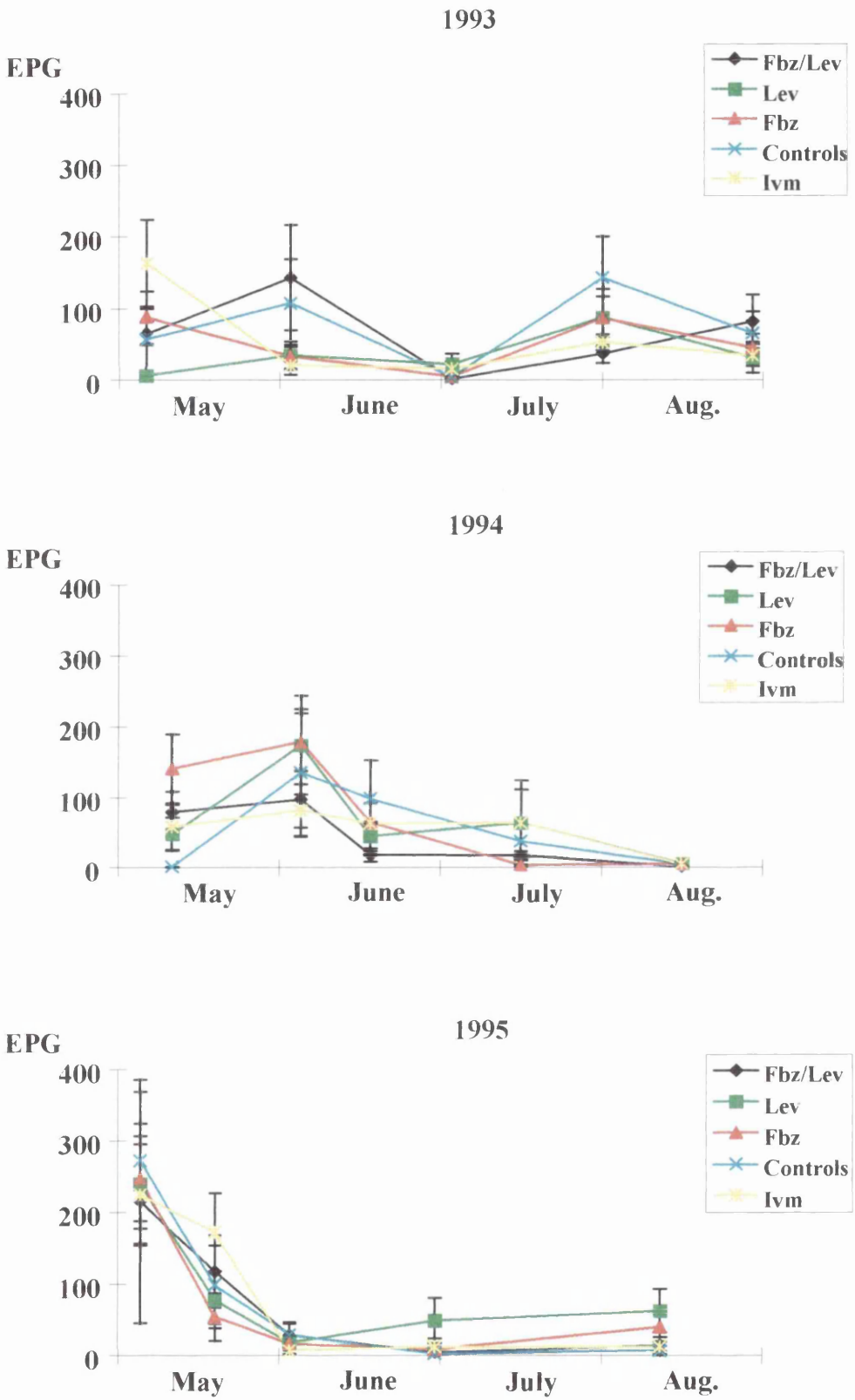

Figure 3.11 *Average trichostrongylid faecal egg counts (± SEM) for the groups of ewes over the three year study*

Table 3.6 *1993 statistical comparison of ewe trichostrongylid faecal egg counts*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
5/5	Lev**	-	-	-	Lev***
1/6	Lev* Fbz* Ivm**	-	-	Lev* Ivm*	-
1/7	-	-	-	-	-
29/7	-	-	-	-	-
26/8	-	-	-	-	-

- no significant differences
* significantly lower egg count than comparator $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3.7 *1994 statistical comparison of ewe trichostrongylid faecal egg counts*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
10/5 #	Controls**	Controls*	Controls*** Lev*	-	-
3/6	-	-	-	-	-
16/6	-	-	-	-	-
23/7	-	-	-	-	-
22/8	-	-	-	-	-

- no significant differences
* significantly lower egg count than comparator $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
# several ewes from the control were 'empty'

Table 3.8 *1995 statistical comparison of ewe trichostrongylid faecal egg counts*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
4/5	-	-	-	-	-
18/5	-	-	-	-	Fbz*
1/6	-	-	-	-	-
28/6	-	-	-	-	-
9/8	-	-	-	-	-

- no significant differences
* significantly lower egg count than comparator $P < 0.05$

Table 3.9 *% species of ewe trichostrongylid eggs identified by dimension measurements over the three year study*

Year	May	June	July	August
1993	93 % Ost/Tc	88 % Ost/Tc	88 % Ost/Tc	78 % Ost/Tc
	7 % Tv	12 % Tv	12 % Tv	22 % Tv
1994	100 % Ost/Tc	93 % Ost/Tc	93 % Ost/Tc	77 % Ost/Tc
		7 % Tv	7 % Tv	23 % Tv
1995	93 % Ost/Tc	93 % Ost/Tc	79 % Ost/Tc	78 % Ost/Tc
	7 % Tv	7 % Tv	21 % Tv	22 % Tv

Ost/Tc, *Ostertagia / T. colubriformis* spp
Tv, *T. vitrinus*

3.3.4.2 Ewe condition scores

The monthly group mean condition scores for ewes in each year are shown in Figure 3.12. There were very few differences in terms of body condition between groups in 1993 and 1994 (the statistics of which are shown in Tables 3.10 and 3.11) and none were apparent in 1995.

Table 3.10 *1993 statistical comparison of ewe condition scores*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
5/5	-	-	-	-	-
1/6	-	-	Lev* Ivm*	-	-
1/7	-	-	Controls*	-	-
29/7	-	-	-	-	-
26/8	-	-	-	-	-

- no significant differences
* significantly higher score than comparator $P < 0.05$

Table 3.11 *1994 statistical comparison of ewe condition scores*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
10/5	Fbz* Controls*	Fbz*	-	-	Fbz*
16/6	-	-	-	-	-
13/7	-	Fbz/Lev*	-	-	-
12/8	-	Fbz/Lev** Ivm*	-	-	-

- no significant differences
* significantly higher score than comparator $P < 0.05$, ** $P < 0.01$

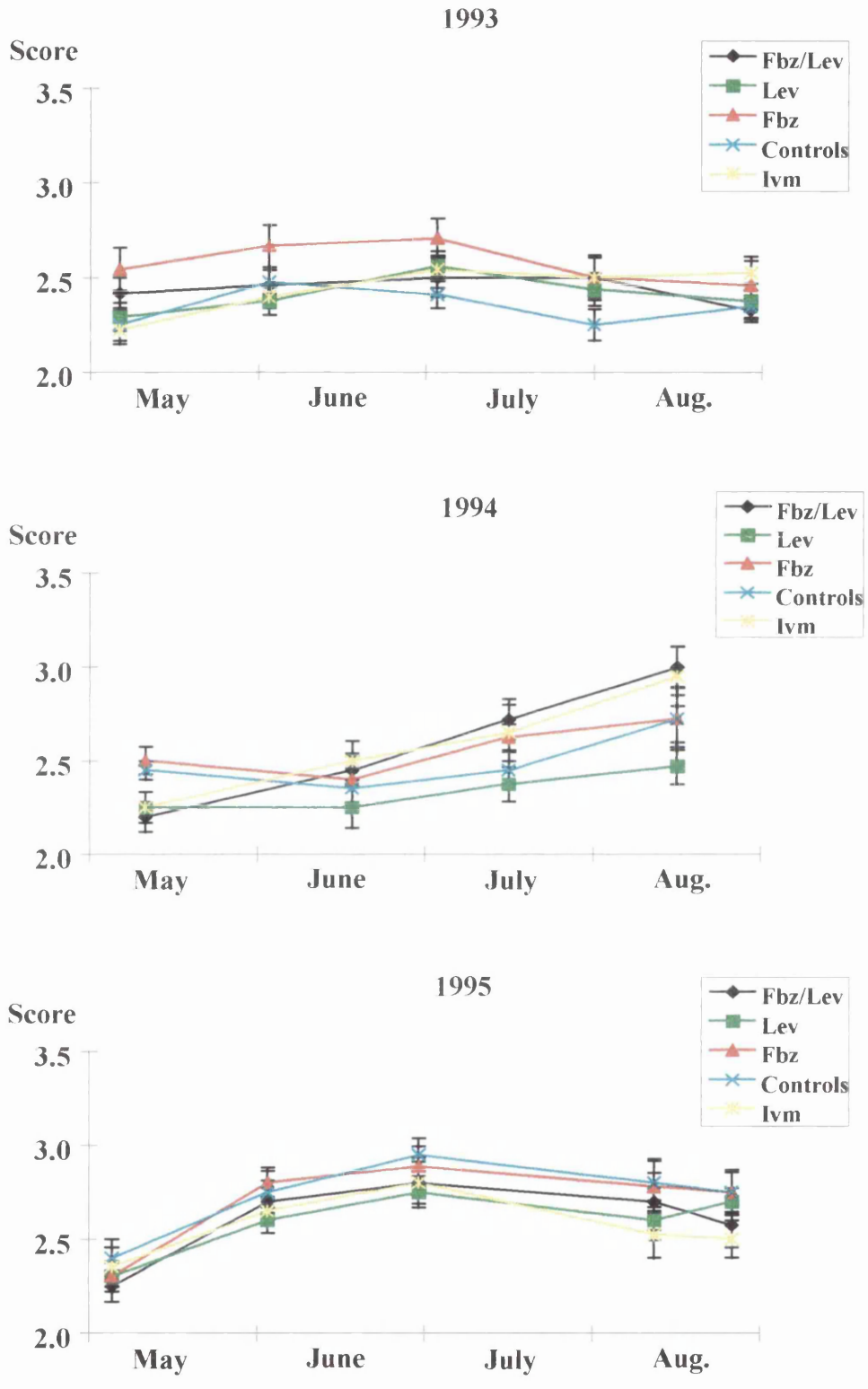

Figure 3.12 *Average ewe condition scores (±SEM) over the three year study*

3.3.4.3 Ewe bodyweights

The average ewe body weights for each group over the three years are shown in Figure 3.13. There were very few differences in terms of bodyweight between groups in 1993 and 1994, although towards the end of the season in 1995 ewes from the control and Fbz groups were heavier than ewes from the Lev group, the statistics of which are shown in Tables 3.12 - 3.14.

Table 3.12 *1993 statistical comparison of ewe bodyweights*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
5/5	-	-	-	-	-
1/6	-	-	-	-	-
1/7	-	Ivm*	-	-	-
29/7	-	-	-	-	-
26/8	-	-	-	-	-

- no significant differences
* significantly heavier than comparator $P < 0.05$

Table 3.13 *1994 statistical comparison of ewe bodyweights*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
10/5	-	-	-	-	-
16/6	-	-	-	Fbz*	-
13/7	-	-	-	-	-
12/8	-	Ivm*	-	-	-

- no significant differences
* significantly heavier than comparator $P < 0.05$

Table 3.14 *1995 statistical comparison of ewe bodyweights*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
4/5	-	-	-	-	-
1/6	Controls*	-	-	-	-
28/6	-	Controls*	-	-	-
9/8	-	Fbz* Controls*	-	-	-
23/8	-	Fbz* Controls*	-	-	-

- no significant differences
* significantly heavier than comparator $P < 0.05$

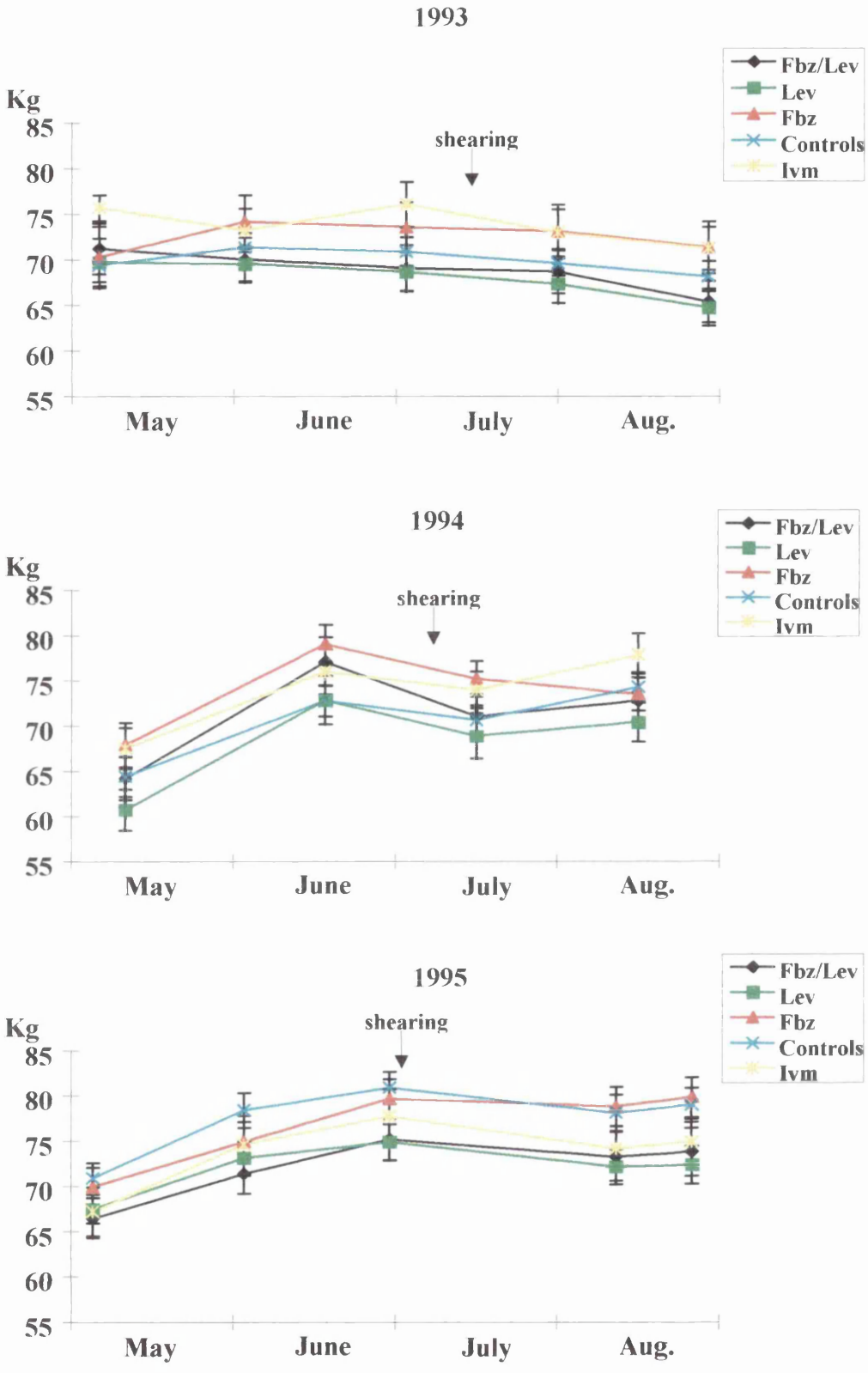

Figure 3.13 *Average ewe bodyweights (±SEM) over the three year study*

3.3.4.4 Ewe fleece weights

The average ewe fleece weights following shearing in July of each year are shown in Figure 3.14. In 1993 and 1995 there were no significant differences between the groups in terms of fleece weight. In 1994, both the Fbz ($P < 0.05$) and control ($P < 0.01$) groups had significantly heavier fleece weights than the Fbz/Lev group. The average fleece weight of the controls was also heavier than the Lev group ($P < 0.05$) in this year.

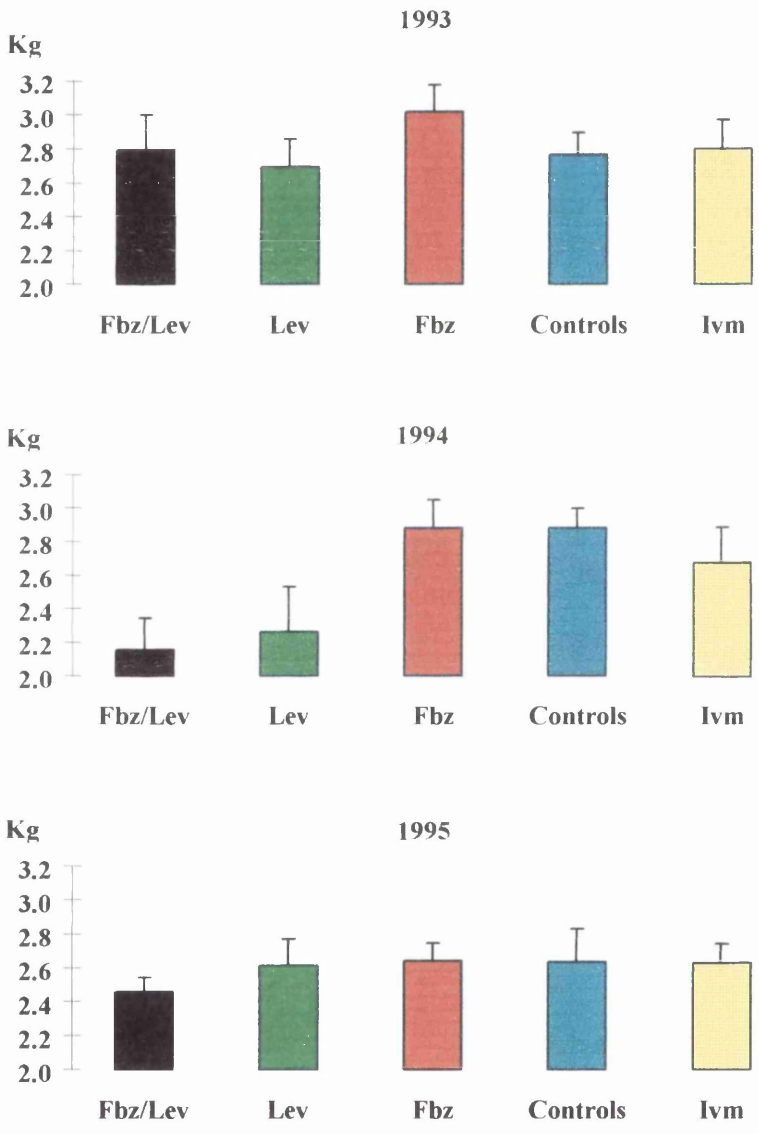

Figure 3.14 *Average ewe fleeceweights (+ SEM) over the three year study*

3.3.5 Lamb parasitological and production results

3.3.5.1 Anthelmintic treatments

The efficacy of the various anthelmintic treatments against *N. battus* and trichostrongyles over the three year study are shown in Figures 3.15 and 3.16 respectively. Treatments administered in May provided good control of *N. battus*, which was susceptible to all of the anthelmintics, and faecal egg counts remained reasonably low throughout the rest of the season. The Lev treatment given in May 1995 however resulted in an efficacy of less than 90 % against this species. Ivm treatment and the Fbz/Lev combination provided good control of trichostrongyles throughout the study. The reduced efficacies seen with Fbz against trichostrongyles confirmed the presence of resistant nematodes against this class of anthelmintic. Results of the faecal egg count reduction tests (FECRTs) also suggest that the Lev treatments given in 1994 and 1995 were not fully effective. Treatment efficacies will be discussed in detail in chapter 4 along with the merits of the FECRT and the end of season controlled efficacy tests (CETs).

3.3.5.2 Lamb faecal egg counts

Figure 3.17 shows the average *N. battus* faecal egg counts for each group whilst Figure 3.18 shows the corresponding trichostrongylid faecal egg counts over the study. The pattern of *N. battus* faecal egg output was similar in 1993 and 1994, peaking in mid-May and remaining minimal following anthelmintic treatment. In 1993 the control group were treated three weeks after the other groups, by which time their *N. battus* faecal egg counts were already declining. In 1995 there was a smaller *N. battus* peak faecal egg count in mid-May which responded well to anthelmintic treatment. Lambs became reinfected with *N. battus* in June 1995 when egg counts averaged 453 EPG in the Fbz/Lev, Lev, Fbz and Ivm treated groups. This reinfection was not evident in the controls which were treated 2 weeks previously. Statistical differences between groups in terms of *N. battus* faecal egg output throughout the early season of each year and overall comparisons are shown in Tables 3.15 to 3.18. In terms of *N. battus* faecal egg output the controls compared

favourably to the other groups, having lower counts up until treatment on several occasions.

The pattern of trichostrongylid faecal egg output was similar in 1993 and 1994, increasing sharply following turnout but, following anthelmintic treatment, tending to increase only slowly. In 1993 the control group were treated three weeks after the other groups, by which time their trichostrongylid faecal egg counts were declining. In the Fbz/Lev, Lev, Fbz and Ivm groups there was a second wave of infection in mid-July of 1993 but these counts subsequently declined. In 1994 the pattern of pasture contamination may have been influenced by the particularly dry spell in May. This would have influenced the pasture larval counts and helps to explain why the second wave of infection in this year was limited. The low numbers of larvae on the pasture at the end of the 1994 season led to relatively low numbers of larvae on pasture the following spring. When the lambs were treated in mid-May of 1995 lamb average trichostrongylid faecal egg counts were lower than the preceding years. A second wave of infection was evident in the Fbz/Lev, Lev and Fbz groups in mid-June of this year which, as with *N. battus* faecal egg output, was not evident in the control group treated 2 weeks previously. These counts subsequently declined and remained low following a mid-seasonal treatment. Statistical differences between groups in terms of trichostrongylid faecal egg counts over the three years are shown in Tables 3.19 to 3.21. Differences between the control group and treated groups were largely governed by the different times of treatment. Despite reduced anthelmintic efficacy, due to the presence of resistant parasites, the Fbz group compared favourably with the other anthelmintic groups in terms of trichostrongylid faecal egg output over the three seasons. Comparison between total trichostrongylid faecal egg output in each year are shown in Table 3.22.

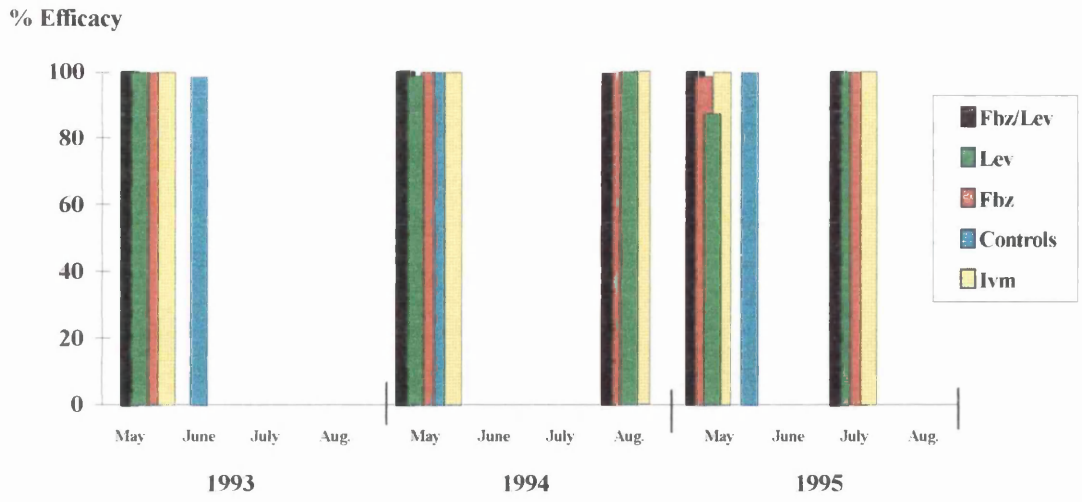

Figure 3.15 *Treatment efficacy against N. battus during the study*

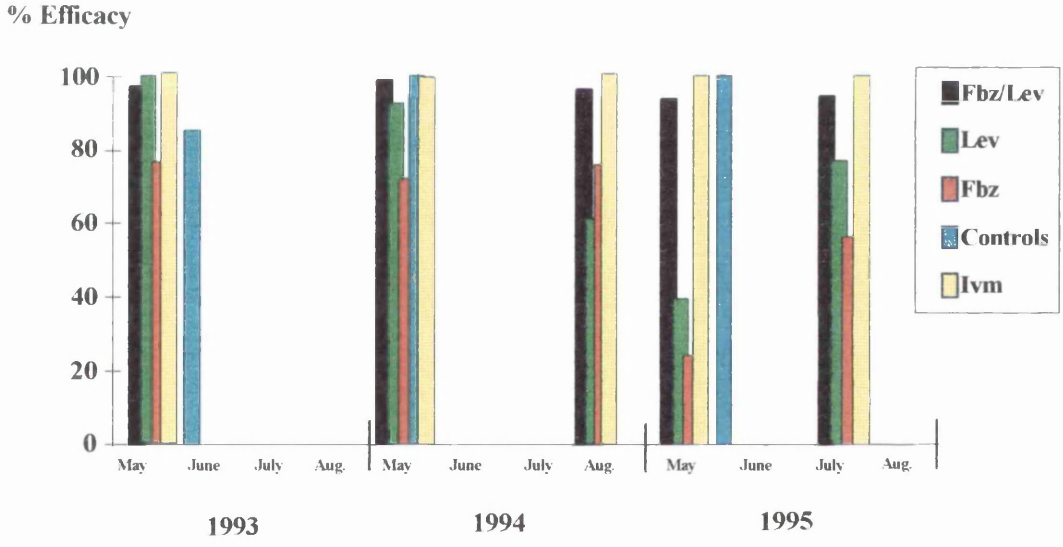

Figure 3.16 *Treatment efficacy against trichostrongyles during the study*

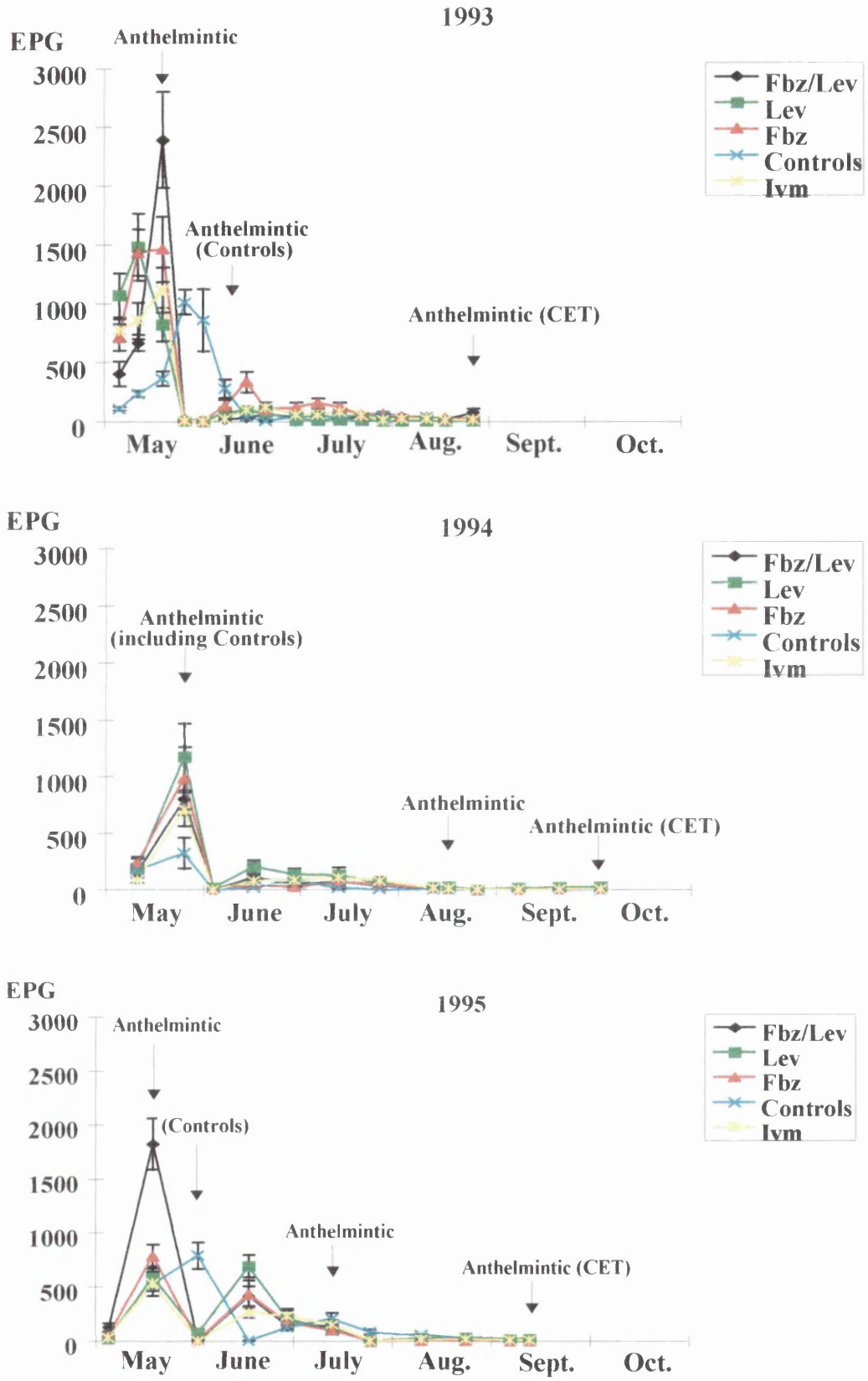

Figure 3.17 *Average lamb N. battus faecal egg counts (±SEM) in each year of the study*

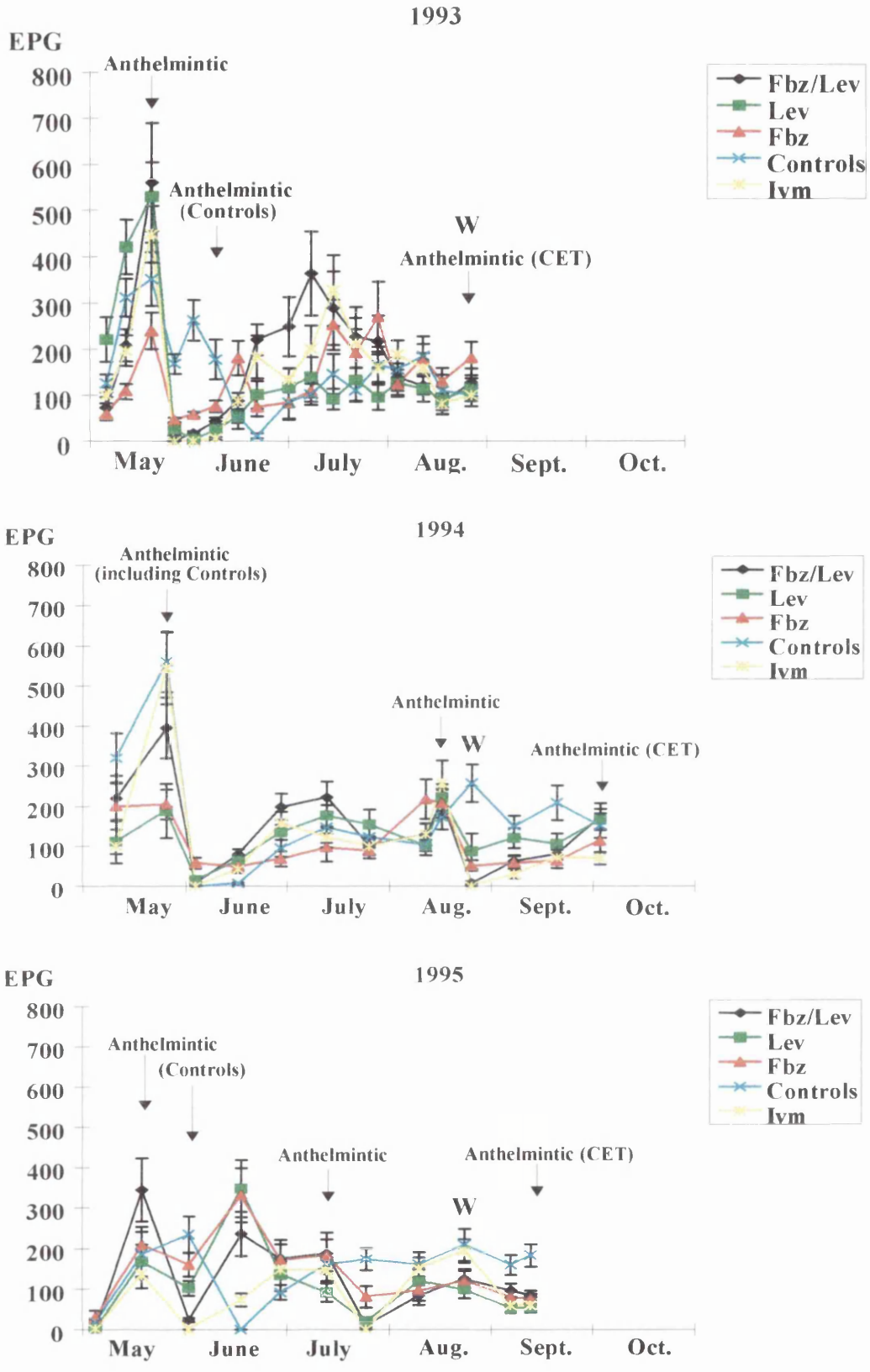

Figure 3.18 *Average lamb trichostrongyle faecal egg counts (±SEM) in each year of the study (W = weaning date)*

Table 3.15 *1993 statistical comparison of lamb N. battus faecal egg counts*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
5/5	-	Controls*** Fbz/Lev**	Controls*** Fbz/Lev**	-	Controls*** Fbz/Lev**
19/5 Anthelmintic (not controls)	Controls**	Controls*	Controls***	-	Controls**
1/6	-	-	Lev* Ivm*	Fbz/Lev*** Lev*** Fbz*** Ivm***	-
15/6 (controls treated 8/6)	Controls*	-	Controls*** Fbz/Lev** Lev*** Ivm**	-	Controls*
1/7	Lev* Ivm*	-	Lev**	-	-

- no significant differences

* significantly lower egg count than comparator P < 0.05, ** P < 0.01, *** P < 0.001

Table 3.16 *1994 statistical comparison of lamb N. battus faecal egg counts*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
10/5	-	-	-	Lev*	-
25/5 Anthelmintic (including controls)	Controls*	Controls**	-	-	-
3/6	-	Controls*** Fbz/Lev*** Fbz*** Ivm***	-	-	-
16/6	-	Controls** Fbz*	-	-	Controls* Fbz*
29/6	-	-	-	-	Controls* Fbz/Lev* Fbz*

- no significant differences

* significantly lower egg count than comparator P < 0.05, ** P < 0.01, *** P < 0.001

Table 3.17 *1995 statistical comparison of lamb N. battus faecal egg counts*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
4/5	-	-	-	-	-
18/5 Anthelmintic (not controls)	Lev*** Fbz** Controls*** Ivm***	-	-	-	-
1/6 (controls treated)	-	Fbz/Lev*** Fbz*** Ivm***	Fbz/Lev** Ivm**	Fbz/Lev*** Lev*** Fbz*** Ivm***	-
16/6	Controls***	Controls*** Fbz/Lev* Fbz* Ivm*	Controls***	-	Controls***
28/6	-	-	-	-	-

- no significant differences

* significantly lower egg count than comparator P < 0.05, ** P < 0.01, *** P < 0.001

Table 3.18 *Statistical comparison of total N. battus faecal egg output in the groups of lambs over each season*

	Comparator				
Year	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
1993	-	-	Lev* Ivm** Controls**	-	-
1994	Controls**	Controls**	-	-	Controls**
1995	Ivm*	-	-	Ivm*	-

- no significant differences

* significantly lower egg count than comparator $P < 0.05$, ** $P < 0.01$

Table 3.19 *1993 statistical comparison of lamb trichostrongylid faecal egg counts*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
5/5	-	Fbz/Lev** Fbz*	-	Fbz/Lev*** Fbz**	Fbz/Lev*
19/5 Anthelmintic (not controls)	-	Fbz**	-	-	Fbz*
1/6	Lev* Ivm***	Ivm***	Fbz/Lev*** Lev*** Ivm***	Fbz/Lev*** Lev*** Fbz** Ivm***	-
15/6 (controls treated 8/6)	Controls*** Lev*	Controls**	Controls*** Lev**	-	Controls***
1/7	Controls* Lev** Fbz**	-	-	-	Fbz*
15/7	Lev*	-	Lev*	-	Lev** Controls*
29/7	-	-	Lev**	-	Lev*
11/8	-	-	Lev*	-	-
26/8	-	-	Ivm*	-	-

- no significant differences

* significantly lower egg count than comparator $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3.20 *1994 statistical comparison of lamb trichostrongylid faecal egg counts*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
10/5	Lev*	-	-	Lev**	Lev**
25/5 Anthelmintic (including controls)	Lev*	-	-	Lev** Fbz*	-
3/6	Controls** Ivm**	Controls** Ivm**	Controls*** Fbz/Lev*** Lev*** Ivm***	-	-
16/6	Controls***	Controls***	Controls***	-	Controls**
29/6	Controls* Fbz**	Fbz*	-	-	Fbz**
13/7	Fbz*	Fbz*	-	-	Fbz*
26/7	-	-	-	-	-
12/8	-	-	-	-	-
17/8 Anthelmintic (not controls)	-	-	-	-	-
26/8	Ivm*	Fbz/Lev** Ivm***	Fbz/Lev*** Ivm***	Fbz/Lev*** Ivm*** Lev*** Fbz***	-
8/9	Ivm*	Ivm**	-	Fbz/Lev* Fbz** Ivm***	-
21/9	-	Fbz*	-	Fbz/Lev** Fbz** Lev* Ivm**	-
4/10	Ivm**	Ivm**	-	Ivm*	-

- no significant differences

* significantly lower egg count than comparator $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3.21 *1995 statistical comparison of lamb trichostrongylid faecal egg counts*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
4/5	-	-	-	-	-
18/5 Anthelmintic (not controls)	Lev* Ivm** Controls*	-	Fbz/Lev* Ivm***	-	-
1/6 (controls treated)	-	Fbz/Lev* Ivm*	Fbz/Lev* Ivm*	Fbz/Lev** Ivm***	-
16/6	Controls*** Ivm**	Controls*** Ivm***	Controls*** Ivm**	-	Controls***
28/6	-	-	-	-	Controls*
12/7 Anthelmintic (not controls)	-	-	-	Lev**	-
24/7	Ivm**	Ivm***	Fbz/Lev*** Lev* Ivm***	Fbz/Lev***Fbz** Lev*** Ivm***	-
9/8	-	-	-	Fbz/Lev* Fbz*	-
23/8	-	-	-	Lev*	Lev*
6/9	Ivm*	-	-	Lev*** Fbz* Ivm**	-
12/9	-	-	-	Fbz/Lev** Fbz** Lev*** Ivm***	-

- no significant differences

* significantly lower egg count than comparator $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3.22 *Statistical comparison of total trichostrongylid faecal egg output in the groups of lambs over each season*

	Comparator				
Year	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
1993	-	-	-	-	-
1994	Fbz**	-	-	-	-
1995	-	-	Ivm**	Ivm**	-

- no significant differences
** significantly lower egg count than comparator $P < 0.01$

Tables 3.23 to 3.25 show the average percentage of the different trichostrongyle species identified by means of egg dimension measurements throughout the season in each year. The patterns seen on each paddock were similar each year with *Ostertagia / T. colubriformis* spp predominating throughout the early season and *T. vitrinus* appearing later in the year. As previously mentioned, results of the lamb CETs carried out at the end of each season identified only *Ostertagia* and *T. vitrinus*, and even though the dimension measurement technique cannot differentiate between *Ostertagia / T. colubriformis* it seems likely that the former species predominated throughout the study.

Table 3.23 *Identification of 1993 lamb trichostrongyle faecal egg counts*

1993	**May**	**June**	**July**	**August**
Fbz/Lev	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	93 % Ost/Tc 7 % Tv
Lev	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	88 % Ost/Tc 12 % Tv
Fbz	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	93 % Ost/Tc 7 % Tv
Controls	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	87 % Ost/Tc 13 % Tv
Ivm	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	94 % Ost/Tc 6 % Tv

Ost/Tc, *Ostertagia /T. colubriformis* spp
Tv, *T. vitrinus*

Table 3.24 *Identification of 1994 lamb trichostrongyle faecal egg counts*

1994	May	June	July	August	September
Fbz/Lev	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	93 % Ost/Tc 7 % Tv	79 % Ost/Tc 21 % Tv
Lev	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	64 % Ost/Tc 36 % Tv
Fbz	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	68 % Ost/Tc 32 % Tv
Controls	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	94 % Ost/Tc 6 % Tv	88 % Ost/Tc 12 % Tv
Ivm	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	90 % Ost/Tc 10 % Tv

Ost/Tc, *Ostertagia /T. colubriformis* spp
Tv, *T. vitrinus*

Table 3.25 *Identification of 1995 lamb trichostrongyle faecal egg counts*

1995	May	June	July	August	September
Fbz/Lev	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	66 % Ost/Tc 34 % Tv
Lev	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	68 % Ost/Tc 32 % Tv
Fbz	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	62 % Ost/Tc 38 % Tv
Controls	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	88 % Ost/Tc 12 % Tv
Ivm	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	100 % Ost/Tc	90 % Ost/Tc 10 % Tv

Ost/Tc, *Ostertagia/ T. colubriformis* spp
Tv, *T. vitrinus*

3.3.5.3 Lamb body weights

Figure 3.19 shows the average cumulative weight gains of the lambs throughout each season whilst Figure 3.20 shows the average daily weight gain of each group over similar periods of the three year study. Statistical differences between groups in terms of cumulative weight gains throughout the season and overall weight gains are shown in Tables 3.26 - 3.29. In 1993 the control group fared less well than the treated groups and were lighter at several points throughout the season. By the end of the 1993 season both the Fbz/Lev and Ivm groups had gained significantly more weight than the Fbz and Lev treated groups ($P<0.05$) and the controls ($P<0.01$). Despite being given anthelmintic on the same date the controls gained weight more slowly than the other groups in the first half of the 1994 season. By the end of 1994 both the Lev and Ivm groups were significantly heavier than the control group ($P<0.05$) but no differences were evident between any of the treated groups. The Fbz group fared less well than the other treated groups at points throughout 1995 but by the end of the season only the Fbz/Lev group had significantly heavier overall weight gains than both the Fbz and control groups ($P<0.05$).

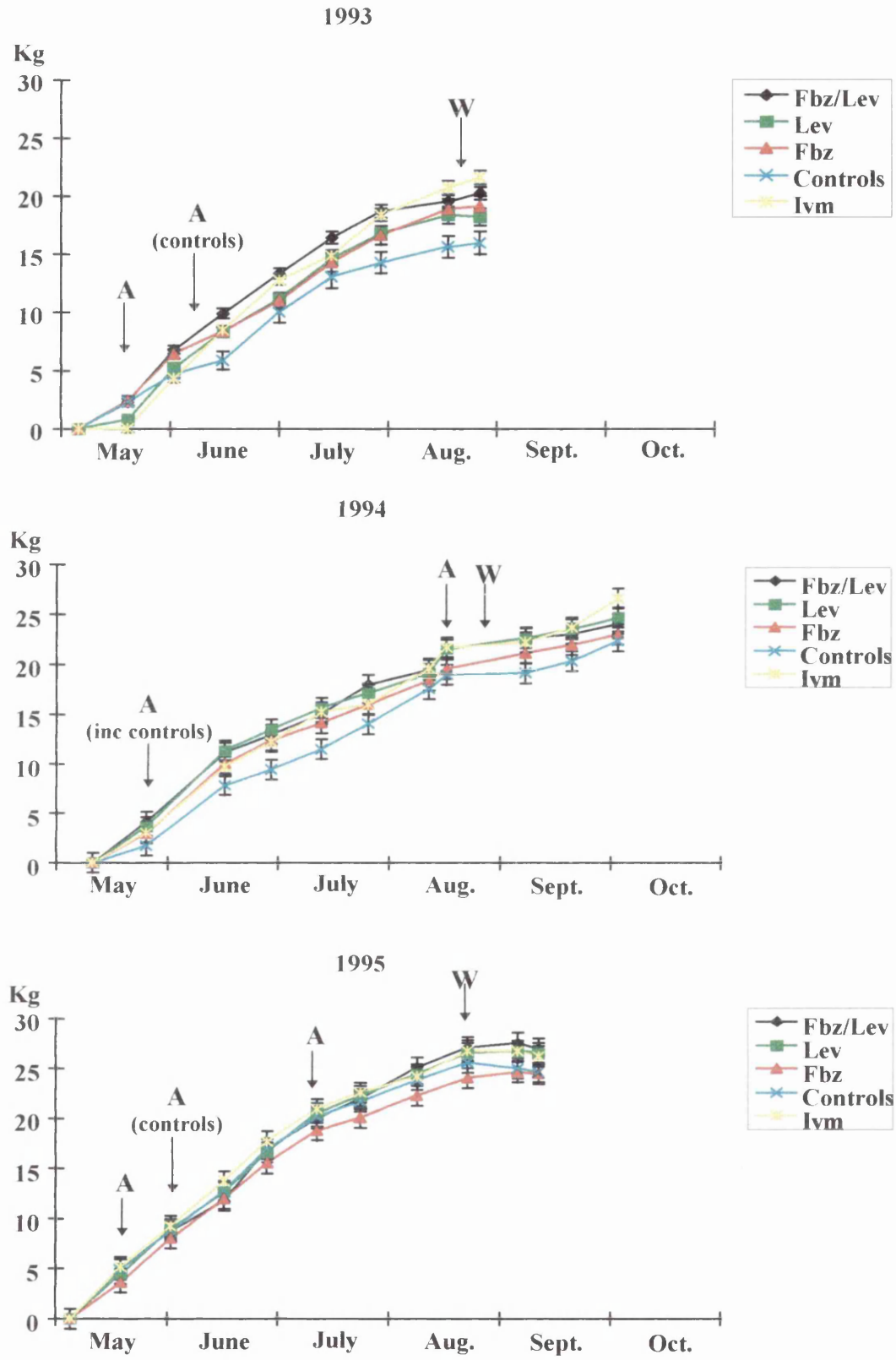

Figure 3.19 *Average lamb cumulative weight gains (±SEM) in each year of the study (A = Anthelmintic, W = weaning date)*

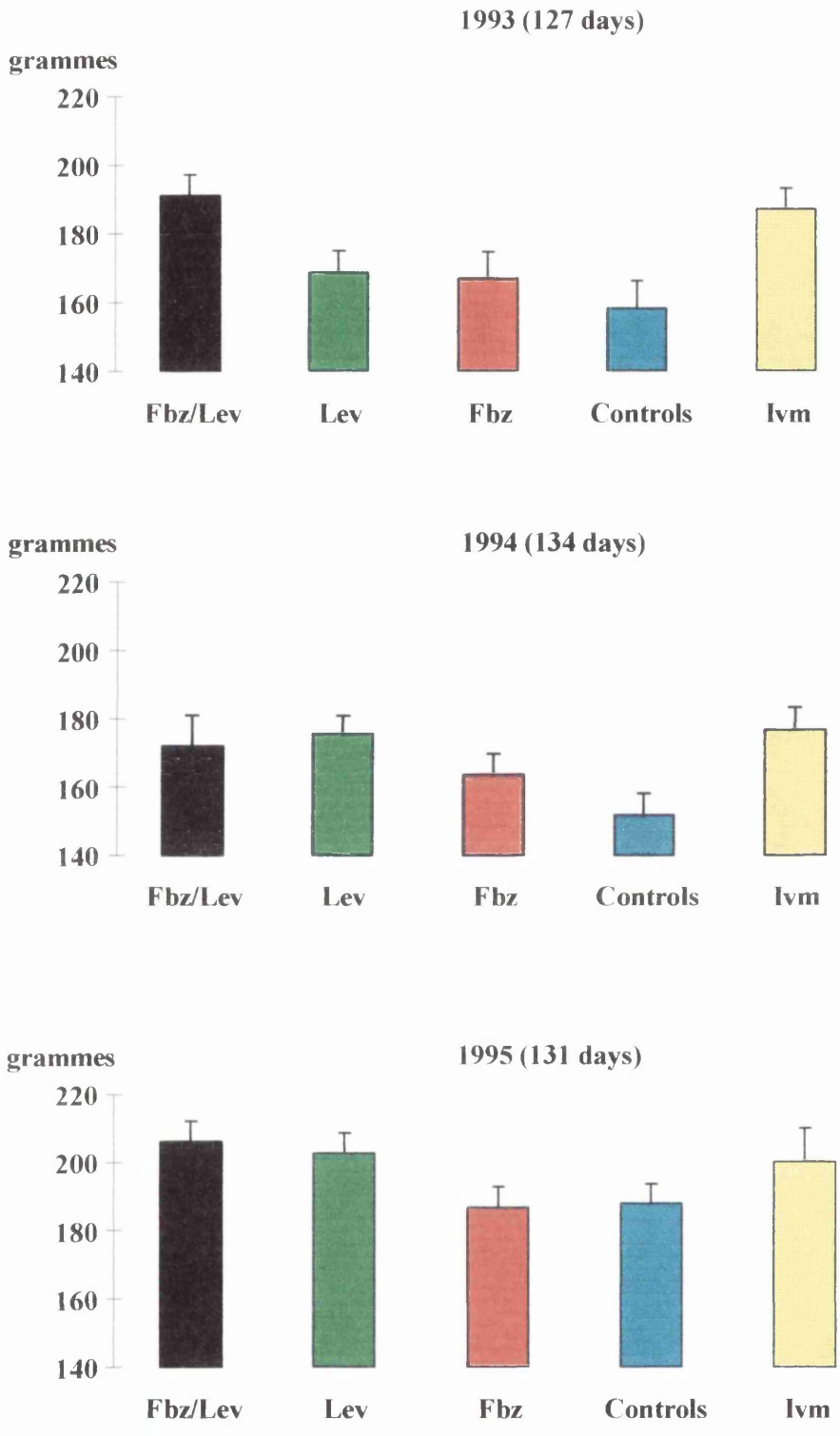

Figure 3.20 *Average daily weight gains (+ SEM) for the groups of lambs over the first 4 months of each season*

Table 3.26 *1993 statistical comparison of lamb cumulative weight gains*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
5/5	-	-	-	-	-
19/5 Anthelmintic (not controls)	-	Controls*** Fbz***	-	-	Controls*** Fbz***
1/6	-	Fbz*	-	Fbz/Lev*** Fbz***	Fbz***
15/6 (controls treated 8/6)	-	-	-	Fbz/Lev*** Lev** Fbz** Ivm***	-
1/7	-	-	Fbz/Lev* Ivm*	Fbz/Lev*** Ivm*	-
15/7	-	-	Fbz/Lev*	Fbz/Lev*** Ivm**	-
29/7	-	-	Fbz/Lev*	Fbz/Lev*** Lev* Fbz* Ivm***	-
11/8	-	-	-	Fbz/Lev*** Lev* Fbz* Ivm***	-
26/8	-	-	Ivm*	Fbz/Lev*** Fbz* Ivm***	-

- no significant differences

* significantly heavier than comparator $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3.27 *1994 statistical comparison of lamb cumulative weight gains*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
10/5	-	-	-	-	-
25/5 Anthelmintic (including controls)	-	-	Fbz/Lev*	Fbz/Lev*** Lev*** Fbz* Ivm*	-
16/6	-	-	Lev**	Fbz/Lev*** Lev*** Fbz*** Ivm**	-
29/6	-	-	-	Fbz/Lev*** Lev*** Fbz*** Ivm***	-
13/7	-	-	-	Fbz/Lev*** Lev*** Fbz** Ivm***	-
26/7	-	-	-	Fbz/Lev*** Lev***	-
12/8	-	-	-	-	-
17/8 Anthelmintic (not controls)	-	-	Lev* Ivm*	Lev* Ivm*	-
8/9	-	-	-	Fbz/Lev* Lev*** Ivm*	-
21/9	-	-	-	Lev** Ivm*	-
4/10	-	-	Ivm**	Lev* Ivm***	-

- no significant differences

* significantly heavier than comparator $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3.28 *1995 statistical comparison of lamb cumulative weight gains*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
4/5	-	-	-	-	-
18/5 Anthelmintic (not controls)	-	-	Controls** Fbz/Lev* Ivm**	-	-
1/6 (controls treated)	-	-	Ivm**	-	-
16/6	Ivm*	-	Ivm*	-	-
28/6	-	-	Fbz/Lev* Ivm**	-	-
12/7 Anthelmintic (not controls)	-	-	Lev*	-	-
24/7	-	-	Lev* Ivm*	-	-
9/8	-	-	Fbz/Lev** Lev*	-	-
23/8	-	-	Fbz/Lev** Lev*	Fbz/Lev*	-
6/9	-	-	Fbz/Lev*	Fbz/Lev*	-
12/9	-	-	Fbz/Lev*	Fbz/Lev*	-

- no significant differences

* significantly heavier than comparator $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3.29 *Statistical comparison of overall cumulative weight gains in the groups of lambs over each season*

	Comparator				
Year	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
1993	-	Fbz/Lev* Ivm*	Fbz/Lev* Ivm*	Fbz/Lev** Ivm**	-
1994	-	-	-	Lev* Ivm*	-
1995	-	-	Fbz/Lev*	Fbz/Lev*	-

- no significant differences

* significantly heavier weight gain than comparator $P < 0.05$, ** $P < 0.01$

3.3.5.4 Lamb plasma pepsinogen values

The average plasma pepsinogen values for the groups of lambs over the three year study are shown in Figure 3.21. Plasma pepsinogen concentrations remained well below 1.0 U in the treated and control groups throughout the study. Statistical analyses (Tables 3.30 - 3.32) showed no clear differences between groups, although the Fbz/Lev treated animals had lower pepsinogen values at several points throughout 1993 and 1994.

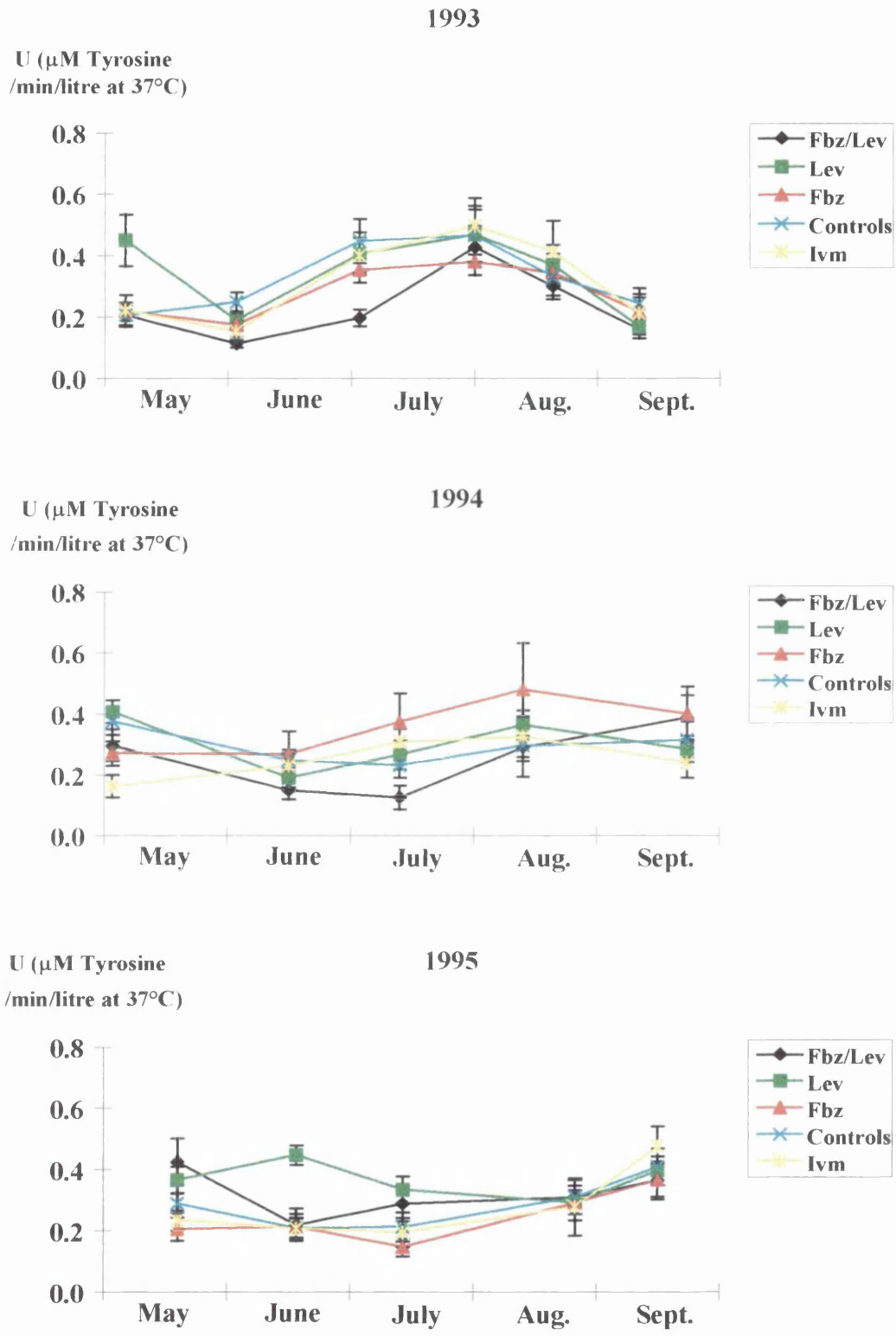

Figure 3.21 *Average pepsinogen values (± SEM) for the groups of lambs during each season*

Table 3.30 *1993 statistical comparison of lamb pepsinogen values*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
5/5	-	Fbz/Lev* Fbz* Controls* Ivm*	-	-	-
1/6	-	Fbz/Lev*	Fbz/Lev**	Fbz/Lev** Ivm*	-
1/7	-	Fbz/Lev*	Fbz/Lev**	Fbz/Lev**	Fbz/Lev**
29/7	-	-	-	-	-
17/8	-	-	-	-	-
8/9	-	-	-	-	-

- no significant differences

* significantly lower pepsinogen value than comparator $P < 0.05$, ** $P < 0.01$

Table 3.31 *1994 statistical comparison of lamb pepsinogen values*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
2/5	Ivm*	Ivm*** Fbz*	-	Ivm**	-
16/6	-	-	-	-	-
13/7	-	Fbz/Lev*	Fbz/Lev*	-	Fbz/Lev**
12/8	-	-	-	-	-
21/9	-	-	-	-	-

- no significant differences

* significantly lower pepsinogen value than comparator $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3.32 *1995 statistical comparison of lamb pepsinogen values*

	Comparator				
Date	**Fbz/Lev**	**Lev**	**Fbz**	**Controls**	**Ivm**
18/5	Fbz* Ivm*	Fbz* Ivm*	-	-	-
16/6	-	Fbz/Lev** Fbz*** Controls*** Ivm***	-	-	-
12/7	Fbz*	Fbz** Ivm*	-	-	-
23/8	-	-	-	-	-
12/9	-	-	-	-	-

- no significant differences

* significantly lower pepsinogen value than comparator $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

3.4 Discussion

There was little evidence of reduced performance in the Fbz treated lambs despite the presence of a Bz resistant isolate of *T. circumcincta* on the paddocks at Firth Mains. The Fbz treated animals performed well throughout the first two seasons with only occasional differences in weight gains compared to the other treated groups. The Fbz treated animals did not perform so well in the early part of 1995 but by the end of the season only the Fbz/Lev group had gained significantly more weight ($P<0.05$). In 1993 a check in growth was evident in the control lambs by the middle of June which persisted for the rest of the season. These lambs were exposed to greater numbers of *N. battus* on pasture and treated with anthelmintic 3 weeks later than the other groups which may explain their reduced performance. Despite being given anthelmintic at the same time as the other groups, the controls gained weight more slowly than the other treated groups in the first half of the 1994 season. This may be explained by the pasture larval counts for *N. battus* and trichostrongylids which were highest on the control paddock in 1994. Interestingly these animals displayed the lowest *N. battus* faecal egg counts in 1994 and there were no differences in trichostrongylid egg output compared to the other treated groups. In 1995 the weight gains of the controls compared well throughout the season despite having only a single treatment in May, which they received two weeks later than the other groups. The timing of treatment resulted in the control animals avoiding a wave of reinfection with both *N. battus* and trichostrongyles which was experienced by the other groups and may account for their comparable performance.

The maximum differences in weight gains between the treated groups at the end of the 1993, 1994 and 1995 seasons were 3.1, 3.6 and 2.6 kgs respectively. Figure 3.22 shows the average total cumulative weight gains over the three seasons for the different treatment groups. The Fbz/Lev and Ivm groups gained significantly more weight than the control group ($P<0.01$ and $P<0.001$ respectively) and the Fbz group ($P<0.01$). The Lev group also gained more weight than the controls ($P<0.05$) but there were no significant differences between these and the Fbz treated animals.

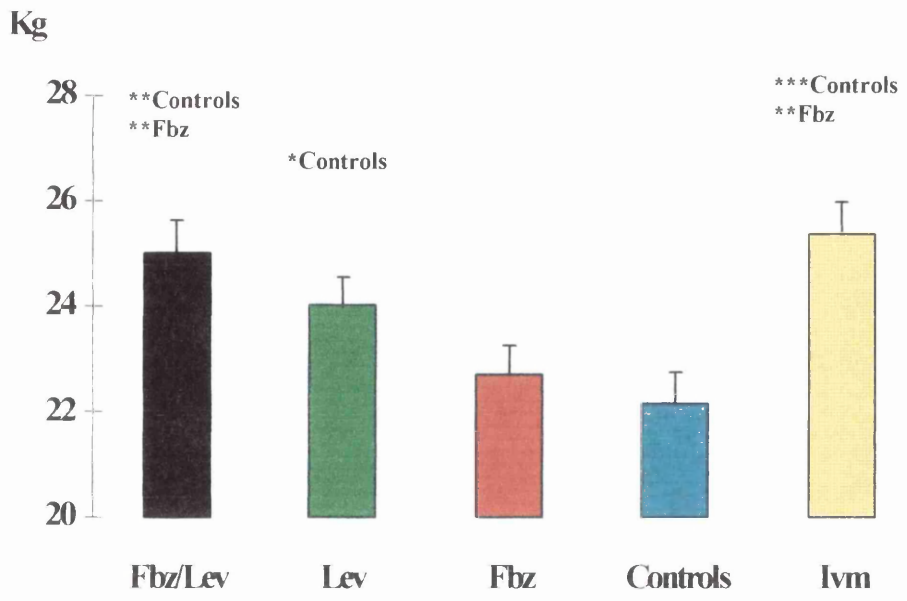

Figure 3.22 *Average total cumulative weight gains (+ SEM) for the different treatment groups over three seasons (* significantly heavier weight gain than comparator $P<0.05$, ** $P < 0.01$, *** $P < 0.001$)*

There were no clinical signs of disease in any of the groups throughout the study. These observations were supported by the plasma pepsinogen concentrations for each of the groups which remained relatively low each season. The highest average plasma pepsinogen concentration reached in the 1993, 1994 and 1995 season was 0.45, 0.35 and 0.40 U respectively. The mean plasma pepsinogen value for 42 uninfected Scottish Blackface sheep aged six-months in the study of Armour *et al.* (1966) was 0.273 U. The normal range for pepsinogen from over 1000 samples collected from parasite-naive sheep was between 0 - 0.454 U in the study of Lawton, Reynolds, Hodgkinson, Pomroy & Simpson (1996) although the age and breed(s) of the sheep were not specified. The field study by Coop *et al.*(1985) is of particular relevance since the same 0.9 hectare paddocks were used in their study with lambs of the same breed and age. The maximal pepsinogen value seen with uninfected control lambs grazing the paddocks when they were minimally contaminated was approximately 0.4 U (Coop *et al.*, 1985). The maximal pepsinogen figures for lambs

given a daily dose of 500, 1,500, 3,000 or 5,000 *T. circumcincta* L_3 on the otherhand were approximately 0.7, 0.8, 1.35 and 1.7 U respectively (Coop *et al.*, 1985). These peak values were reached following 6 weeks of daily dosing but by week 10 the average plasma pepsinogen values for all of these groups had fallen to approximately 0.4, 0.5, 0.6 and 0.65 U respectively (Coop *et al.*, 1985). It is possible therefore that pepsinogen values may well have been higher at a particular point in the early part of each season and were inadvertently missed since the lambs were only monitored on a monthly basis. Nevertheless, the plasma pepsinogen values remained relatively low throughout the season and suggests that the lambs in this study were not facing a particularly high level of larval challenge from pasture.

The density of coccidial oocysts was notably higher at the start of sampling in May 1995 compared to the previous two years. Of the 11 species of *Eimeria* described in the UK only *E. ovinoidalis* and *E. crandallis* are pathogenic. The other 9 species are not known to be very harmful, but their occurrence in mixed infections can pose problems in diagnosis (Catchpole, Norton & Gregory, 1993). Whereas very young lambs are relatively resistant to infection, susceptibility has been shown to increase progressively up to at least 4 weeks of age (Gregory & Catchpole, 1989). There are two possible explanations for the increased counts in 1995. Firstly, lambs may not have been exposed to challenge at housing and secondly, the lambs went onto pasture a week earlier in this year which may have affected their exposure pattern.

Concurrent infection in a rodent model with coccidia and *Nippostrongylus brasiliensis* has been shown to produce increased egg production and extended patency of the nematode infection (Bristol, Piñon & Mayberry, 1983). An interaction between coccidia and *N. battus* has also been shown to exist in lambs on pasture (Catchpole & Harris, 1989). These workers demonstrated that simultaneous infection with pathogenic coccidia and *N. battus* increased both the clinical symptoms and the numbers of nematode eggs. Faecal consistencies tended to be lower in May 1995 and it is possible that this may have exerted some influence on apparent *N. battus* faecal egg counts. However, it was in this year that weight gains in all groups were greatest despite the increased coccidial counts and reduced faecal consistencies. The species of coccidia present were not identified and it is possible

therefore that neither pathogenic species featured in this study. There was a wave of reinfection in the treated groups with increased *N. battus* FECs in mid June and it is possible that the interaction between coccidia and *N. battus* may have affected the development of immunity in some way. A more likely explanation is that the timing of anthelmintic treatment affected the development of immunity towards *N. battus* in this year. The control group were treated two weeks later than the other groups in 1995 and no second wave of infection with *N. battus* was evident in these animals following anthelmintic treatment.

As previously mentioned the field study by Coop *et al.*(1985) is of particular relevance since the same 0.9 hectare paddocks were used in their study with lambs of the same breed and age. These workers demonstrated average daily growth rates for minimally parasitised control animals of 155 grammes per day over the first nine weeks of a three month period commencing in July. Table 3.33 shows the average daily liveweight gains of the treated groups in each year of the present study over a comparable nine week period.

Table 3.33 *Average daily liveweight gain in grammes (± SD) of the groups of lambs over a nine week period from July - September of each year*

Year	Fbz/Lev	Lev	Fbz	Ivm
1993	157.9 (47.8)	148.5 (40.0)	147.3 (48.2)	159.4 (54.6)
1994	136.8 (44.9)	128.9 (34.9)	127.1 (35.4)	135.6 (30.8)
1995	153.2 (29.2)	145.1 (27.8)	128.6 (32.1)	129.3 (54.9)

Although the gains seen by Coop *et al.* (1985) offer a valuable contrast, the cumulative weight gains of the animals before July should also be taken into consideration when making comparisons. The average cumulative weight gains of the treated groups before this period in 1993, 1994 and 1995 were 12.1 kg, 9.4 kg and 16.7 kg respectively. Consequently, although lambs in 1995 seem to have fared less favourably over the nine weeks, it was in this year that the highest initial gains were achieved in all groups. The capacity for maximal growth should be taken into account therefore when assessing gains over such a restricted period.

Nevertheless, if we consider the gain of 155 grammes seen by Coop *et al.* (1985) as an index of the maximal potential growth rate on pasture of that age, quality and location when the paddocks were minimally contaminated, the rates seen over the three year study compare favourably despite seasonal differences in climate, herbage availability and parasite contamination. In 1994 for example, when gains in all groups were poorest over this period, the figure for the Fbz treated lambs equates to a weight gain of less than 1 kg per month below that seen with the minimally parasitized animals of Coop *et al.* (1985).

There were very few differences between the dams of the treated and control lambs with no evidence of reduced body weight, condition score or fleece weight over the three year study. Sampling commenced in early May of each year and in 1993 and 1994 the PPRI in ewes was relatively insignificant. The predominant species of eggs derived from reinfection post-turnout were from *Ostertagia*. Egg counts for *N. battus* did not feature in the post parturient rise despite heavy pasture contamination and remained low or negative throughout the season confirming work by Thomas (1959b). The PPRI phenomenon was most evident in 1995 when pasture larval counts were at their lowest. Recent work suggests that nutrition may play an important role in governing periparturient immunity (M. van Houtert, personal communication). It is possible that the ewes were under some nutritional stress in 1995 which would explain their response despite lower pasture contamination. Nevertheless, no compromise in ewe production was evident compared to previous years and it was in 1995 that lambs in all groups performed best.

From the lamb trichostrongylid egg counts over the three years (Figure 3.18) it can be seen that the peak count in May was lowest in 1995. It could be argued that the lamb treatments were administered at a more strategic point in this season before counts had reached a 'maximum'. However, the control lambs were treated two weeks later without any further increase in egg count suggesting that pasture contamination may have been lower in this year. This is supported by the average pasture larval counts for trichostrongylids seen on the paddocks (Figure 3.10) and may help to explain why the initial cumulative weight gains for all groups were greatest in 1995.

Faecal egg counts are recognized as an insensitive quantitative measure of pathogenicity of parasitic infections and there is no simple relationship between them and worm burdens, clinical signs or liveweight gains (Chiejina & Sewell, 1974b). They serve to confirm that worms are reproductively active but do not convey quantitative significance in terms of effect on animal performance (Coop *et al.*, 1976). In the case of *Ostertagia* species the most pathogenic stage is caused by emerging fourth stage larvae (Holmes, 1985) and therefore, on this criterion alone, faecal egg counts are of limited value in assessing effects on performance (Coop *et al.*, 1977). Moreover, despite five-fold differences in larval intake and large differences between groups in effects on performance, faecal egg counts were similar in all groups in the study conducted by Coop *et al.* (1977). This stereotypic effect seen with *Ostertagia* species has been noted by several other workers (Michel, 1969; Jackson & Christie, 1979 and 1984; Coop *et al.*, 1976 and 1985) and should be taken into consideration when assessing the significance of faecal egg counts with this species.

Nevertheless, faecal egg counts do offer a convenient and valuable tool in the monitoring of parasitic infection, especially in young lambs. In a study undertaken to determine the relationship between egg and worm counts McKenna (1981) studied the gastrointestinal tracts of 190 'young' (up to 12 months of age) and 131 'old' (over 12 months of age) sheep harbouring mixed infections. By comparing strongyle species other than *Nematodirus*, McKenna (1981) showed that faecal egg counts were closely correlated with worm burdens in young outbred sheep with a weaker correlation in older animals. Faecal egg counts were also found to be a good indicator of the potential pathogenicity of the burden in both young and older sheep but it was concluded that their diagnostic significance should never be considered in isolation. Rather they should always be interpreted in relation to the history and management of the flock and be supported by clinical signs such as anaemia, diarrhoea and illthrift. Stear, Bishop, Duncan, McKellar & Murray (1995) found that despite only moderate repeatability, faecal egg counts were the parasitological parameter most strongly associated with worm burdens in comparison with eosinophil counts and plasma pepsinogen concentrations during experimental infections with *O. circumcincta* in lambs aged 6 and 9 months. Similarly,

Williamson, Blair, Garrick, Pomroy & Douch (1994) found a reasonable relationship between worm burden and faecal egg count in fleeceweight-selected and control sheep using mixed infections, which was stronger in unselected animals.

Between season differences in climate and herbage availability make it difficult to make direct comparisons of the results from each year. The epidemiological pattern of trichostrongyle pasture contamination in 1994 and 1995 was markedly different to that of 1993 which saw a characteristic biphasic pattern typically seen on 'dirty' pasture (Thomas & Boag, 1972). This was a consequence of the particularly dry spells in May 1994 and 1995 which limited the size of the early season suprapopulation peak in late June of these years, thereby affecting the pattern of contamination throughout the rest of the season. Differences between the average minimum daily grass temperatures and rainfall were also evident, both of which would be expected to affect the microclimate and consequently the epidemiology of pasture contamination. The temperature requirement for maximal larval development of free-living stages of *T. circumcincta* has recently been shown to vary depending upon moisture levels (Rossanigo & Gruner, 1995) adding further to the complexities of comparisons.

This study has provided evidence that drugs against which resistance has been selected may be used in the management of PGE, at least when it involves less pathogenic species with a low biotic potential such as *Teladorsagia* (*Ostertagia*). However, one major question that has to be considered is the relative pathogenicity and immunogenicity of the particular Bz-resistant isolate of *T. circumcincta* at Firth Mains. Since the selection process inevitably produces resistant parasites which are genetically restricted, it is possible that they may differ in their pathogenicity and/or immunogenicity in comparison to unselected susceptible isolates. It is for this reason that a study was undertaken to compare the responses of different resistant isolates of *T. circumcincta* to that of a susceptible isolate, the results of which will be discussed in chapter 5.

CHAPTER 4

Field study on the control of a fenbendazole resistant isolate of *Teladorsagia* (*Ostertagia*) - treatment efficacy

4.1 Introduction

As the results from chapter 3 demonstrate there may be a small penalty upon production associated with the continued use of drugs against which resistance already exists. However, there was no evidence that the penalty increased successively over the three seasons that were studied. Continuing to use resistance selected drugs (RSDs) may also have the drawback of selecting resistant populations of other target species (Coles & Rouch, 1992). The faecal egg count reduction test (FECRT) and controlled efficacy test (CET) are commonly practiced *in vivo* techniques used to detect resistance but are known to be relatively insensitive (Martin *et al.*, 1989). Consequently they are only likely to provide evidence of gross changes in resistance within a population. Although *in vitro* bioassays such as the egg hatch assay have the potential to provide data on the resistance of a population there is a requirement for known susceptible isolates (Hunt & Taylor, 1989). In practice egg hatch assays are also flawed since it is known that the expression of resistance to the Bz's varies during the course of an infection (Borgsteede & Couwenberg, 1987; Kerboeuf & Hubert, 1987). The lack of sensitivity of the resistance detection methods suggests that it will be necessary to use genetic markers associated either directly or indirectly with resistance in order to obtain accurate measurements of changes at the population level. The current study provides the opportunity to examine within and between season variation in efficacy in animals carrying susceptible and resistant populations.

Results of chapter 3 have also demonstrated the effectiveness of a Bz-Lev combination in the control of the Bz-resistant isolate of *Teladorsagia* on the paddocks at Firth Mains. It has been suggested that the risk of selecting for multiple resistance in parasites already resistant to one of the components of a Bz-Lev combination is unlikely to be greater than that which would otherwise develop from the use of the other component separately (McKenna, 1990b). The success of this strategy was also examined in terms of treatment efficacies using FECRTs and CETs throughout the three year study.

The investigation also provided an ideal model system for examining ways of improving the efficacy of RSDs. The increasing prevalence of anthelmintic resistance has focused attention on the need to maximize the useful life span of

present and any future anthelmintic compounds. As a result, recent research has investigated means of increasing drug bioavailability in order to enhance efficacy against resistant species. Extending the period of drug administration by dividing the dose has been shown to increase the efficacy of Bz anthelmintics. Bogan *et al.* (1987), working with goats, demonstrated that the repetition of three administrations of oxfendazole at 24 hour intervals produced a significant increase in the area under the plasma concentration-time curve (AUC) compared to a single equivalent total dose. A similar enhancement of drug bioavailability produced increased efficacies against Bz-resistant isolates of *H.contortus*, *Ostertagia* spp and *Trichostrongylus* spp (Sangster *et al.*, 1991b). In addition, greater efficacy was recorded by Sangster *et al.* (1991b) when the oxfendazole doses were separated by a 12 compared to a 24 hour interval.

Altering feed intake has also been shown to enhance Bz efficacy. Recent evidence suggests that Bz anthelmintics associate strongly with particulate digesta in the rumen (Hennessy, Ali & Tremain, 1994) and that drug availability is significantly influenced by the rate of passage of digesta (Ali & Chick, 1992; Taylor, Mallon, Blanchflower, Kennedy & Green, 1992). Ali & Hennessy (1993) found that halving feed intake of sheep for 36 hours before and after treatment slowed digesta flow rate and prolonged the period for metabolite absorption/availability. As a result, the activity of oxfendazole was significantly increased against Bz-resistant isolates of *H.contortus* and *T.colubriformis*. Increased efficacy appears to be influenced by drug-digesta particle association in the rumen which, by regulating the rate and duration of metabolite availability, is a major determinant of the pharmacokinetic disposition of oxfendazole in ruminants (Hennessy *et al.*, 1994). Similar results against Bz-resistant isolates of *H.contortus* and *T.colubriformis* have been demonstrated with albendazole despite intrinsic pharmacokinetic differences compared to oxfendazole (Hennessy, Ali & Sillince, 1995).

Modification of drug metabolism is another method which has been shown to enhance treatment efficacy against resistant parasites. In a laboratory study, using the Firth Mains Bz-resistant isolate of *T.circumcincta*, Benchaoui & McKellar (1994) demonstrated an increase in worm reduction of over 80 % compared to conventional Fbz treatment by co-administering piperonyl butoxide, a novel Bz synergist.

Piperonyl butoxide is a metabolic inhibitor of cytochrome P450. This enzyme system is found in liver microsomes and is involved in the mixed-function oxidization of xenobiotic compounds in order to facilitate their excretion. As a result of piperonyl butoxide co-administration, the metabolism of Fbz and its active sulphoxide metabolite were inhibited, resulting in extended bioavailability of these metabolites and increased treatment efficacy.

4.2 Materials and methods

4.2.1 Experimental Design

The experimental design was outlined in chapter 3 with the treatment allocation, stocking details and treatment dates in Tables 3.1 and 3.2. A non-suppressive control regime was adopted consisting of an initial anthelmintic treatment in May to control *Nematodirus* and a summer drench to control other gastrointestinal nematodes when necessary. Lambs were treated at the manufacturer's recommended dose (MRD) for their allocated drug on the basis of liveweight. Lambs within the control group were treated only when required on welfare grounds with ivermectin (Oramec, MSD Agvet UK). At weaning the ewes were removed from the paddocks and the lambs grazed until of marketable size. At the end of each season a number of lambs from each treatment group were used in a CET.

4.2.2 Faecal egg count reduction tests (FECRTs)

Lambs were faecal sampled on day 0 and treated on the basis of liveweight at the relevant MRD for sheep before returning to pasture. Since the study was primarily concerned with monitoring effects on production it was not feasible to include untreated control animals as recommended by WAAVP guidelines (Coles *et al.*, 1992) for the anthelmintic treatments given in May of each year. Faecal egg counts were determined using a flotation technique described in chapter 2.2.3. Eggs from each group were pooled and identified by means of an image shearing technique, described in chapter 2.2.4. Treatment efficacies were calculated using the formula:

% Reduction = 100 (1 - Xt / Xo), where Xo and Xt are the treated group arithmetic mean egg counts at days 0 and 10-14 respectively.

Confidence intervals (95 %) were calculated according to WAAVP guidelines (Coles *et al.*, 1992) although day 0 faecal egg counts for the treated groups were used to make comparisons rather than day 10-14 counts of untreated control animals.

4.2.3 Controlled efficacy tests (CETs)

At the end of each season groups of animals (n = 6) from each paddock were used in CETs based on their previous anthelmintic exposure. All animals were faecal sampled and weighed two days before treatment and allocated into balanced groups such that each group had members with similar faecal egg count and bodyweight. The animals were faecal sampled again on day 0 and treated on the basis of liveweight at the relevant MRD for sheep. Lambs remained housed following treatment to avoid the possibility of post-treatment re-infection. FECRTs using untreated control animals were performed in conjunction with the CETs, the efficacies of which were calculated according to WAAVP guidelines (Coles *et al.*, 1992) using the formula:

% Reduction = 100 (1 - Xt / Xc), where Xt and Xc are the treated and control group arithmetic mean egg counts respectively at 10 -14 days.

Following slaughter the gastrointestinal tract was removed and the abomasal and small intestinal worm burdens estimated using 2 % sub-samples as described in chapter 2.3. Efficacies resulting from treatment were calculated according to WAAVP guidelines (Wood *et al.*, 1995) using geometric mean data in the following formula:

% Reduction = 100 (1 - Xt / Xc), where Xt and Xc are the respective treated and control group geometric mean worm counts at slaughter.

Concerns have been raised as to whether the use of geometric mean worm burdens is appropriate in the calculation of drug efficacy (Dash, Hall & Barger, 1988). Therefore % reductions were also calculated using arithmetic mean worm data as a comparison. In an effort to reduce the costs of the study all lambs, except those of the Fbz group in 1994, were slaughtered with the co-operation of a local abattoir (Stobbarts, Gorgie, Edinburgh). In 1993 all animals were slaughtered on day

14 post treatment whereas in 1994 and 1995 this date was extended by one week due to new regulations on withdrawal periods.

4.2.4 Reduced feed intake and divided dosing

The effect of feed withdrawal upon the treatment efficacy of Fbz was investigated at the end of season CET in 1993. The effects of feed withdrawal and divided-dosing upon the treatment efficacy of Fbz were investigated during the mid-seasonal FECRT in 1994. The synergistic effects of feed withdrawal and divided dosing upon the treatment efficacy of Fbz were investigated in the 1995 CET. The effect of feed withdrawal upon the treatment efficacy of Lev was also investigated in the 1995 CET.

All animals were faecal sampled and weighed two days before treatment and allocated into balanced groups such that each group had members with similar faecal egg count and bodyweight. Group treatments were assigned randomly and the animals in the feed reduction group housed 24 hours prior to drug administration. This group was allowed water *ad libitum* but denied access to feed whilst the other groups remained at pasture. Faecal samples were taken again on day 0 and groups treated on the basis of liveweight with the relevant MRD for sheep.

In the 1994 FECRT, the Fbz dose was divided with half the MRD given at time 0 and half after a 12 hour interval (2.5 + 2.5 mg kg^{-1}), with the animals returning to pasture during this interval. In the 1995 CET, lambs were given half of a double-MRD following feed withdrawal at time 0 and the other half after a 24 hour interval (5 + 5 mg kg^{-1}), with the animals remaining housed until slaughter.

4.2.5 Piperonyl butoxide

The effect of the co-administration of piperonyl butoxide upon the treatment efficacy of Fbz was investigated at the end of season CET in 1994. Eight lambs were drafted in from the control group, two of which were assigned to each of the groups in order to provide sufficient animals for statistical comparisons. All animals were faecal sampled and weighed two days before treatment and allocated into balanced groups such that each group had members with similar faecal egg count and bodyweight. Group treatments were assigned randomly and faecal samples re-taken

on day 0 before treating animals on the basis of liveweight at the relevant dose rate. Group 1 lambs were treated with Fbz at the MRD for sheep (5 mg kg^{-1}) whereas those of group 2 remained as the untreated controls. Group 3 were orally administered piperonyl butoxide at a dose rate of 63 mg kg^{-1} and acted as controls for this compound. Group 4 were co-administered both Fbz (5 mg kg^{-1}) and piperonyl butoxide (63 mg kg^{-1}). Since the piperonyl butoxide treated lambs were unsuitable for commercial use the four Fbz groups were slaughtered in-house on day 14 whereas the other anthelmintic groups in the 1994 CET were slaughtered at the abattoir on day 21.

4.2.6 Statistical analyses

Differences between treatment group faecal egg count and worm burden reductions were determined by Log_{10} (x+1) transformation prior to analysis of variance (Minitab, version 10.0).

4.3 Results

4.3.1 FECRTs

A graphical representation of the treatment efficacies against both *N. battus* and other strongyles during the study was shown in chapter 3 (Figures 3.15 and 3.16). Tables 4.1 to 4.5 show the pre- and post-treatment group arithmetic mean faecal egg counts for each group and the resulting % efficacies of treatment against *N. battus*, including 95 % confidence intervals where appropriate. At the time of anthelmintic treatment in August 1994 the faecal egg counts for *N. battus* in all groups were minimal and efficacies against this species have not been included.

Table 4.1 *N. battus faecal egg count reductions in May 1993*

Treatment	EPG (± SD) day 0	EPG (± SD) day 12	% Reduction	95 % CIs
Fbz/Lev	2392.2 (1995.5)	2.4 (5.1)	99.90	99.7, 100
Lev	825.0 (702.6)	0.4 (0.8)	99.95	99.9,100
Fbz	1461.7 (1352.3)	5.2 (11.9)	99.64	99.0, 99.9
Ivm	1117.4 (935.5)	0.3 (0.7)	99.97	99.9, 100

Table 4.2 *N. battus faecal egg count reductions in May 1994*

Treatment	EPG (± SD) day 0	EPG (± SD) day 10	% Reduction	95 % CIs
Fbz/Lev	803.6 (590.0)	0.0 (0.0)	100	-
Lev	1174.5 (1104.2)	16.2 (31.2)	98.62	96.3, 99.5
Fbz	986.0 (951.8)	0.5 (1.1)	99.94	99.9, 100
Ivm	713.2 (660.6)	0.2 (0.4)	99.97	99.9, 100

Table 4.3 *N. battus faecal egg count reductions in August 1994*

Treatment	EPG (± SD) day 0	EPG (± SD) day 10	% Reduction	95 % CIs
Fbz/Lev	28.3 (67.2)	0.2 (0.7)	99.29	94.5, 99.9
Lev	22.6 (37.8)	0.0 (0.0)	100	-
Fbz*	-	-	-	-
Ivm	12.3 (27.7)	0.0 (0.0)	100	-

* feed reduction and divided dose study undertaken

Table 4.4 *N. battus faecal egg count reductions in May 1995*

Treatment	EPG (± SD) day 0	EPG (± SD) day 14	% Reduction	95 % CIs
Fbz/Lev	1823.6 (1065.7)	1.3 (3.3)	99.93	99.8, 100
Lev	588.2 (471.9)	76.1 (114.8)	87.06	71.7, 94.1
Fbz	784.9 (498.9)	12.6 (19.1)	98.39	96.6, 99.2
Ivm	534.5 (323.1)	1.3 (4.2)	99.76	98.9, 99.9

Table 4.5 *N. battus faecal egg count reductions in July 1995*

Treatment	EPG (± SD) day 0	EPG (± SD) day 10	% Reduction	95 % CIs
Fbz/Lev	143.2 (197.2)	0.0 (0.0)	100	-
Lev	102.9 (185.7)	0.1 (0.2)	99.90	99.6, 100
Fbz	98.2 (161.7)	0.0 (0.0)	100	-
Ivm	152.6 (197.3)	0.0 (0.0)	100	-

The anthelmintic treatments administered in May of each year, and July 1995, provided good control of *N. battus* with each compound approaching 100 % efficacy against this species. The Lev treatment given in May 1995 however resulted in an efficacy of less than 90 %.

Tables 4.6 to 4.10 show the group arithmetic mean faecal egg counts pre- and post-treatment for each group and the resulting % efficacies of treatment against trichostrongyles over the study including 95 % confidence intervals.

Table 4.6 *Trichostrongylid faecal egg count reductions in May 1993*

Treatment	EPG (± SD) day 0	EPG (± SD) day 12	% Reduction	95 % CIs
Fbz/Lev	560.3 (631.6)	18.0 (17.7)	96.78	94.0, 98.3
Lev	529.8 (363.2)	4.4 (4.5)	99.17	98.6, 99.5
Fbz	239.6 (196.9)	58.6 (27.6)	75.54	63.7, 83.5
Ivm	449.1 (297.2)	0.2 (0.5)	99.95	99.9, 100

Table 4.7 *Trichostrongylid faecal egg count reductions in May 1994*

Treatment	EPG (± SD) day 0	EPG (± SD) day 10	% Reduction	95 % CIs
Fbz/Lev	396.0 (313.4)	7.5 (10.1)	98.10	96.1, 99.1
Lev	188.8 (253.6)	14.8 (27.6)	92.16	77.5, 97.3
Fbz	204.1 (128.1)	57.6 (42.1)	71.78	56.1, 81.8
Ivm	544.1 (398.5)	0.2 (0.7)	99.97	99.8, 100

Table 4.8 *Trichostrongylid faecal egg count reductions in August 1994*

Treatment	EPG (± SD) day 0	EPG (± SD) day 10	% Reduction	95 % CIs
Fbz/Lev	211.3 (127.7)	8.12 (17.0)	96.16	89.6, 98.6
Lev	222.0 (151.4)	86.9 (195.8)	60.86	0, 86.7
Fbz*	-	-	-	-
Ivm	257.3 (243.8)	0.1 (0.5)	99.96	99.7, 100

* feed reduction and divided dose study undertaken

Table 4.9 *Trichostrongylid faecal egg count reductions in May 1995*

Treatment	EPG (± SD) day 0	EPG (± SD) day 14	% Reduction	95 % CIs
Fbz/Lev	345.4 (349.0)	23.8 (24.2)	93.11	86.7, 96.4
Lev	168.1 (190.4)	102.0 (84.6)	39.32	0, 68.1
Fbz	209.6 (197.4)	159.9 (127.7)	23.71	0, 56.7
Ivm	136.0 (151.5)	0.0 (0.0)	100	-

Table 4.10 *Trichostrongylid faecal egg count reductions in July 1995*

Treatment	EPG (± SD) day 0	EPG (± SD) day 10	% Reduction	95 % CIs
Fbz/Lev	188.0 (230.1)	11.1 (27.2)	94.10	79.3, 98.3
Lev	91.7 (103.7)	21.4 (28.2)	76.66	48.2, 89.4
Fbz	183.8 (174.6)	81.3 (120.6)	55.77	0.9, 80.3
Ivm	145.9 (115.0)	0.1 (0.5)	99.93	99.5, 100

Pre- and post-treatment egg measurements identified 100 % *Teladorsagia* for all of the groups confirming that the paddocks were contaminated predominantly with this species at these points in the season. According to WAAVP guidelines (Coles *et al.,* 1992), resistance is present if the percentage reduction is less than 95 % and the lower confidence interval is less than 90 %. If only one of these two criteria is met then resistance is suspected. Under these assumptions, the efficacy of Ivm against trichostrongylids remained highly effective over the three year study. The Fbz/Lev combination was fully effective for the treatments given in May 1993 and May 1994 but resistance was suspected for the treatment given in August of 1994. Efficacies calculated for the FECRT suggest that resistance was also present to this drug combination for the treatments administered in May and July of 1995. The Lev treatment given in May 1993 was fully effective against trichostrongylids but results of remaining FECRTs suggest that resistance to this compound was present. The faecal egg count reduction of 75 % seen with Fbz in May 1993 confirmed the presence of resistant species to this drug upon commencement of the study. Subsequent FECRTs also indicated Bz-resistance, but efficacies did not follow any consistent pattern.

4.3.1.1 Reduced feed intake and divided dosing

As part of the August treatment in 1994 to control trichostrongyles the opportunity was taken to investigate the effects of feed withdrawal and divided dosing upon the treatment efficacy of Fbz. FECRT calculated efficacies for the different methods of drug presentation are shown in Table 4.11. Pre- and post-treatment egg measurements identified 100 % *Teladorsagia* confirming that the paddock was contaminated predominantly with this species at this point in the season. Dividing the dose with a 12 hour interval resulted in an increased efficacy of 28.8 % compared to conventional treatment although this was not statistically significant. Administering the MRD following a 24 hour period of food withdrawal on the otherhand resulted in a significant increase compared to conventional treatment of 39.7 %.

Table 4.11 *Faecal egg count reductions in sheep naturally infected with Bz-resistant T. circumcincta treated with Fbz either conventionally, dividing the dose with a 12 hour interval or following 24 hours of food withdrawal. Including 95 % confidence intervals and differences compared to conventional treatment*

Group (n=6)	Treatment	EPG (± SD) day -2	EPG (± SD) day 0	EPG (± SD) day 10	% Reduction	95 % CIs	P value
1	Conventional (5 mg kg^{-1})	218 (198)	171 (126)	86.7 (77)	49.3	0, 81	-
2	Divided dose (2.5 + 2.5 mg kg^{-1}) (12 hour interval)	211 (243)	155 (115)	34 (45)	78.1	21, 94	0.13
3	24 hour food withdrawal followed by 5 mg kg^{-1}	223 (235)	318 (196)	35 (50)	89.0*	59, 97	0.03

* Significantly different ($P < 0.05$)

4.3.2 CETs

4.3.2.1 CET faecal egg count reductions

Trichostrongylid FECRTs performed in conjunction with the 1993, 1994 and 1995 CETs, determined according to WAAVP guidelines, are shown in Tables 4.12-4.14. Reductions calculated using FECs of the treated group on day 0 are also included for comparison. Invariably the FECs of the untreated control lambs

increased markedly for each of the treatment groups upon housing. Efficacies calculated using treated and untreated control mean FECs on day 10-14 (as suggested by WAAVP guidelines) were higher than those calculated using treated counts on day 0. Only Ivm treatment was effective in reducing FECs of the housed animals by more than 95 %. Calculated efficacies (WAAVP method) for the Fbz/Lev group ranged from 82 - 92 % whilst that of the conventionally treated Lev and Fbz groups between 20 - 51 and 0 - 72 % respectively. As with the field FECRTs the calculated efficacies did not follow any consistent pattern.

Table 4.12 *1993 CET faecal egg count reductions calculated using mean egg counts of the treatment and control groups on day 10-14, WAAVP, or by comparison to the treatment group count on day 0*

Treatment (n = 6)	Weight (kg) (± SD) day -2	EPG (± SD) day -2	EPG (± SD) day 0	EPG (± SD) day 13	% Reduction WAAVP (day 0)
Fbz/Lev					
Untreated controls	38.5 (5.2)	97 (69)	151 (121)	470 (178)	-
Conventional (5 + 7.5 mg kg^{-1})	39.2 (5.7)	88 (55)	179 (119)	47 (47)	**90.0 (73.7)**
Ivm					
Untreated controls	40.8 (2.4)	101 (136)	95 (61)	602 (495)	-
Conventional (0.2 mg kg^{-1})	41.0 (1.7)	99 (139)	90 (56)	1 (2)	**99.8 (98.9)**
Lev					
Untreated controls	40.0 (3.5)	121 (84)	154 (98)	338 (271)	-
Conventional (7.5 mg kg^{-1})	40.0 (2.8)	123 (88)	146 (152)	271 (202)	**19.9 (0)**
Fbz					
Untreated controls	38.3 (5.6)	128 (109)	185 (160)	745 (542)	-
Conventional (5 mg kg^{-1})	38.2 (3.0)	129 (101)	151 (122)	210 (159)	**71.8 (0)**
No feed (24 hours) followed by (5 mg kg^{-1})	38.5 (3.9)	113 (106)	127 (103)	137 (116)	**81.6 (0)**

Table 4.13 *1994 CET faecal egg count reductions calculated using mean egg counts of the treatment and control groups on day 10-14, WAAVP, or by comparison to the treatment group count on day 0*

Treatment (n = 6)	Weight (kg) (± SD) day -2	EPG (± SD) day -2	EPG (± SD) day 0	EPG (± SD) day 14	% Reduction WAAVP (day 0)
Fbz/Lev					
Untreated controls	38.8 (5.1)	142 (103)	195 (190)	564 (338)	-
Conventional (5 + 7.5 mg kg^{-1})	38.8 (9.6)	145 (99)	162 (116)	98 (162)	**82.6 (39.5)**
Ivm					
Untreated controls	44.8 (6.8)	104 (63)	87 (53)	207 (184)	-
Conventional (0.2 mg kg^{-1})	45.3 (5.1)	94 (74)	95 (108)	0.0	**100 (100)**
Lev					
Untreated controls	39.4 (6.5)	121 (48)	188 (76)	602 (306)	-
Conventional (7.5 mg kg^{-1})	39.2 (4.1)	117 (59)	120 (58)	323 (212)	**46.4 (0)**
Fbz					
Untreated controls	40.1 (3.3)	107 (82)	114 (115)	498 (255)	-
Piperonyl butoxide (63 mg kg^{-1})	39.3 (6.9)	115 (134)	163 (106)	362 (167)	-
Conventional (5 mg kg^{-1})	40.0 (6.3)	113 (158)	70 (94)	449 (262)	**0 (0)***
Fbz (5 mg kg^{-1}) + Piperonyl butoxide (63 mg kg^{-1})	39.4 (4.2)	105 (104)	145 (142)	201 (113)	**53.2 (0)***

*Using pooled faecal egg counts of untreated and PB controls

Table 4.14 *1995 CET faecal egg count reductions calculated using mean egg counts of the treatment and control groups on day 10-14, WAAVP, or by comparison to the treatment group count on day 0*

Treatment (n = 6)	Weight (kg) (± SD) day -2	EPG (± SD) day -2	EPG (± SD) day 0	EPG (± SD) day 14	% Reduction WAAVP (day 0)
Fbz/Lev					
Untreated controls	40.9 (5.2)	88 (52)	67 (56)	410 (203)	-
Conventional (5 + 7.5 mg kg^{-1})	40.9 (4.3)	90 (45)	95 (80)	34 (37)	**91.9 (64.2)**
Ivm					
Untreated controls	42.6 (4.0)	56 (24)	55 (36)	335 (136)	-
Conventional (0.2 mg kg^{-1})	42.4 (7.2)	55 (40)	78 (69)	0.2 (0.4)	**99.9 (99.7)**
Lev					
Untreated controls	41.0 (3.9)	62 (57)	61 (44)	333 (179)	-
Conventional (7.5 mg kg^{-1})	40.5 (5.8)	49 (25)	74 (65)	163 (142)	**51.2 (0)**
No feed (24 hours) followed by (7.5 mg kg^{-1})	40.3 (5.5)	52 (22)	97 (74)	90 (86)	**73.0 (7.2)**
Fbz					
Untreated controls	40.7 (3.2)	74 (60)	56 (35)	365 (118)	-
Conventional (5 mg kg^{-1})	38.1 (6.7)	70 (53)	75 (41)	267 (174)	**26.7 (0)**
No feed (24 hours) followed by divided dose (5 + 5 mg kg^{-1}) (24 hour interval)	40.1 (5.8)	71 (54)	92 (101)	306 (183)	**16.2 (0)**

4.3.2.2 Abomasal worm burdens

Only *T.circumcincta* worms were identified from lamb in these studies. Figure 4.1 shows the *T.circumcincta* burdens for the Fbz/Lev, Lev and Ivm treated groups in the 1993 end of season CET. The effect of feed withdrawal upon the treatment efficacy of Fbz was also investigated in this year and is shown in Figure 4.2. Individual *T.circumcincta* burdens for the treatment groups in 1993 are shown in Tables 4.15 - 4.18

Figure 4.3 shows the *T.circumcincta* burdens for the Fbz/Lev, Lev and Ivm treated groups in the 1994 end of season CET. The effect of co-administering piperonyl butoxide upon the treatment efficacy of Fbz was also investigated in 1994 and is shown in Figure 4.4. Individual *T.circumcincta* burdens for the treatment groups in 1994 are shown in Tables 4.19 - 4.22.

Figure 4.5 shows the *T.circumcincta* burdens for the Fbz/Lev and Ivm treated groups in the 1995 end of season CET. The synergistic effect of feed withdrawal and divided dosing upon the treatment efficacy of Fbz and feed withdrawal upon the treatment efficacy of Lev were also investigated in this year and are shown in Figures 4.6 and 4.7. Individual *T.circumcincta* burdens for the treatment groups in 1995 are shown in Tables 4.23 - 4.26.

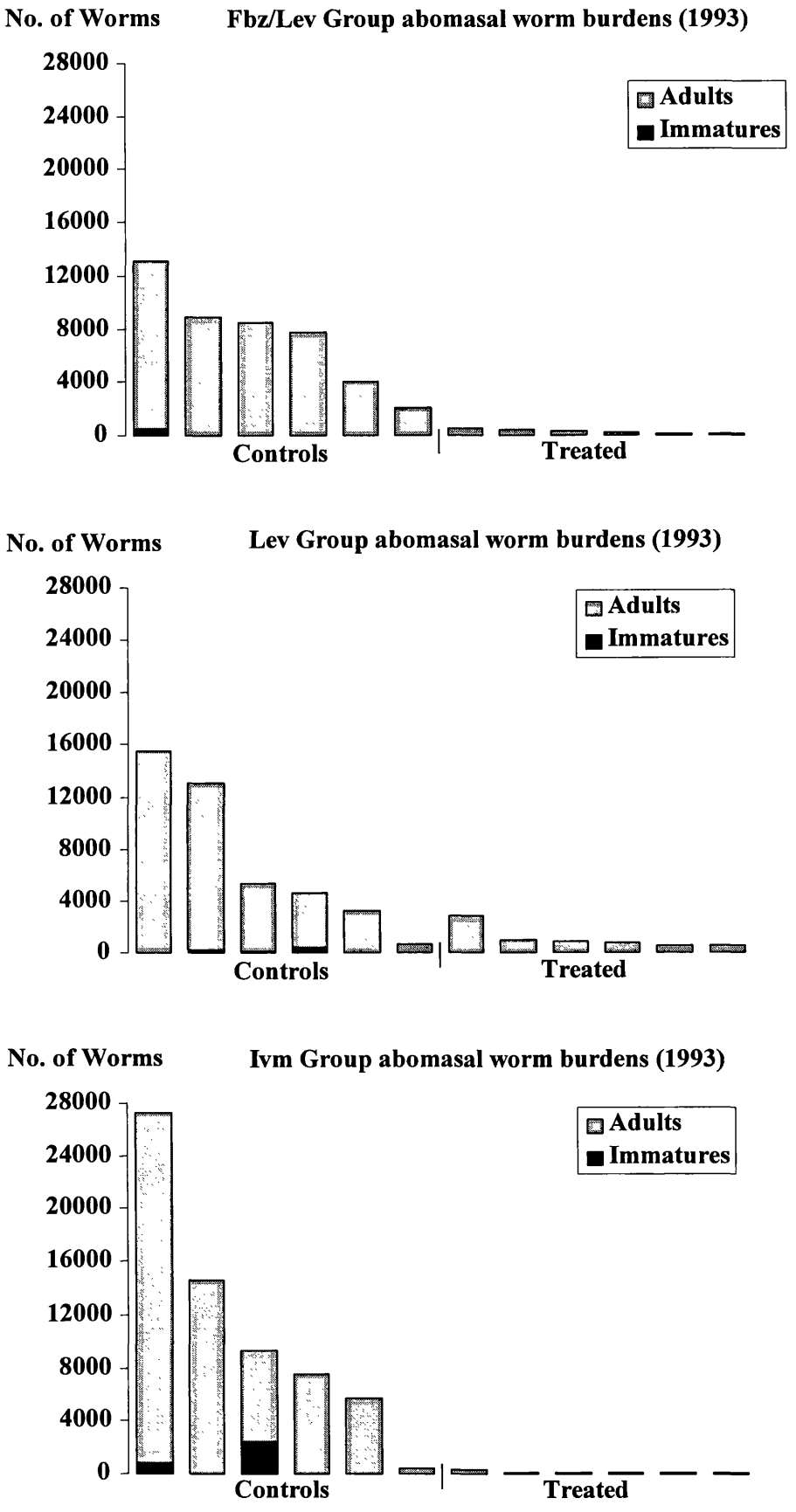

Figure 4.1 *T.circumcincta burdens for the Fbz/Lev, Lev and Ivm treated groups in the 1993 end of season CET (Day 14)*

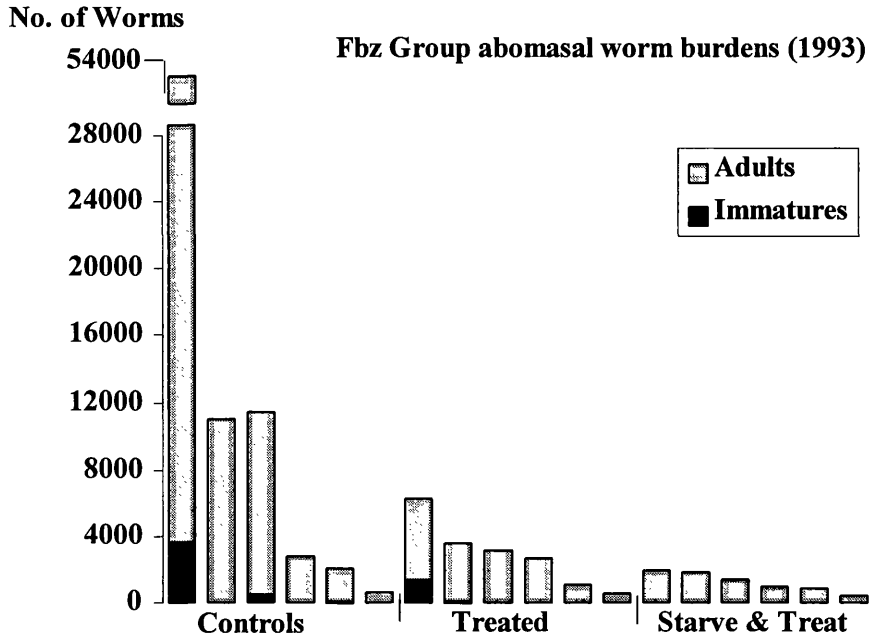

Figure 4.2 *Abomasal worm burdens of sheep naturally infected with Bz-resistant T. circumcincta, treated with Fbz either conventionally or following 24 hours of feed withdrawal in the 1993 end of season CET (Day 14)*

Table 4.15 *1993 CET Fbz/Lev group individual T.circumcincta burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	969	3300	5600	0	8900
	1004	1400	2650	0	4050
	1197	5350	6750	500	12600
Untreated controls	1198	2900	4800	0	7700
	1258	800	1150	50	2000
	1259	4300	4050	50	8400
	Mean	**3008**	**4167**	**100**	**7275 (± 3759)**
	970	150	400	0	550
	1001	100	100	0	200
	1003	100	250	50	400
Fbz/Lev	1005	100	200	0	300
	1257	0	50	0	50
	1260	50	100	0	150
	Mean	**83**	**183**	**8**	**275 (± 181)**

Table 4.16 *1993 CET Lev group individual T.circumcincta burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	358	150	500	0	650
	362	5900	7050	200	13150
	363	1550	2650	400	4600
Untreated controls	368	2450	2700	150	5300
	397	7550	7950	50	15550
	876	1200	2000	50	3250
	Mean	**3133**	**3808**	**142**	**7083 (± 5898)**
	359	200	300	0	500
	360	150	300	50	500
	361	150	550	0	700
Lev	369	400	450	50	900
	396	1050	1750	0	2800
	875	400	400	0	800
	Mean	**392**	**625**	**17**	**1033 (± 880)**

Table 4.17 *1993 CET Ivm group individual T.circumcincta burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	888	5950	8650	0	14600
	901	50	400	0	450
	902	2700	4850	0	7550
Untreated controls	918	2000	3650	50	5700
	959	11500	14900	850	27250
	960	3350	3500	2450	9300
	Mean	**4258**	**5992**	**558**	**10808 (± 9284)**
	889	50	50	0	100
	919	0	0	0	0
	920	150	150	0	300
Ivm	921	0	50	0	50
	936	0	0	0	0
	937	0	0	0	0
	Mean	**33**	**42**	**0**	**75 (± 117)**

Table 4.18 *1993 CET Fbz group individual T.circumcincta burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	898	900	1900	0	2800
	926	3900	7050	0	10950
	927	21450	27750	3650	52850
Untreated controls	930	600	1300	100	2000
	944	5900	4950	550	11400
	965	300	350	0	650
	Mean	**5508**	**7217**	**717**	**13442 (± 19855)**
	899	200	350	0	550
	925	1350	1800	0	3150
	932	1100	1600	0	2700
Fbz	933	1500	1950	100	3550
	945	1800	3000	1400	6200
	955	600	500	0	1100
	Mean	**1092**	**1533**	**250**	**2875 (± 2009)**
	924	1000	950	0	1950
	931	250	200	0	450
	956	300	550	50	900
No Feed (24 hours)	962	450	550	0	1000
Fbz	963	700	1100	0	1800
	966	350	1000	50	1400
	Mean	**508**	**725**	**17**	**1250 (± 573)**

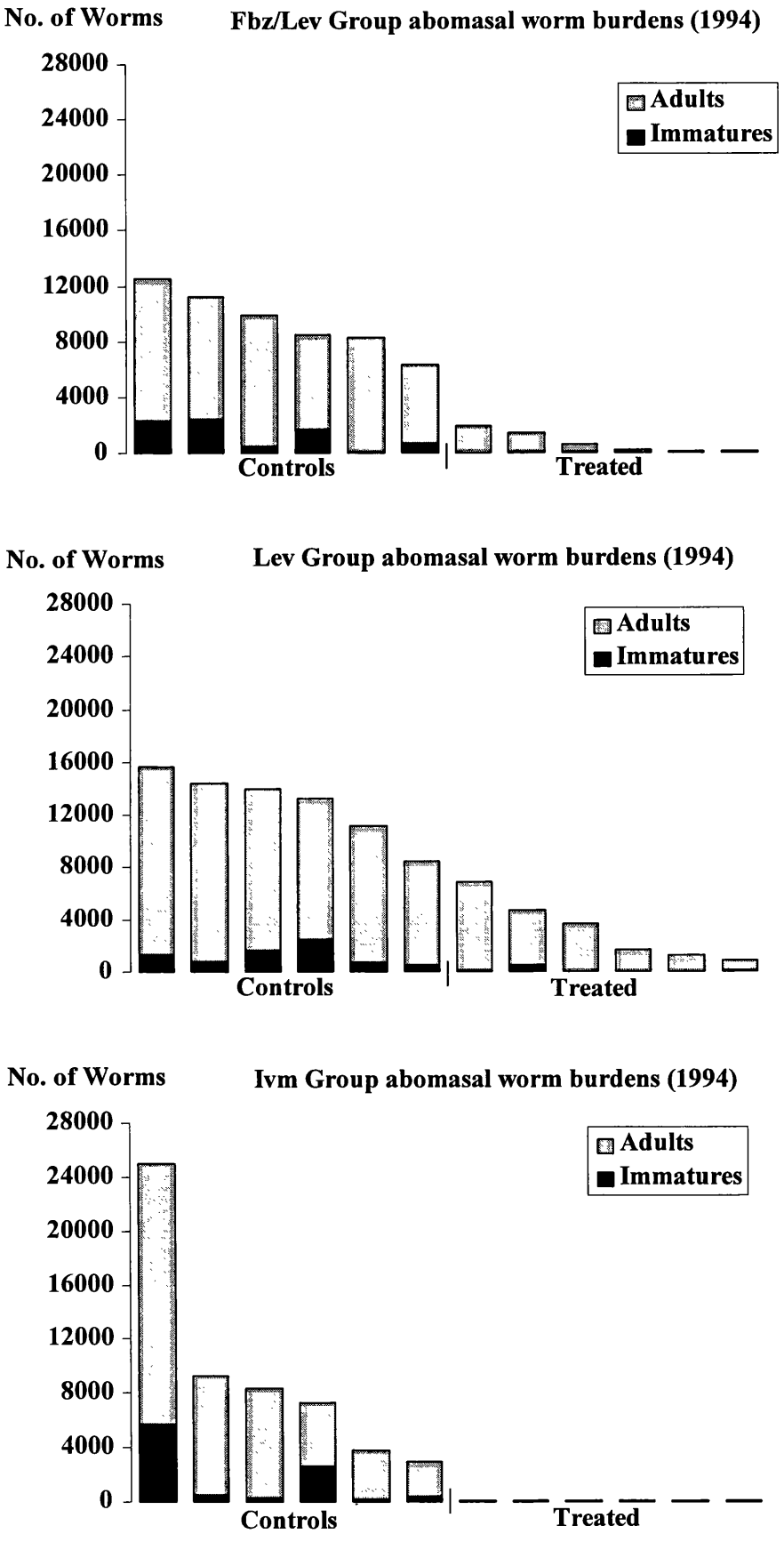

Figure 4.3 *T.circumcincta burdens for the Fbz/Lev, Lev and Ivm treated groups in the 1994 end of season CET (Day 21)*

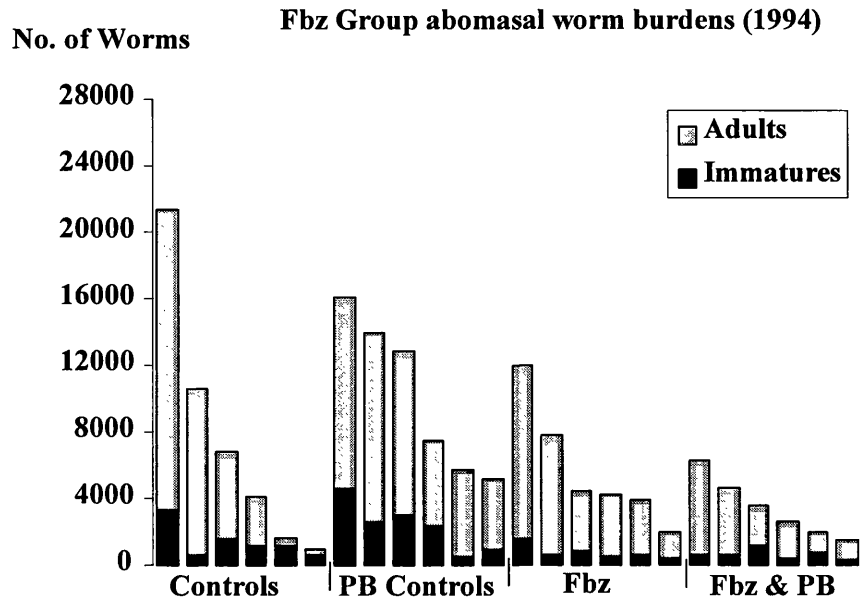

Figure 4.4 *Abomasal worm burdens of sheep naturally infected with Bz-resistant T. circumcincta, treated conventionally with Fbz (5 mg kg^{-1}), with piperonyl butoxide (63 mg kg^{-1}) or a combination (5 + 63 mg kg^{-1}) in the 1994 end of season CET (Day 14)*

Table 4.19 *1994 CET Fbz/Lev group individual T.circumcincta burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1268	3900	5550	500	9950
	1319	2650	4100	1750	8500
	1324	3750	6550	2300	12600
Untreated controls	1517	2950	5200	100	8250
	1538	2100	3450	750	6300
	1726	4250	4450	2500	11200
	Mean	**3267**	**4883**	**1317**	**9467 (± 2258)**
	1260	700	650	100	1450
	1320	0	50	0	50
	1325	0	0	0	0
Fbz/Lev	1539	150	50	50	250
	1547	750	1150	50	1950
	1552	50	400	150	600
	Mean	**275**	**383**	**58**	**717 (± 806)**

Table 4.20 *1994 CET Lev group individual T.circumcincta burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1215	3250	4650	550	8450
	1254	6350	7950	1350	15650
	1524	4050	6350	750	11150
Untreated controls	1578	5650	6650	1700	14000
	1579	6400	7250	800	14450
	1696	4650	6150	2500	13300
	Mean	**5058**	**6500**	**1275**	**12833 (± 2614)**
	1152	200	500	100	800
	1255	2350	4400	150	6900
	1525	500	700	50	1250
Lev	1668	1100	2550	50	3700
	1694	300	1350	50	1700
	1729	1650	2500	550	4700
	Mean	**1017**	**2000**	**158**	**3175(± 2366)**

Table 4.21 *1994 CET Ivm group individual T.circumcincta burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1154	1350	2250	250	3850
	1169	8050	11150	5800	25000
	1184	2000	2600	2700	7300
Untreated controls	1226	4300	4400	550	9250
	1227	1150	1400	400	2950
	1276	3250	4750	350	8350
	Mean	**3350**	**4425**	**1675**	**9450 (± 8016)**
	1155	0	0	0	0
	1160	0	0	0	0
	1170	50	0	0	50
Ivm	1201	0	50	0	50
	1202	0	50	0	50
	1560	100	0	0	100
	Mean	**25**	**17**	**0**	**42 (± 38)**

Table 4.22 *1994 CET Fbz group individual T.circumcincta burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1174	8150	9850	3400	21400
	1197	3900	5950	700	10550
	1253	2700	2550	1600	6850
Untreated controls	1256	100	300	1200	1600
	1257	1050	1950	1150	4150
	1540	100	250	600	950
	Mean	**2667**	**3475**	**1442**	**7583(± 7640)**
	1134	5150	6350	4650	16150
	1179	4850	6500	2550	13900
	1204	2050	2100	1000	5150
PB Controls	1265	2400	2650	2400	7450
	1530	4950	4950	3000	12900
	1565	1900	3250	550	5700
	Mean	**3550**	**4300**	**2358**	**10208(± 4684)**
	1043	1150	2050	650	3850
	1133	3500	6900	1650	12050
	1203	1400	2300	550	4250
Fbz	1266	400	1150	400	1950
	1488	2150	4950	700	7800
	1541	1250	2300	850	4400
	Mean	**1642**	**3275**	**800**	**5717(± 3633)**
	1042	650	550	350	1550
	1196	850	1300	450	2600
	1240	1150	1200	1200	3550
Fbz + PB	1252	2350	3300	650	6300
	1529	1600	2350	700	4650
	1567	400	800	800	2000
	Mean	**1167**	**1583**	**692**	**3442(± 1789)**

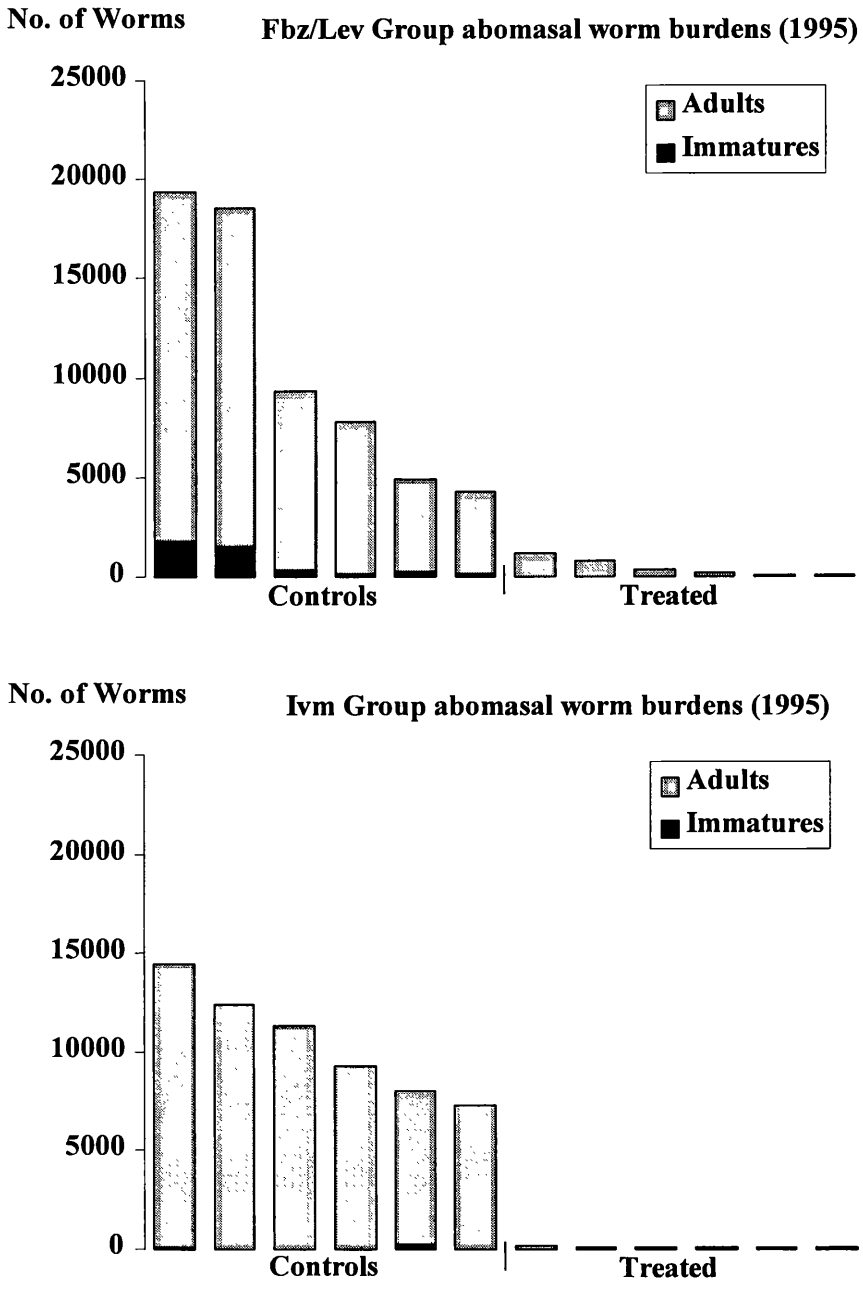

Figure 4.5 *T.circumcincta burdens for the Fbz/Lev and Ivm treated groups in the 1995 end of season CET (Day 21)*

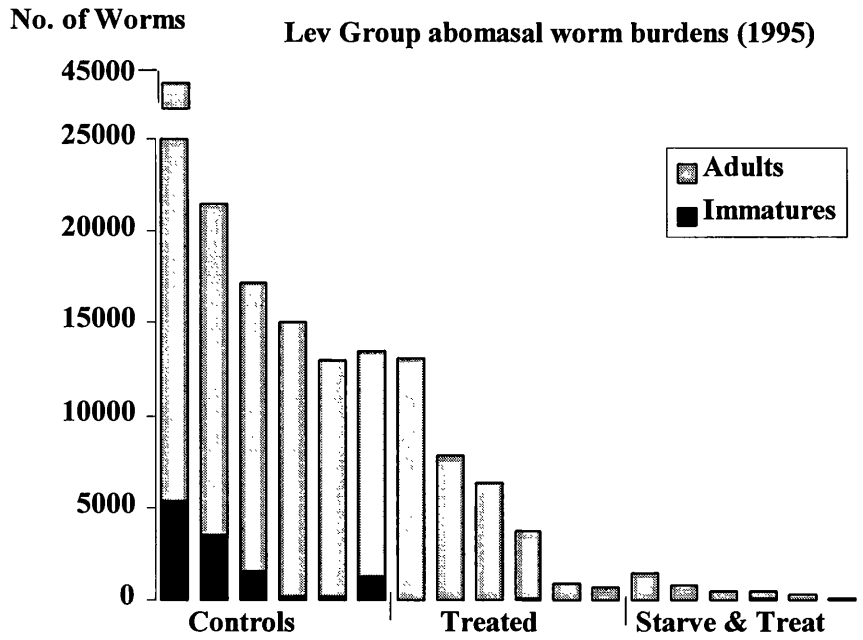

Figure 4.6 *T.circumcincta burdens of naturally infected sheep treated with Lev either conventionally or following 24 hours of feed withdrawal in the 1995 end of season CET (Day 21)*

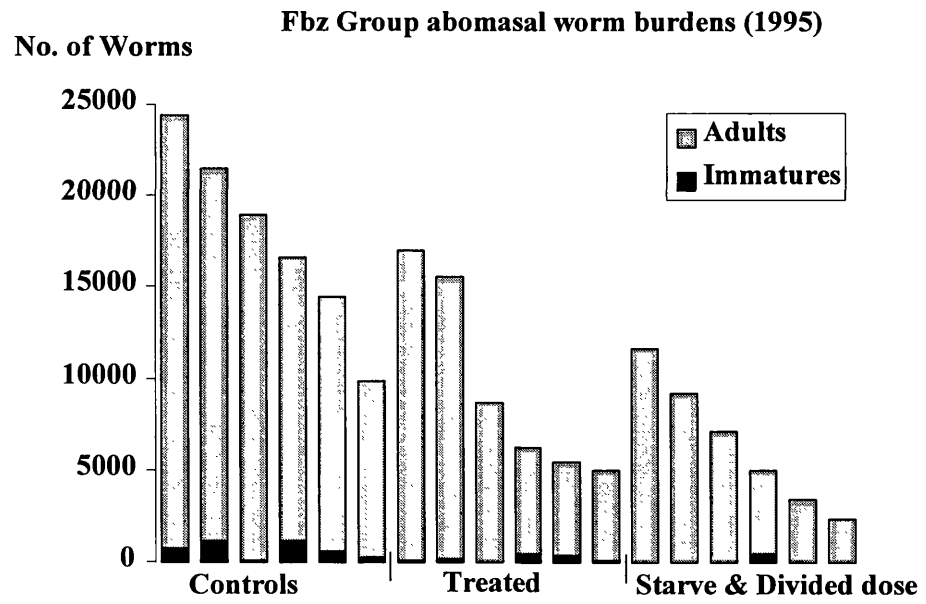

Figure 4.7 *Abomasal worm burdens of sheep naturally infected with Bz-resistant T. circumcincta, treated with Fbz either conventionally (5 mg kg^{-1}) or as a divided double dose following 24 hours of feed withdrawal using a 24 hour treatment interval (5 + 5 mg kg^{-1}), in the 1995 end of season CET (Day 21)*

Table 4.23 *1995 CET Fbz/Lev group individual T.circumcincta burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1429	1900	2700	300	4900
	1486	8450	9150	1800	19400
	1488	1300	2800	150	4250
Untreated controls	1502	8100	8950	1550	18600
	1547	3200	4400	200	7800
	1551	3650	5350	350	9350
	Mean	**4433**	**5558**	**725**	**10717 (± 6687)**
	1485	100	100	0	200
	1501	100	300	0	400
	1511	400	800	0	1200
Fbz/Lev	1525	50	0	0	50
	1526	250	600	0	850
	1545	50	50	0	100
	Mean	**158**	**308**	**0**	**467 (± 462)**

Table 4.24 *1995 CET Ivm group individual T.circumcincta burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1454	3850	5400	0	9250
	1510	3350	4400	250	8000
	1518	5550	6850	0	12400
Untreated controls	1532	6300	8050	100	14450
	1538	5250	6050	0	11300
	1566	3000	4250	0	7250
	Mean	**4550**	**5833**	**58**	**10442 (± 2764)**
	1453	0	0	0	0
	1509	0	50	0	50
	1519	150	50	0	200
Ivm	1531	0	50	0	50
	1539	0	0	0	0
	1553	0	0	0	0
	Mean	**25**	**25**	**0**	**50 (± 77)**

Table 4.25 *1995 CET Lev group individual T.circumcincta burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1375	6350	8450	200	15000
	1440	5250	7500	200	12950
	1465	7650	10300	3500	21450
Untreated controls	1477	4800	7350	1300	13450
	1490	6750	8800	1600	17150
	1797	16700	20600	5400	42700
	Mean	**7917**	**10500**	**2033**	**20450 (± 11331)**
	1387	450	400	0	850
	1438	3100	4750	0	7850
	1439	5500	7550	0	13050
Lev	1458	1550	2100	50	3700
	1489	2950	3350	0	6300
	1498	150	550	0	700
	Mean	**2283**	**3117**	**8**	**5408 (± 4712)**
	1374	250	0	0	250
	1386	0	50	0	50
	1436	300	200	0	500
No Feed (24 hours)	1466	400	1100	0	1500
Lev	1478	200	600	0	800
	1499	100	350	50	500
	Mean	**208**	**383**	**8**	**600 (± 509)**

Table 4.26 *1995 CET Fbz group individual T.circumcincta burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1397	7650	11200	50	18900
	1416	11200	12400	800	24400
	1449	6600	8800	1200	16600
Untreated controls	1455	8550	11700	1200	21450
	1460	4250	5400	250	9900
	1464	5650	8250	550	14450
	Mean	**7317**	**9625**	**675**	**17617 (± 5157)**
	1358	1950	2950	100	5000
	1360	2150	3000	350	5500
	1422	2350	3400	500	6250
Fbz	1441	7900	9050	50	17000
	1448	3550	5150	0	8700
	1459	7450	7950	150	15550
	Mean	**4225**	**5250**	**192**	**9667 (± 5294)**
	1357	4900	6750	0	11650
	1359	2900	4200	0	7100
	1415	3800	5350	0	9150
No Feed (24 hours)	1442	1700	2800	450	4950
Divided dose Fbz	1456	1150	2250	0	3400
	1463	1100	1250	0	2350
	Mean	**2592**	**3767**	**75**	**6433 (± 3553)**

Table 4.27 shows the CET calculated efficacies against abomasal species using geometric and arithmetic mean worm burden data for each of the conventionally treated groups. The efficacy of Ivm, calculated using either geometric or arithmetic mean worm burdens remained highly effective (> 99 %) over the three year study. The Fbz/Lev combination, although not as effective, remained above 95 % when calculated using geometric mean data. Similar figures were achieved using

arithmetic mean data in 1993 and 1995, although in 1994 the combination was only 92.4 % effective. Generally, efficacies calculated using arithmetic mean worm burden data were lower than those resulting from geometric data for each of the anthelmintic treatments. The efficacy of Lev calculated with either method remained at about 75 - 80 % suggesting that resistance to this compound may have been present at the end of the first season. The presence of resistant species to Fbz upon commencement of the study was confirmed with a geometric efficacy of 59 % although the figure achieved using arithmetic data was reasonably higher at 78.6 %. The efficacy for Fbz was lower in 1994 with reductions of 25.7 and 35.7 % using geometric and arithmetic mean data respectively. Efficacies for Fbz were slightly higher in 1995 with reductions in worm burdens of approximately 45 % using either method.

Table 4.27 *End of season CET % abomasal worm reductions following conventional anthelmintic treatment, calculated using geometric (or arithmetic) mean data*

Treatment		1993	1994	1995
		% Efficacy	% Efficacy	% Efficacy
Fbz/Lev	(5 + 7.5 mg kg^{-1})	96.5 (96.2)	98.2 (92.4)	97.0 (95.6)
Lev	(7.5 mg kg^{-1})	82.1 (85.4)	80.7 (75.3)	82.0 (73.5)
Fbz	(5 mg kg^{-1})	59.1 (78.6)	25.7 (35.7)*	49.4 (45.1)
Ivm	(0.2 mg kg^{-1})	99.8 (99.3)	99.8 (99.6)	99.9 (99.5)

* Using pooled worm burdens of untreated and piperonyl butoxide controls

The CET calculated efficacies, using geometric and arithmetic mean data against abomasal species following feed withdrawal, divided dosing and the co-administration of piperonyl butoxide are shown in Table 4.28. Using geometric mean data, withholding feed for 24 hours before drug administration produced an increase in Fbz efficacy of 20 % compared to conventional treatment. Although this increase was only 12 % using arithmetic data, the efficacy of 90.7 % that resulted

from feed withdrawal was somewhat higher than the 79.1 % calculated using geometric data. An increase of over 27 % using geometric data was calculated following the co-administration of the novel Bz synergist piperonyl butoxide. A similar increase was seen using arithmetic data although efficacies were approximately 10 % higher using these figures. A period of feed withdrawal followed by a divided dose, using double the MRD for Fbz and a 24 hour treatment interval, resulted in an increase of approximately 18 % using geometric mean data. The calculated efficacies were very similar using arithmetic mean data as was the increase compared to conventional treatment. The enhancement of approximately 16% seen with Lev treatment following 24 hour feed withdrawal resulted in significantly fewer worms ($P<0.05$) than the conventionally treated lambs. The efficacy of conventional Lev treatment was somewhat lower using arithmetic mean data with a resulting increase in efficacy of approximately 24 % following feed withdrawal.

Table 4.28 *Abomasal worm reductions as a result of feed withdrawal, divided dosing and the co-administration of piperonyl butoxide, calculated using geometric (or arithmetic) mean data*

1993	% Efficacy	1994	% Efficacy	1995	% Efficacy
Fbz		**Fbz**		**Fbz**	
Conventional (5 mg kg^{-1})	59.1 (78.6)	Conventional (5 mg kg^{-1})	25.7* (35.7)*	Conventional (5 mg kg^{-1})	49.4 (45.1)
No feed for 24 hours followed by 5 mg kg^{-1}	79.1 (90.7)	Fbz (5 mg kg^{-1}) + piperonyl butoxide (63 mg kg^{-1})	53.1* (61.3)*	No feed for 24 hours followed by divided dose 5 + 5 mg kg^{-1} (24 hour interval)	67.1 (63.5)
				Lev	
				Conventional (7.5 mg kg^{-1})	82.0 (73.5)
				No feed for 24 hours followed by 7.5 mg kg^{-1}	$97.9^{\dagger}$ $(97.1)^{\dagger}$

* Using pooled worm burdens of untreated and piperonyl butoxide controls

† Significantly fewer worms than conventional treatment $P < 0.05$

4.3.2.3 Small intestinal worm burdens

Only *Trichostrongylus* spp were identified from lambs in these studies and examination of the male worms confirmed that they were *T.vitrinus*. Figure 4.8 shows the *T.vitrinus* burdens for the Fbz/Lev, Lev and Ivm treated groups in the 1993 end of season CET. The effect of feed withdrawal upon the treatment efficacy of Fbz was also investigated in this year and is shown in Figure 4.9. Individual *T.vitrinus* burdens for the treatment groups in 1993 are shown in Tables 4.29 - 4.32.

Figure 4.10 shows the *T.vitrinus* burdens for the Fbz/Lev, Lev and Ivm treated groups in the 1994 end of season CET. The effect of co-administering piperonyl butoxide upon the treatment efficacy of Fbz was also investigated in this year and is shown in Figure 4.11. Individual *T.vitrinus* burdens for the treatment groups in 1994 are shown in Tables 4.33 - 4.36.

Figure 4.12 shows the *T.vitrinus* burdens for the Fbz/Lev and Ivm treated groups in the 1995 end of season CET. The synergistic effect of feed withdrawal and divided dosing upon the treatment efficacy of Fbz and feed withdrawal alone upon the treatment efficacy of Lev were also investigated in this year and are shown in Figures 4.13 and 4.14. Individual *T.vitrinus* burdens for the treatment groups in 1995 are shown in Tables 4.37 - 4.40.

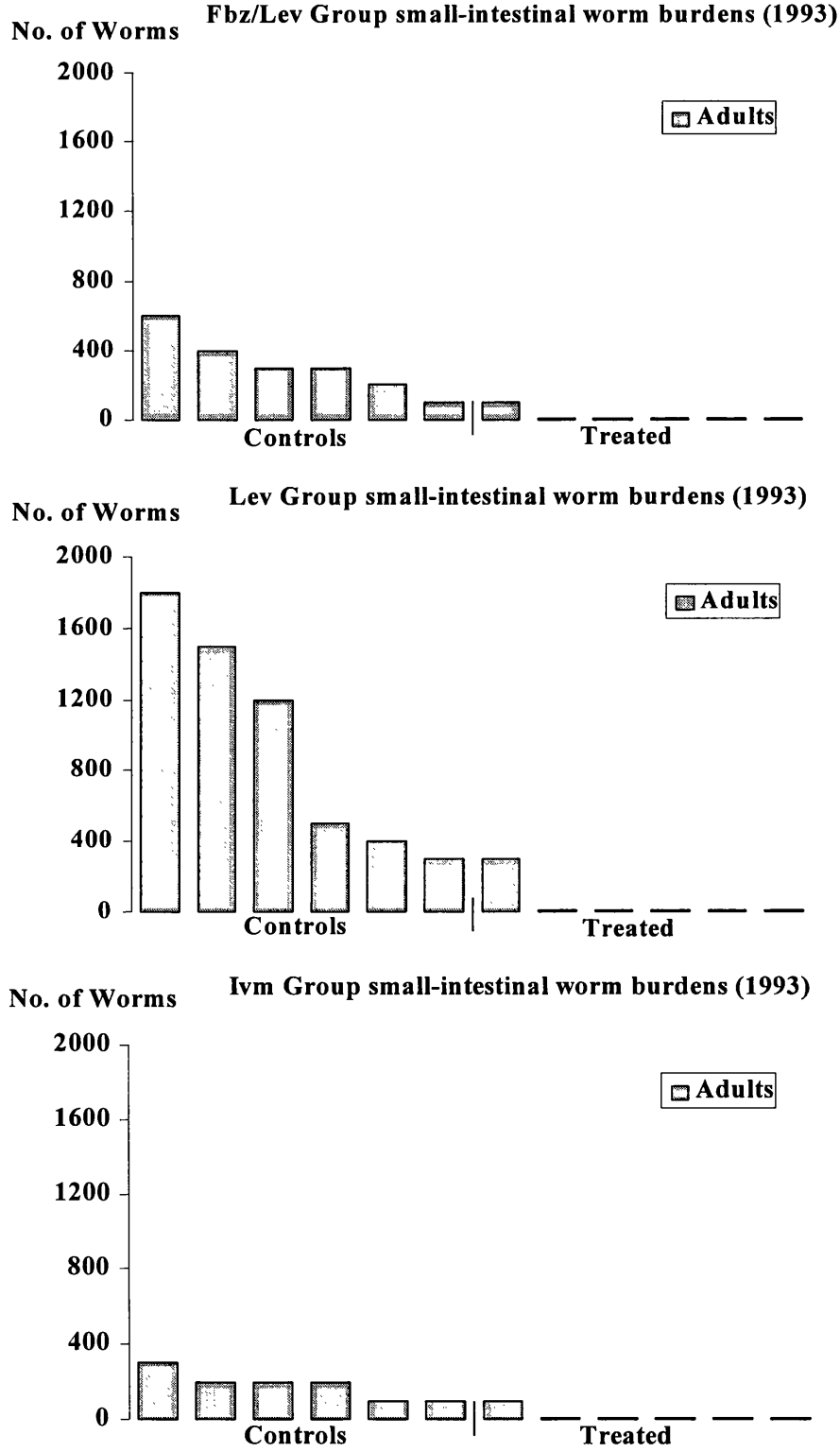

Figure 4.8 *T.vitrinus burdens for the Fbz/Lev, Lev and Ivm treated groups in the 1993 end of season CET (Day 14)*

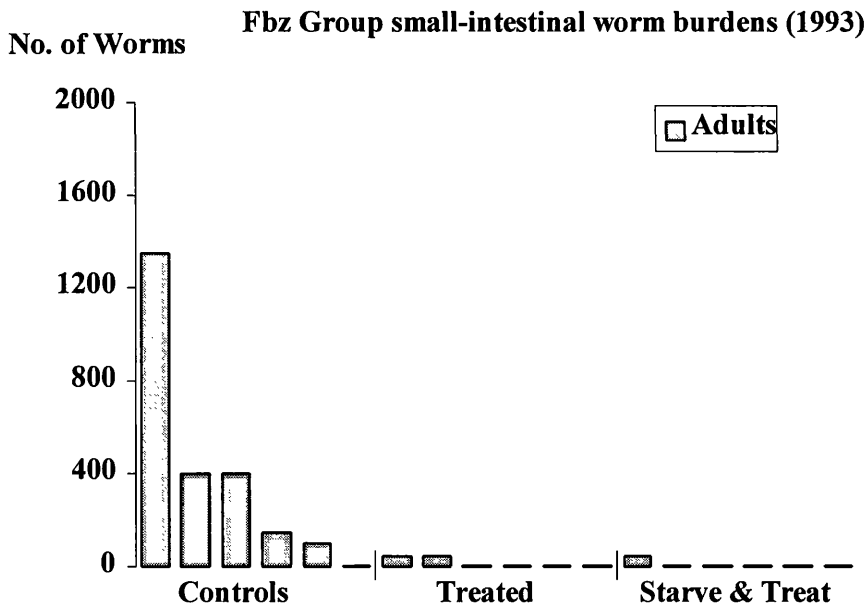

Figure 4.9 *T.vitrinus burdens of naturally infected sheep treated with Fbz either conventionally or following 24 hours of feed withdrawal in the 1993 end of season CET (Day 14)*

Table 4.29 *1993 CET Fbz/Lev group individual T.vitrinus burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	969	200	100	0	300
	1004	100	100	0	200
	1197	400	200	0	600
Untreated controls	1198	300	100	0	400
	1258	0	100	0	100
	1259	100	200	0	300
	Mean	**183**	**133**	**0**	**317 (± 172)**
	970	0	100	0	100
	1001	0	0	0	0
	1003	0	0	0	0
Fbz/Lev	1005	0	0	0	0
	1257	0	0	0	0
	1260	0	0	0	0
	Mean	**0**	**17**	**0**	**17 (± 41)**

Table 4.30 *1993 CET Lev group individual T.vitrinus burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	358	200	100	0	300
	362	800	400	0	1200
	363	800	1000	0	1800
Untreated controls	368	200	200	0	400
	397	400	100	0	500
	876	700	800	0	1500
	Mean	**517**	**433**	**0**	**950 (± 635)**
	359	0	0	0	0
	360	0	0	0	0
	361	0	0	0	0
Lev	369	0	0	0	0
	396	0	0	0	0
	875	300	0	0	300
	Mean	**50**	**0**	**0**	**50 (± 122)**

Table 4.31 *1993 CET Ivm group individual T.vitrinus burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	888	0	200	0	200
	901	100	0	0	100
	902	200	0	0	200
Untreated controls	918	100	200	0	300
	959	200	0	0	200
	960	0	100	0	100
	Mean	**100**	**83**	**0**	**183 (± 75)**
	889	0	0	0	0
	919	0	0	0	0
	920	100	0	0	100
Ivm	921	0	0	0	0
	936	0	0	0	0
	937	0	0	0	0
	Mean	**17**	**0**	**0**	**17 (± 41)**

Table 4.32 *1993 CET Fbz group individual T.vitrinus burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	898	0	0	0	0
	926	200	200	0	400
	927	450	900	0	1350
Untreated controls	930	50	50	0	100
	944	150	250	0	400
	965	50	100	0	150
	Mean	**150**	**250**	**0**	**400 (± 493)**
	899	0	0	0	0
	925	0	0	0	0
	932	0	0	0	0
Fbz	933	0	50	0	50
	945	50	0	0	50
	955	0	0	0	0
	Mean	**8**	**8**	**0**	**17 (± 26)**
	924	0	0	0	0
	931	0	0	0	0
	956	0	0	0	0
No Feed (24 hours)	962	50	0	0	50
Fbz	963	0	0	0	0
	966	0	0	0	0
	Mean	**8**	**0**	**0**	**8 (± 20)**

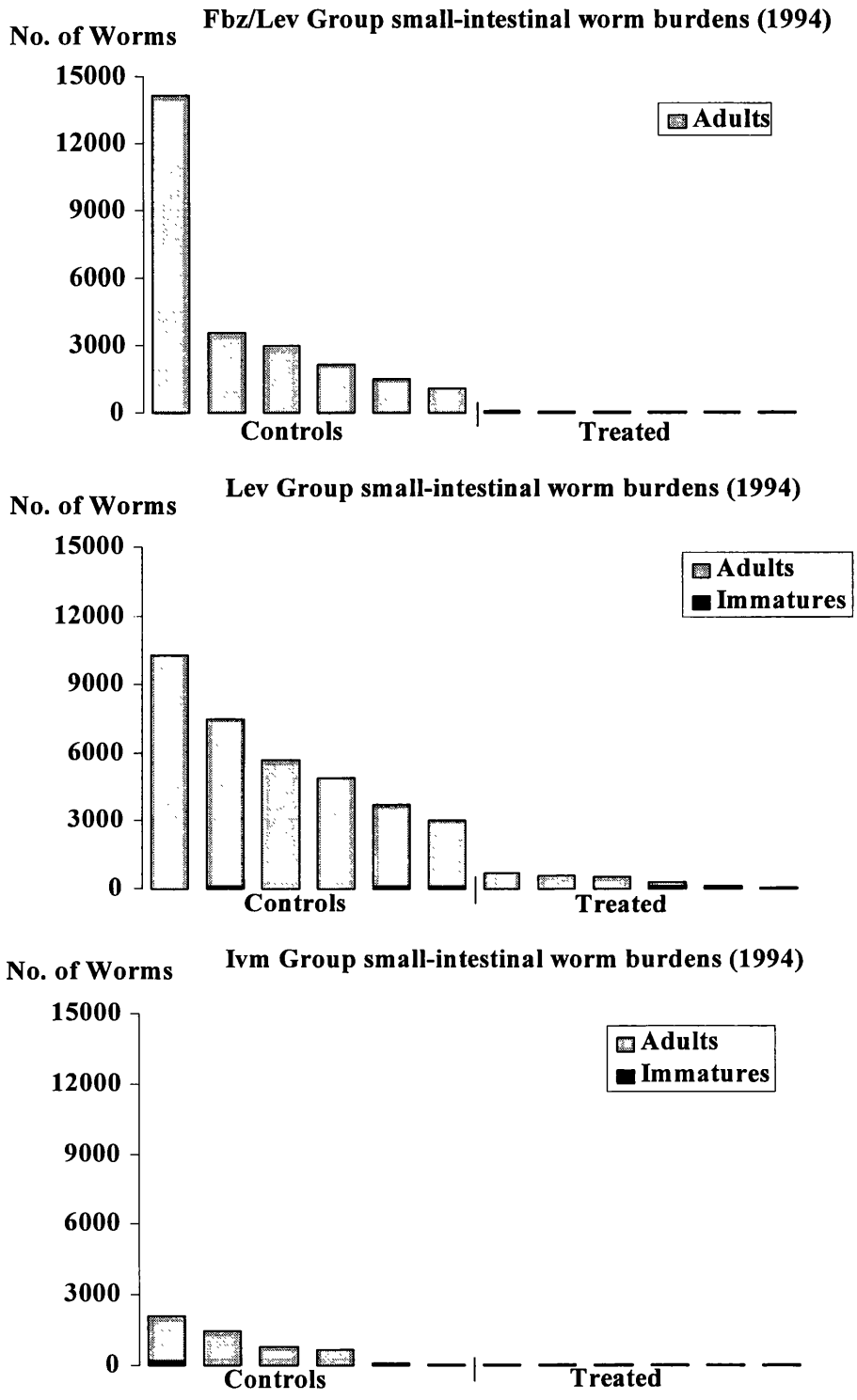

Figure 4.10 *T.vitrinus burdens for the Fbz/Lev, Lev and Ivm treated groups in the 1994 end of season CET (Day 21)*

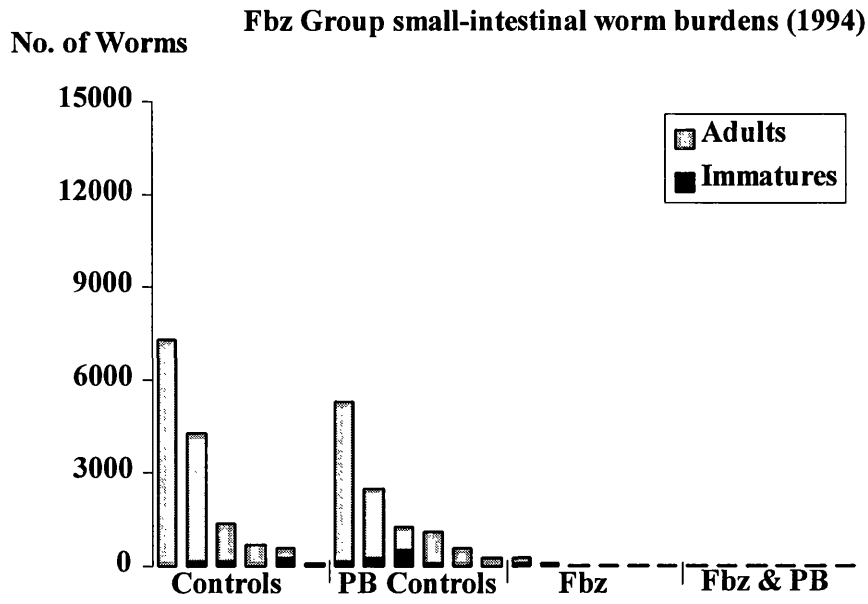

Figure 4.11 *T.vitrinus burdens of naturally infected sheep treated conventionally with Fbz (5 mg kg^{-1}), with piperonyl butoxide (63 mg kg^{-1}) or a combination (5 + 63 mg kg^{-1}) in the 1994 end of season CET (Day 14)*

Table 4.33 *1994 CET Fbz/Lev group individual T.vitrinus burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1268	600	500	0	1100
	1319	900	2100	0	3000
	1324	1200	2400	0	3600
Untreated controls	1517	600	900	0	1500
	1538	600	1500	100	2200
	1726	6700	7500	0	14200
	Mean	**1767**	**2483**	**17**	**4267 (± 4953)**
	1260	0	0	0	0
	1320	0	0	0	0
	1325	0	0	0	0
Fbz/Lev	1539	0	0	0	0
	1547	0	100	0	100
	1552	0	0	0	0
	Mean	**0**	**17**	**0**	**17 (± 41)**

Table 4.34 *1994 CET Lev group individual T.vitrinus burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1215	1300	1600	100	3000
	1254	4100	6200	0	10300
	1524	2000	2900	0	4900
Untreated controls	1578	2000	3700	0	5700
	1579	1400	2200	100	3700
	1696	4000	3400	100	7500
	Mean	**2467**	**3333**	**50**	**5850 (± 2691)**
	1152	100	100	100	300
	1255	200	400	0	600
	1525	100	0	0	100
Lev	1668	400	100	0	500
	1694	0	0	0	0
	1729	200	500	0	700
	Mean	**167**	**183**	**17**	**367 (± 280)**

Table 4.35 *1994 CET Ivm group individual T.vitrinus burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1154	400	400	0	800
	1169	1300	600	200	2100
	1184	1000	500	0	1500
Untreated controls	1226	0	0	0	0
	1227	0	100	0	100
	1276	200	500	0	700
	Mean	**483**	**350**	**33**	**867 (± 812)**
	1155	0	0	0	0
	1160	0	0	0	0
	1170	0	0	0	0
Ivm	1201	0	0	0	0
	1202	0	0	0	0
	1560	0	0	0	0
	Mean	**0**	**0**	**0**	**0 (± 0)**

Table 4.36 *1994 CET Fbz group individual T.vitrinus burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1174	300	0	300	600
	1197	100	0	0	100
	1253	2700	4600	0	7300
Untreated controls	1256	200	500	0	700
	1257	1200	2900	200	4300
	1540	600	600	200	1400
	Mean	**850**	**1433**	**117**	**2400 (± 2830)**
	1134	1200	1000	300	2500
	1179	200	100	0	300
	1204	200	400	0	600
PB Controls	1265	100	900	100	1100
	1530	2200	2900	200	5300
	1565	300	400	600	1300
	Mean	**700**	**950**	**200**	**1850 (± 1852)**
	1043	0	0	0	0
	1133	0	0	0	0
	1203	0	0	0	0
Fbz	1266	0	0	0	0
	1488	0	100	0	100
	1541	100	100	100	300
	Mean	**17**	**33**	**17**	**67 (± 121)**
	1042	0	0	0	0
	1196	0	0	0	0
	1240	0	0	0	0
Fbz + PB	1252	0	0	0	0
	1529	0	0	0	0
	1567	0	0	0	0
	Mean	**0**	**0**	**0**	**0 (± 0)**

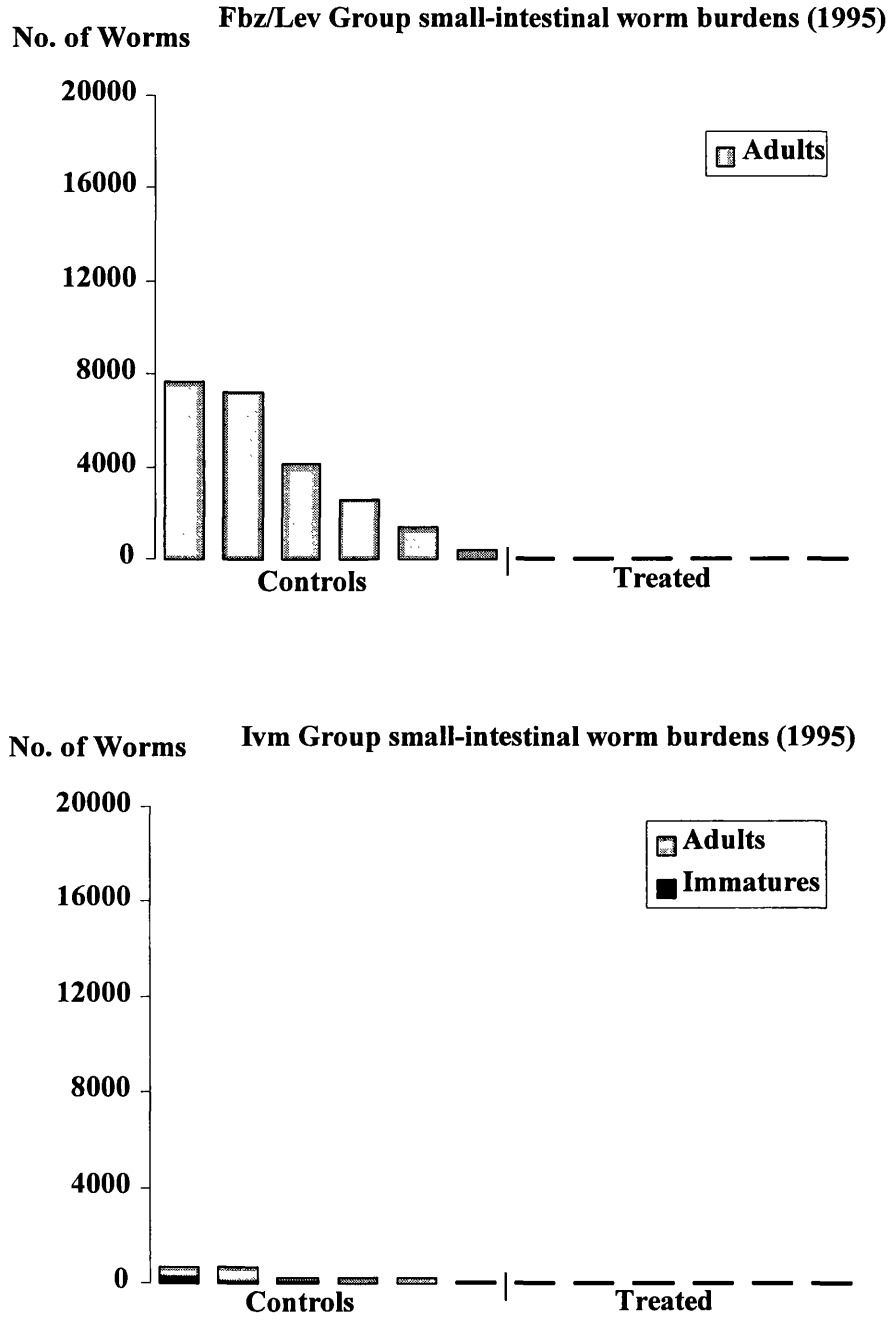

Figure 4.12 *T.vitrinus burdens for the Fbz/Lev and Ivm treated groups in the 1995 end of season CET (Day 21)*

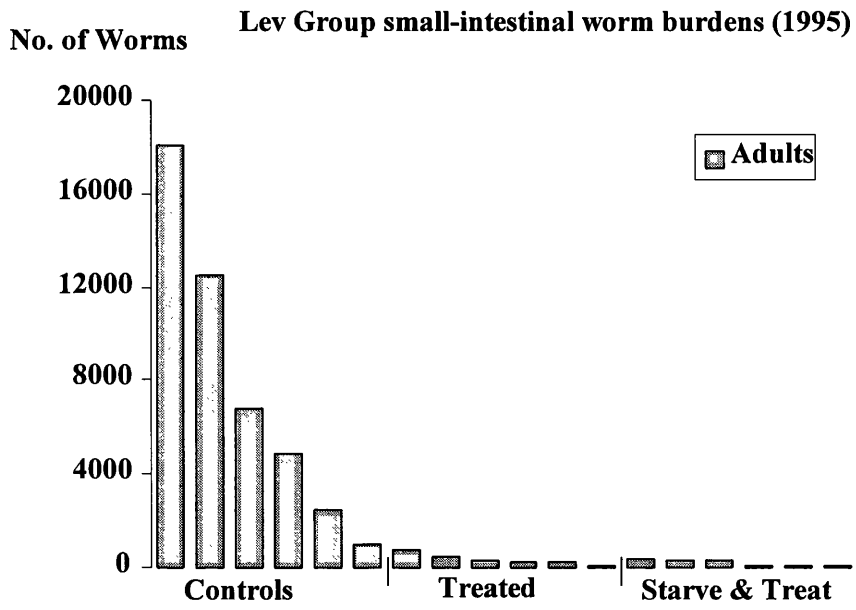

Figure 4.13 *T.vitrinus burdens of naturally infected sheep treated with Lev either conventionally or following 24 hours of feed withdrawal in the 1995 end of season CET (Day 21)*

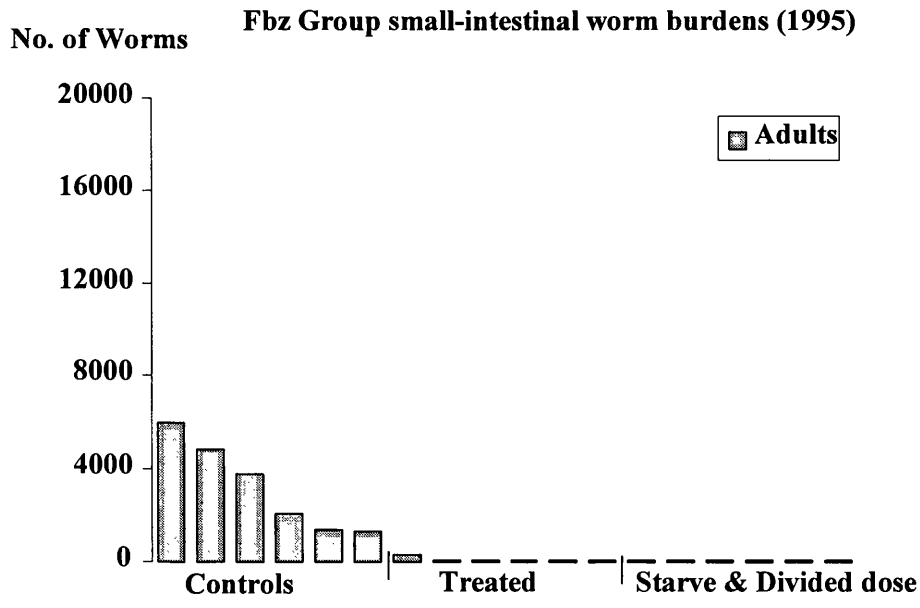

Figure 4.14 *T.vitrinus burdens of naturally infected sheep treated with Fbz either conventionally (5 mg kg^{-1}) or as a divided double dose following 24 hours of feed withdrawal using a 24 hour treatment interval (5 + 5 mg kg^{-1}), in the 1995 end of season CET (Day 21)*

Table 4.37 *1995 CET Fbz/Lev group individual T.vitrinus burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1429	1600	1000	0	2600
	1486	2000	2100	0	4100
	1488	400	1000	0	1400
Untreated controls	1502	2900	4300	0	7200
	1547	100	300	0	400
	1551	3000	4300	400	7700
	Mean	**1667**	**2167**	**67**	**3900 (± 3019)**
	1485	0	0	0	0
	1501	100	0	0	100
	1511	0	0	0	0
Fbz/Lev	1525	0	0	0	0
	1526	100	0	0	100
	1545	0	0	0	0
	Mean	**33**	**0**	**0**	**33 (± 52)**

Table 4.38 *1995 CET Ivm group individual T.vitrinus burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1454	0	100	300	400
	1510	0	0	0	0
	1518	0	0	100	100
Untreated controls	1532	200	0	0	200
	1538	100	400	100	600
	1566	0	200	0	200
	Mean	**50**	**117**	**83**	**250 (± 217)**
	1453	0	0	0	0
	1509	0	0	0	0
	1519	0	0	0	0
Ivm	1531	0	0	0	0
	1539	0	0	0	0
	1553	0	100	0	100
	Mean	**0**	**17**	**0**	**17 (± 41)**

Table 4.39 *1995 CET Lev group individual T.vitrinus burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1375	1300	900	300	2500
	1440	2400	2300	200	4900
	1465	5100	7400	0	12500
Untreated controls	1477	2800	3500	500	6800
	1490	400	600	0	1000
	1797	6200	6000	5900	18100
	Mean	**3034**	**3450**	**1150**	**7634 (± 6507)**
	1387	400	100	0	500
	1438	200	0	0	200
	1439	300	500	0	800
Lev	1458	100	100	0	200
	1489	200	100	0	300
	1498	0	0	0	0
	Mean	**200**	**134**	**0**	**334 (± 280)**
	1374	0	0	0	0
	1386	0	0	0	0
	1436	200	100	0	300
No Feed (24 hours)	1466	400	0	0	400
Lev	1478	100	0	0	100
	1499	100	200	0	300
	Mean	**134**	**50**	**0**	**184 (± 172)**

Table 4.40 *1995 CET Fbz group individual T.vitrinus burdens*

Group	Lamb No.	Males	Females	Immatures	Total (± SD)
	1397	600	800	0	1400
	1416	2500	2400	0	4900
	1449	2700	3300	0	6000
Untreated controls	1455	1000	1100	0	2100
	1460	500	800	0	1300
	1464	1700	2100	0	3800
	Mean	**1500**	**1750**	**0**	**3250 (± 1956)**
	1358	0	0	0	0
	1360	0	0	0	0
	1422	0	100	0	100
Fbz	1441	100	200	0	300
	1448	0	0	0	0
	1459	100	0	0	100
	Mean	**34**	**50**	**0**	**84 (± 116)**
	1357	0	0	0	0
	1359	0	0	0	0
	1415	0	0	0	0
No Feed (24 hours)	1442	0	0	0	0
Divided dose Fbz	1456	0	0	0	0
	1463	0	0	0	0
	Mean	**0**	**0**	**0**	**0 (± 0)**

Table 4.41 shows the CET calculated efficacies against *T.vitrinus* using geometric and arithmetic mean worm burden data for each of the conventionally treated groups. The calculated efficacies for each of the anthelmintic treatments was greater than 97.5 % using geometric mean data. These figures were lower using arithmetic mean data and in some cases were below 95 % suggesting that drug resistant *T.vitrinus* may well be present on the paddocks. The CET calculated efficacies, using geometric and arithmetic mean data against *T.vitrinus* following

feed withdrawal, divided dosing and the co-administration of piperonyl butoxide are shown in Table 4.42. Although the conventional treatments in these cases were effective, > 95% using either geometric or arithmetic data, small increases in efficacy were evident using each of the enhancement methods.

Table 4.41 *T.vitrinus reductions (%) following conventional anthelmintic treatment at the end of season CETs, calculated using geometric (or arithmetic) mean data*

Treatment		1993	1994	1995
		% Efficacy	% Efficacy	% Efficacy
Fbz/Lev	(5 + 7.5 mg kg^{-1})	99.6 (94.7)	99.9 (99.6)	99.9 (99.1)
Lev	(7.5 mg kg^{-1})	99.8 (94.7)	97.5 (93.7)	97.5 (95.6)
Fbz	(5 mg kg^{-1})	97.8 (95.8)	99.6 (96.9)	99.6 (97.4)
Ivm	(0.2 mg kg^{-1})	99.3 (90.9)	100 (100)	97.8 (92.9)

Table 4.42 *T.vitrinus reductions as a result of feed withdrawal, divided dosing and the co-administration of piperonyl butoxide compared to conventional treatment, calculated using geometric (or arithmetic) mean data*

1993	% Efficacy	1994	% Efficacy	1995	% Efficacy
Fbz		**Fbz**		**Fbz**	
Conventional (5 mg kg^{-1})	97.8 (95.8)	Conventional (5 mg kg^{-1})	99.6* (96.9)*	Conventional (5 mg kg^{-1})	99.6 (97.4)
No feed for 24 hours followed by 5 mg kg^{-1}	99.2 (97.9)	Fbz (5 mg kg^{-1}) + piperonyl butoxide (63 mg kg^{-1})	100* (100)*	No feed for 24 hours followed by divided dose 5 + 5 mg kg^{-1} (24 hour interval)	100 (100)
				Lev	
				Conventional (7.5 mg kg^{-1})	97.5 (95.6)
				No feed for 24 hours followed by 7.5 mg kg^{-1}	99.3 (97.6)

* Using pooled worm burdens of untreated and piperonyl butoxide controls

Comparisons between the geometric mean abomasal and small intestinal worm burdens of the untreated control animals for each treatment group at the end of each season are shown in Tables 4.43 to 4.45. The % of the total worm burdens which were *T. vitrinus* are also included.

Table 4.43 *1993 CET geometric mean worm burdens, including % of total burden which were T. vitrinus, of untreated control lambs from each treatment paddock*

Comparator	Abomasal worm burden (± SD)	Small intestinal worm burden (± SD)	% *T. vitrinus*
Fbz/Lev	6235 (3759)	275 (172)	4.2
Lev	4682 (5898)	762 (635) Fbz/Lev* Ivm**	14.0
Fbz	5372 (19855)	122 (493)	2.2
Ivm	6445 (9284)	170 (75)	2.6

*Significantly fewer worms than comparator $P < 0.05$, ** $P < 0.01$

Table 4.44 *1994 CET geometric mean worm burdens, including % of total burden which were T. vitrinus, of untreated control lambs from each treatment paddock*

Comparator	Abomasal worm burden (± SD)	Small intestinal worm burden (± SD)	% *T. vitrinus*
Fbz/Lev	9235 (2258)	2869 (4953)	23.7
Lev	12582 (2614) Fbz/Lev*	5369 (2691) Ivm* Fbz*	29.9
Fbz †	6545 (6195)	1166 (2298)	15.1
Ivm	7368 (8016)	238 (812)	3.1

* Significantly fewer worms than comparator $P< 0.05$

† Using pooled worm burdens of untreated and piperonyl butoxide controls

Table 4.45 *1995 CET geometric mean worm burdens, including % of total burden which were T. vitrinus, of untreated control lambs from each treatment paddock*

Comparator	Abomasal worm burden (± SD)	Small intestinal worm burden (± SD)	% *T. vitrinus*
Fbz/Lev	9046 (6687)	2631 (3019) Ivm*	22.5
Lev	18573 (11331) Fbz/Lev* Ivm*	5161 (6508) Ivm**	21.7
Fbz	16924 (5157) Ivm*	2746 (1956) Ivm**	14.0
Ivm	10139 (2764)	100 (217)	1.0

*significantly fewer worms than comparator $P < 0.05$, ** $P < 0.01$

By the end of the first grazing season there were no differences in the *T.circumcincta* burdens of the untreated control lambs from each of the treatment paddocks. However, untreated control animals from the Lev treatment paddock did have significantly more *T. vitrinus* burdens than those from the Fbz/Lev ($P<0.05$) and Ivm paddocks ($P<0.01$). The abomasal and small intestinal worm burdens of the untreated lambs from each of the treatment paddocks had increased at the end of season CET in 1994. The increases seen with *T. vitrinus* burdens were significantly higher ($P<0.01$) for each of the untreated groups in 1994 compared to those of the untreated animals in 1993 except for the Ivm group. Untreated lambs from the Lev group had significantly higher ($P<0.05$) *T.circumcincta* burdens than the Fbz/Lev group and *T. vitrinus* burdens than both the Fbz and Ivm groups. Post mortem examination of the untreated lambs in the 1995 end of season CET revealed similar levels of abomasal and small intestinal worm burdens to those of 1994. However, untreated lambs from the Ivm group once again displayed lower *T. vitrinus* burdens which were significantly less ($P<0.05$) than each of the other treatment group controls. In addition, the Ivm controls were carrying significantly fewer ($P<0.05$) *T.circumcincta* than the Fbz and Lev controls, the latter of which had significantly more ($P<0.05$) worms than the Fbz/Lev group.

4.4 Discussion

The anthelmintic treatments administered in May of each year, and July 1995, provided good control of *N. battus* with each compound approaching 100 % efficacy against this species. The Lev treatment given in May 1995 however resulted in an efficacy of less than 90 %. This may be explained by the post-treatment sample being taken on day 14 which is close to the prepatent period for this species. This is supported by the second seasonal treatment given in July of this year which, on day 10 post-treatment, resulted in an efficacy of 99.9 %.

Early and mid-season FECRTs could not be calculated using day 10-14 post-treatment faecal egg counts of untreated control animals, as suggested by WAAVP guidelines (Coles *et al.*, 1992), due to constraints of the study design. Nevertheless,

in May 1993 and 1995 the control group were treated at a later date and, although the faecal egg counts of these animals are not directly comparable, they do give some indication of the dynamics of egg production over these periods. Comparisons can also be made for the mid-seasonal treatments given in 1994 and 1995 since the controls were not treated on these dates. Table 4.46 shows the trichostrongylid faecal egg count reductions for each group calculated using counts of either the treated animals on day zero or according to WAAVP guidelines (Coles *et al.*, 1992) using counts for the untreated control lambs on day 10 - 14.

Table 4.46 *Anthelmintic efficacies against trichostrongyles calculated by comparing arithmetic mean faecal egg counts of the treatment groups on day 0 or to the untreated control group on day 10-14 (WAAVP)*

Treatment	May 1993	August 1994	May 1995	July 1995
	day 0 (WAAVP)	day 0 (WAAVP)	day 0 (WAAVP)	day 0 (WAAVP)
Fbz/Lev	96.8 (93.2)	96.2 (96.9)	93.1 (89.8)	94.1 (93.7)
Lev	99.2 (98.1)	60.9 (66.3)	39.3 (56.6)	76.7 (87.4)
Fbz	75.5 (77.6)	72.1* (80.2)*	23.7 (31.9)	55.8 (53.7)
Ivm	99.9 (99.9)	99.9 (99.9)	100 (100)	99.9 (99.9)

* Using average faecal egg counts of feed withdrawal, divided dose and conventional Fbz treatments

The efficacies calculated using either approach correlate very well and lend support to the validity of the values obtained using pre-treatment faecal egg counts. McKenna (1990a) also found a very good relationship between faecal egg count reductions calculated using pre-treatment counts compared to post-treatment control counts in his study which analysed the data of 149 published trials.

Results of the trichostrongylid FECRTs have conclusively demonstrated the presence of Bz resistance on the paddocks at Firth Mains and suggest that resistance to Lev has developed since the start of the study. Resistance to the Fbz/Lev combination was also suspected in the latter treatments of the study. Ivm on the otherhand remained fully effective throughout the study with faecal egg count reductions in excess of 99.9 % on each occasion. In every case where faecal egg count reductions were less than 95% the lower 95% confidence limit was below 90%

and, as suggested by McKenna (1994), is of little practical purpose when dealing with reductions below this level.

The FECRTs indicated that the magnitude of resistance increased with successive Lev treatments. However, caution must be taken when assessing efficacies with Lev since there is a potential for misinterpretation of FECRT results. Grimshaw *et al.* (1996) noted that faecal egg counts taken 11 or more days after treatment with Lev may allow time for development of immature stages to egg producing adults. This may account for the particularly poor efficacy seen in May 1995 which was calculated on day 14 post treatment. Grimshaw *et al.* (1996) concluded that it may be necessary to take faecal samples no later than 7 days post-treatment to avoid false positive results with Lev. It is questionable therefore, whether resistance was present to this class of drug throughout the study since faecal egg counts were determined, at the earliest, on day 10. The average pre-treatment faecal egg counts for the Lev group in July 1995 were less than 100 EPG which would also affect the reliability of the test on this occasion.

The present study has shown that FECRTs do have their limitations when dealing with naturally infected animals, particularly when applied to species with a relatively low fecundity such as *Teladorsagia* whose egg counts tend to be stereotypic (Michel 1969; Jackson & Christie 1979; Barger, 1987). Furthermore, the fecundity of parasites capable of surviving anthelmintic treatment appears to be variable, thereby reducing the value of the test as a quantitative assay. For example, McKellar, Bogan, Horspool & Reid (1988) noted that the numbers of eggs *in utero* of *Cooperia* were markedly reduced following treatment with Ivm. Scott, Baxter & Armour (1991) on the otherhand noted that the numbers of eggs *in utero* increased in adult multiple resistant *Haemonchus* within 7 days post-treatment with Ivm but were lower following the administration of oxfendazole. Ovosuppression not only occurs in response to anthelmintic treatment but may also be a feature of host immunoregulatory responses. Faecal egg counts are unlikely therefore to provide an accurate measure of the number of parasites present and as a result FECRTs can provide evidence of resistance but not a measure of its extent.

Infection levels of the animals under study should also be considered when evaluating FECRTs and CETs. Compared to non-parasitised animals, Marriner,

Evans & Bogan (1984/85) observed reduced AUCs of Fbz in parasitized sheep. These workers suggested that the reduced bioavailability was due to a combination of increased gut motility and increased abomasal pH in the parasitized animals, resulting in a shorter residence time for drug absorption and reduced solubility at the higher pH's.

Despite its inherent weaknesses the FECRT offers the considerable advantages of simplicity and applicability and is also relatively inexpensive. The results of the feed withdrawal and divided dosing studies carried out in August 1994 clearly demonstrate the value of the technique in field investigations upon Fbz treatment efficacy. Administering the MRD as two 2.5 mg kg^{-1} bodyweight doses divided by a 12 hour interval resulted in an increase in efficacy of over 28 %, whereas withholding feed for 24 hours before treatment at the MRD resulted in an increase of 39.7 % ($P < 0.05$) compared to conventionally treated sheep. One criticism of this study is that each of the treatment groups consisted of only 6 lambs whereas 15 animals and an untreated control group are required to satisfy WAAVP guidelines (Coles *et al.*, 1992). Although this was not possible due to the overall study design, as discussed, the use of treatment counts on day 0 correlated very well with that of untreated control counts in this study.

The FECRTs performed in conjunction with the 1993, 1994 and 1995 CETs, determined according to WAAVP guidelines provided interesting results. Invariably the FECs of the untreated control lambs increased markedly upon housing. The FECs of the Fbz and Lev treated animals also increased upon housing with consequent decreases in calculated efficacy. FECs did not increase to the same degree in the Fbz/Lev treated animals but efficacies were still compromised as a result. Only Ivm treatment was effective in reducing FECs by more than 95 % in these housed studies. It should be noted that in 1993, as a result of one of the untreated control lambs (927x) in the Fbz group having an extremely high faecal egg count on day 14 (1737 EPG), the treatment efficacies for this drug are likely to be an overestimation in this year. Increases in faecal egg count following the cessation of larval dosing has been noted in previous experimental infections with *Teladorsagia* species (Coop *et al.*, 1982 and 1985). These authors suggested that the antigenic stimulus from incoming larvae may have depressed the egg laying capacity of

existing adult worm populations and/or retarded the development of larvae through to the adult stage. The possibility of dietary changes influencing faecal egg concentration was also considered. Faecal consistencies did not change upon housing in this study and it seems more likely that the increase in FECs may have been caused by a relaxation in immunity resulting from the removal of any antigenic stimulus. Furthermore, since it is well established that there are density dependent effects upon faecal egg production with *Teladorsagia* spp (Michel 1969; Jackson & Christie 1979; Barger, 1987) the validity of such reductions is questionable.

The definitive test for determining drug susceptibility/resistance to all stages of development is the CET. However, as with the FECRTs, the immunoresponsiveness of the lambs will exert some influence upon calculated treatment efficacies. This was particularly evident from the range of worm burdens seen in the untreated control animals in the CETs conducted at the end of each grazing season. CET treatment efficacies are likely to be underestimated when using older naturally infected control animals compared to parasite naive lambs. This statement is supported by the study of Benchaoui & McKellar (1994) who demonstrated an increase in worm reduction of over 80% compared to conventional Fbz treatment by co-administering piperonyl butoxide whereas the comparable figure was just over 27% in this study. The parasite naive lambs used by Benchaoui & McKellar (1994) were also Suffolk-cross, but more importantly the Bz-resistant *T. circumcincta* used for artificial infection was isolated from the same pastures at Firth Mains.

The question as to whether geometric or arithmetic mean worm burden data should be used in the calculation of CET treatment efficacies has been raised by Dash *et al.* (1988). The credibility of the WAAVP recommendation (Wood *et al.*, 1995) regarding the use of geometric means is undermined by their contradictory support for the use of arithmetic means in the FECRT (Coles *et al.*, 1992). In view of the similarity in the aims of these two procedures Dash *et al.*(1988) concluded that arithmetic data would provide a better estimate of anthelmintic efficacy. Reductions calculated using arithmetic mean values in this present study generally provided a more conservative estimate of anthelmintic efficacy. However, when the drugs were less effective the use of arithmetic estimations of worm burden tended to favour treatment efficacies. It seems therefore, that there is no simple or clear cut

relationship and that a common sense approach is required when evaluating worm burden data. If all the animals in a treatment group have zero burdens apart from one animal then obviously using arithmetic data from this animal would lower the apparent efficacy of a drug. In this case it is likely that the individual is not representative due to some peculiarity in pharmacokinetics or maladministered drug and the use of geometric means would offer a more realistic measure of effectiveness. Resistance is likely to be indicated where several treated animals have worm burdens and under these circumstances geometric or arithmetic data should suffice. It seems logical therefore, that both calculations should be quoted so that large discrepancies can be identified and examined more carefully. This would prevent not only false impressions of anthelmintic success but also misguided conceptions of resistance.

Despite the fact that treatment efficacies are likely to be underestimated when using older naturally infected control animals, increases in Fbz efficacy as a result of feed withdrawal were evident in the end of season CETs. These findings are similar to those seen with FECRTs using smaller treatment groups than WAAVP recommendations. Withholding feed for 24 hours before treatment with Fbz at the MRD resulted in an increase in geometric mean abomasal worm reduction of 20 % compared to conventionally treated sheep. A similar regimen with Lev resulted in an increase in abomasal worm reduction of nearly 16 % compared to conventional treatment which was statistically significant ($P < 0.05$).

A 24 hour period of feed withdrawal followed by double the MRD for Fbz (5 + 5 mg kg^{-1}), with a 24 hour treatment interval produced an increase in abomasal worm reduction of nearly 20 % compared to conventional treatment. This is a similar figure to that achieved using a single MRD of Fbz (5 mg kg^{-1}) following feed withdrawal alone. The reason why no cumulative effect upon Fbz treatment efficacy was seen by combining feed withdrawal and divided dosing may be explained by the extended treatment interval. Administering the MRD in August 1994 as two 2.5 mg kg^{-1} bodyweight doses with a 12 hour interval resulted in an increase in faecal egg count reduction of over 28 % compared to conventional treatment. Although this value was not statistically significant, no increase was seen when a 24 hour interval was used in a previous study with sheep at Moredun (Jackson, Rugutt, Jackson, Coop

& Russell, 1993). Similar findings have also been reported from a study using goats naturally infected with *Haemonchus*, *Ostertagia* and *Trichostrongylus* in which reducing the administration interval for oxfendazole from 24 to 12 hours markedly increased efficacy (Sangster *et al.* 1991b). A 24 hour treatment interval exposes the worms to two discrete intervals of drug which apparently is not as effective as sustained exposure achieved by using a 12 hour interval. In hindsight therefore, a 12 hour interval may have resulted in a cumulative increase following feed withdrawal but consideration must also be given to the practicality of such an approach. It was for this reason that twice the MRD was administered using the 24 hour treatment interval, although no further increase in efficacy resulted. However, simply increasing the dosage rate does not appear to offer a viable solution to the problem of resistant strains. In a previous Australian study, using animals naturally infected with resistant gastrointestinal nematodes, efficacies in a FECRT using groups of goats given single doses of oxfendazole at 10 and 20 mg kg^{-1} increased from 53 to only 66% (Sangster *et al.* 1991b). Considering that the clearance of a single dose follows first order principles (Hennessy 1994), increasing the dosage increases the peak plasma concentration but may not significantly extend drug persistence. Nevertheless, the fact that immunoresponsive lambs were used in the study should also be considered and the use of such a regimen in naive animals deserves further investigation.

The results of using the Fbz/Lev combination, in terms of lamb performance (discussed in chapter 3) and treatment efficacy was very promising despite the presence of Bz-resistant *Teladorsagia* spp on the paddocks at Firth Mains. Reduced efficacy of this combination was evident in the 1995 FECRTs with calculated efficacies of just under 95 % on each occasion. However, results of the CETs against abomasal species saw geometric (arithmetic) reductions of 96.5 (96.2), 98.2 (92.4) and 97.0 (95.6) % in 1993, 1994 and 1995 respectively. The significance of the continued effectiveness of this combination is further emphasized by the suspected development of Lev resistance on the paddock at Firth Mains where this drug was employed. These results support the view of McKenna (1990b) that the selection for multiple resistance is unlikely to be greater than that which would otherwise develop from the use of each component separately. Therefore, although advocated for

slowing down the development of anthelmintic resistance, the benefits of using such combinations when resistance is present are clear. Furthermore, evidence from Australia (Anderson, Martin & Jarrett, 1991; Overend *et al.*, 1994) and New Zealand (McKenna *et al.*, 1996) have shown that the use of Bz-Lev combinations may also be effective on some properties where resistance is present against both anthelmintics.

Analysis of the geometric mean worm burdens of the untreated control lambs from each paddock which were used in the CETs conducted at the end of each season provided some interesting results. The proportion of the total burden that was *T.vitrinus* was highest in all of the untreated groups when the CET was conducted later in the season, a finding in agreement with the epidemiological studies of Boag & Thomas (1977). The untreated control lambs from the Lev treatment paddock had the highest proportion of *T.vitrinus* at the end of the first season. This may have also reflected the numbers of *T.circumcincta* since previous studies have suggested a physiologically mediated negative interaction between these two species (Jackson *et al.*, 1992b). Nevertheless, the untreated control animals in the Lev group had the highest *T.vitrinus* burdens in each year suggesting that the pasture contamination with this species was greatest on this paddock. The proportion of *T.vitrinus* burdens at the end of each season also increased in the untreated control lambs grazing the Fbz/Lev and Fbz treatment paddocks. Interestingly however, the same pattern was not seen in lambs grazing the Ivm treatment paddock where the proportion of *T.vitrinus* in the untreated controls actually decreased over the three year study. In view of these results it is tempting to speculate that *T. vitrinus* may be particularly sensitive to Ivm and to suggest that this class of anthelmintic may offer better control of this species.

Besides highlighting the limitations of both FECRTs and CETs the study has clearly demonstrated the presence of a Bz-resistant isolate of *T. circumcincta* on the paddocks at Firth Mains. Whether the expression of resistance that results from continued use of Bz drugs has increased over the study is very difficult to establish. Whilst it is possible to estimate a resistance factor for a strain by comparing its susceptibility to that of a known susceptible strain (Hunt & Taylor, 1989), this can only be applied to artificial infections. In the case of natural infections the apparent extent of resistance, measured in terms of efficacy, can be influenced by seasonally

variable factors such as climate, pasture contamination and timing of treatment. Also, as demonstrated by the feed withdrawal studies, nutrition can also exert quantitative and qualitative effects upon treatment efficacy (Taylor *et al.*, 1992). Another point that should be considered when assessing resistance, particularly mid- and end of season treatments, is the effect of acquired immunity. As shown in chapter 3, there was evidence that faecal egg output was regulated from mid-season with counts remaining low despite increasing pasture contamination. Therefore, it is not possible to make direct within-season comparisons of anthelmintic efficacies. Furthermore, treatment efficacies in CETs are likely to be underestimated when using older naturally infected control animals. Therefore, although the efficacies of Lev were just over 80 % in the CETs each year, whether or not resistance to this class of drug was present is unclear. Towards the end of the study an attempt was made to resolve these questions and confirm resistance levels for each anthelmintic. Larvae were collected from donor lambs taken from each of the treatment paddocks and used to infect parasite naive lambs, the results of which will be discussed in chapter 6.

CHAPTER 5

Pathogenicity and immunogenicity of different isolates of *Teladorsagia* (*Ostertagia*)

5.1 Introduction

It is possible that the genetic restriction that occurs during the selection of resistance may produce genotypes whose pathogenicity and/or immunogenicity varies from that of unselected isolates. Previous studies (Kelly, Whitlock, Thompson, Hall, Martin & Le Jambre, 1978; Le Jambre, Martin & Jarrett, 1982; MacLean, Lewis & Holmes, 1987; Martin, Anderson, Brown & Miller, 1988; Maingi, Scott & Prichard, 1990) have suggested that changes in the physiological characteristics of a parasite population may be associated with alleles that confer resistance to anthelmintics. In addition to a higher infectivity rate, Kelly *et al.* (1978) reported more severe pathological changes, as measured by packed cell volume, plasma protein concentration and haemoglobin levels, in sheep infected with a Bz-resistant isolate of *H. contortus* compared to that of a susceptible isolate. The development and survival of eggs and free-living stages on pasture and the faecal egg output for the resistant isolate were also higher, as was the exsheathment rate in ruminal fluid. MacLean *et al.* (1987), on the otherhand, reported completely opposite results (lower establishment, fecundity and pathogenicity) when comparing a Bz-resistant to that of a Bz-susceptible isolate of *T. colubriformis*. The view that distinct populations of a parasite may show significant differences in immunogenicity was demonstrated by Goyal & Wakelin (1993). Using two different in-bred strains of mice these workers noted considerable variation in immunogenicity (worm recovery, mast cell, eosinophil and antibody responses) towards three different isolates of *Trichinella spiralis*. Whether the same variability occurs in sheep is more difficult to establish due to host heterogeneity in the immune responses of these animals.

The results of the studies reported in chapter 3 showed that the Bz resistant populations at Firth Mains had limited effects upon lamb performance and elicited a fairly rapid immune response. Since the selection process inevitably produces resistant parasites which are genetically restricted, it is possible that they may vary in their pathogenicity and/or immunogenicity in comparison to unselected susceptible isolates. Previous studies at Moredun using a Bz-susceptible isolate of *T.circumcincta*, Moredun ovine susceptible isolate (MOSI), showed that a daily dose of 4000 L_3 produced reduced growth rates in naive lambs following 3-4 weeks of

infection (Sykes & Coop, 1977). Resistance to the establishment of incoming worms developed between 4-8 weeks in parasite naive lambs given the MOSI at a daily dose of 1000 L_3 (Seaton *et al.*, 1989b). The aim of the present study therefore, was to investigate the effects on performance and development of immunity in lambs infected with different Bz-resistant isolates of *T. circumcincta* compared to that of the MOSI.

5.2 Materials and methods

5.2.1 Infective larvae

The methods used for larval culture, recovery, storage and infection were those described in chapter 2.2.5. Details of the *T. circumcincta* isolates used in the study are described in chapter 2.2.6. The Bz-resistant isolate is referred to as the Moredun ovine resistant isolate (MORI). The multiple resistant (Bz + Ivm) isolate of *T. circumcincta* is referred to as the Sourhope caprine resistant isolate (SCRI). The Bz-susceptible isolate of *T. circumcincta* is referred to as the Moredun ovine susceptible isolate (MOSI).

5.2.2 Experimental Design

Details of the experimental design are summarized in Table 5.1. Twenty six parasite naive Suffolk-cross lambs (average weight 30 kg), aged six months, were allocated into three groups of 6 animals and a control group consisting of 8 animals, balanced in terms of bodyweight and sex. The different *T. circumcincta* isolates were randomly assigned to groups of lambs, each of which received 4000 L_3 *per os* daily for eight weeks. The lambs were fed a ruminant ration daily and hay was supplied *ad libitum*. On days 56 and 57 all of the lambs, including the uninfected controls, were treated with anthelmintic on the basis of liveweight (Levamisole 7.5 mg kg^{-1}, Norbrook Animal Health, UK) in an effort to ensure the removal of all worms including histotrophic stages. One week later the lambs were challenged with 10,000 L_3 of their designated isolate. Previous work at Moredun suggests that the establishment and persistence of incoming larvae and histotrophic stages is relatively

consistent and populations of immature *Teladorsagia* larvae appear to be remarkably stable (W. D. Smith, unpublished data). Similar results have been shown by Armour *et al.* (1966) in their serial kill of lambs infected with 100,000 *O.circumcincta.* Since only 8 worm-free animals were available for controls it was decided that 4 would be challenged with the susceptible isolate 10,000 L_3 (MOSI) and the other 4 with the Bz-resistant isolate (MORI). The lambs were necropsied 10 days post challenge and their abomasa removed and worm burdens estimated using 2% sub-samples (described in chapter 2.3.1). Worms were staged and sexed according to the methods described in chapter 2.3.2.

Table 5.1 *Experimental design of the immunogenicity and pathogenicity study of lambs infected with different isolates of T. circumcincta*

Group (n = 6)	**Primary Infection**	**Day 0 - 56** **Daily dosage**	**Day 56 + 57** **Anthelmintic**	**Day 63** **Challenged**
1*	Controls	–	Levamisole	10,000 L_3 MORI (4)
				10,000 L_3 MOSI (4)
2	MORI	4,000 L_3	Levamisole	10,000 L_3 MORI
3	SCRI	4,000 L_3	Levamisole	10,000 L_3 SCRI
4	MOSI	4,000 L_3	Levamisole	10,000 L_3 MOSI
	FEC - twice weekly Bodyweight - weekly Pepsinogen - fortnightly			Day 73 - post mortem (worm burdens) (mucosal mast cells)

* n = 8

MORI = Moredun ovine resistant isolate (Bz-resistant)
SCRI = Sourhope caprine resistant isolate (Bz + Ivm resistant)
MOSI = Moredun ovine susceptible isolate

5.2.3 Parasitological and production parameters

Lambs were sampled *per rectum* twice weekly with faecal consistencies and egg counts determined using the methods described in chapter 2.2. The animals were weighed weekly (chapter 2.4.1), blood samples were taken fortnightly (chapter 2.4.2) and plasma stored for subsequent pepsinogen analysis (chapter 2.5.1). At slaughter small sections of an abomasal fold were taken from each lamb and the number of mast cells counted as described in chapter 2.8.1. The enumeration of mast cells was

kindly performed by John Huntley from the immunopathology department at Moredun.

5.2.4 Statistical analyses

Differences between treatment group faecal egg count and worm burden reductions were determined by Log_{10} (x+1) transformation prior to analysis of variance (Minitab, version 10.0). Differences between group faecal consistencies, liveweight gains, pepsinogen values, and mucosal mast cells were determined by analysis of variance of untransformed data (Minitab, version 10.0).

5.3 Results

5.3.1 Faecal consistencies and egg counts

The average faecal consistency scores for the different groups are shown in Figure 5.1. The MORI (Bz-resistant) group had significantly softer faeces compared to the other challenged groups ($P < 0.05$) when the animals were sampled on days 7 and 14 but by day 21 faecal consistencies were similar in all of the groups including the controls. The faecal egg counts (FECs) of the different groups are shown in Figure 5.2. One lamb (No. 2444) in the SCRI (Bz + Ivm) group had an extremely high faecal egg count compared to the other lambs although it's faecal scores were no different to the other members in the group. The average FECs for the groups omitting lamb No. 2444 are shown in Figure 5.3. Patent infections were apparent in some of the lambs by day 14, with the MOSI (susceptible) group having significantly higher ($P < 0.05$) counts than the other two infected groups. By day 21, FECs were similar in all the infected groups although they tended to be lower in the SCRI (Bz + Ivm) group if the counts of lamb No. 2444 were disregarded. Nevertheless, with or without this particular animal, there were no significant differences between the FECs of the infected groups from day 21 onwards. By day 52, FECs had declined for all of the infected groups and there were no differences between them and the control group.

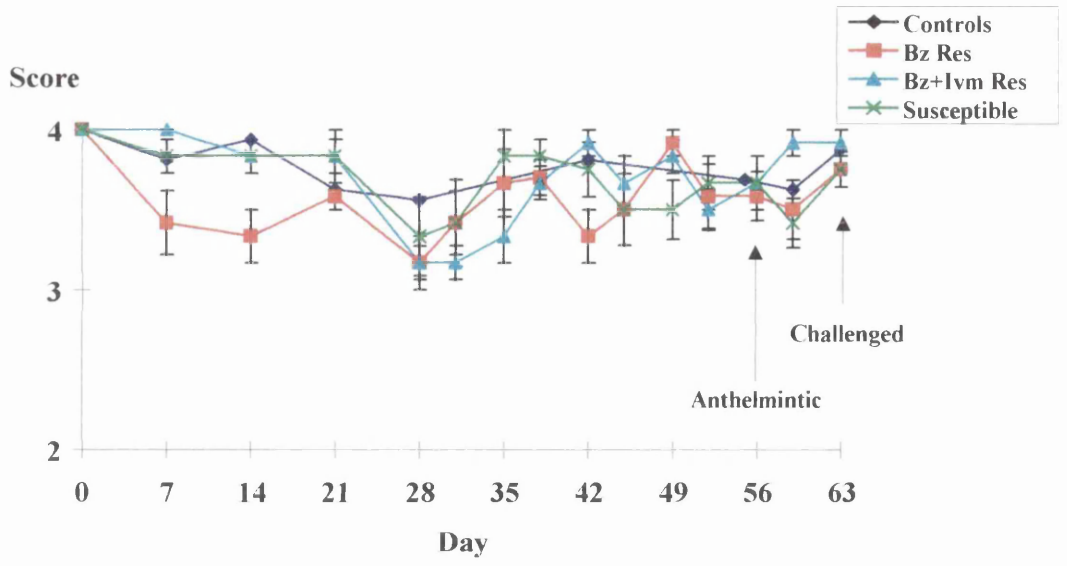

Figure 5.1 *Average faecal consistencies (±SEM) for groups of lambs infected daily with 4000 L_3 of different isolates of T. circumcincta*

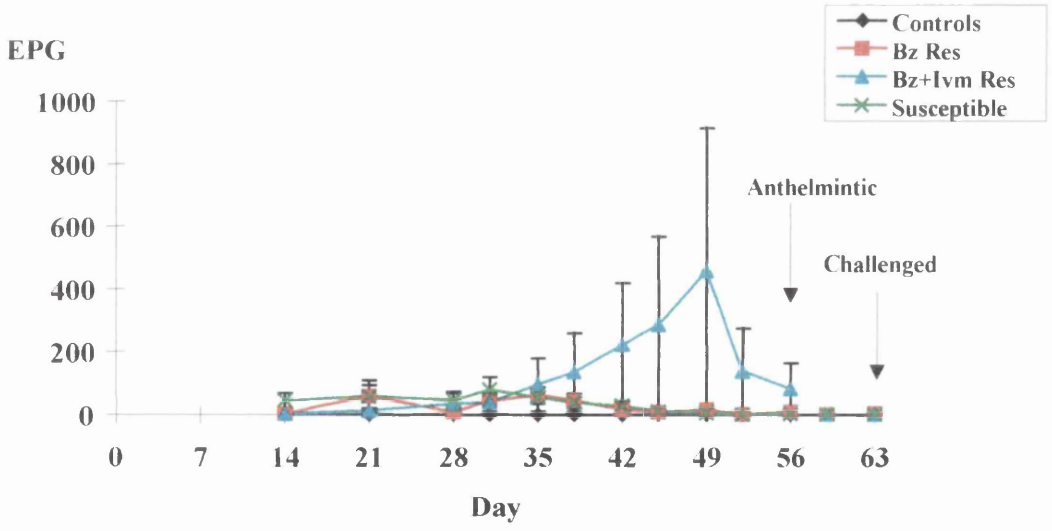

Figure 5.2 *Faecal egg counts (±SEM) of lambs infected daily with 4000 L_3 of different isolates of T. circumcincta.* **Note: the EPG scale is 0 - 1000*

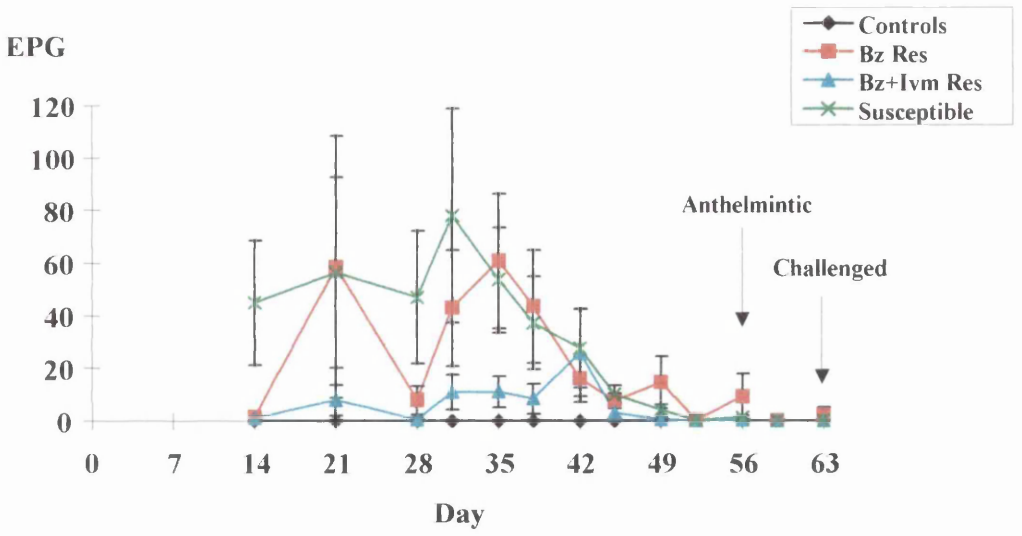

Figure 5.3 *Faecal egg counts (±SEM) of lambs infected daily with 4000 L_3 of different isolates of T. circumcincta (omitting lamb No. 2444 from the Bz + Ivm group) *Note: the EPG scale is 0 - 120*

5.3.2 Liveweight gains

The cumulative weight gains of the different groups are shown in Figure 5.4 whilst the average daily weight gains are shown in Figure 5.5. Lambs in all of the infected groups showed varying degrees of liveweight gain and there were no statistical differences between them and the uninfected control group at any point in the study. Animal No. 2444 from the SCRI (Bz + Ivm) group fared less well, gaining only 2 kg over the 8 week study compared to an average of 9.7 kg for the other animals in this group. Nevertheless, there were still no statistical differences in terms of weight gains between the different groups whether omitting this animal or not.

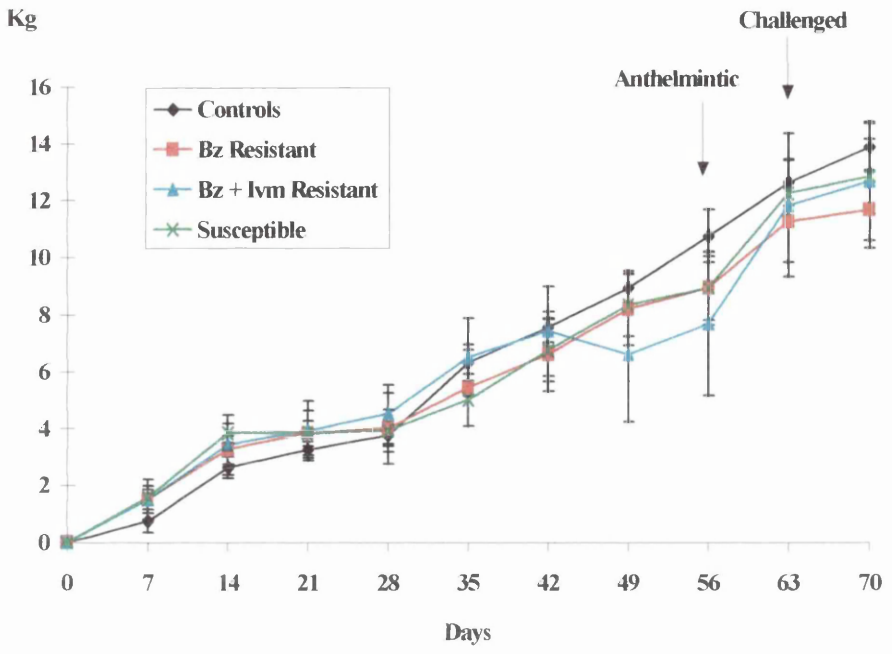

Figure 5.4 *Average cumulative weight gains (±SEM) of lambs infected daily with 4000 L_3 of different isolates of T. circumcincta*

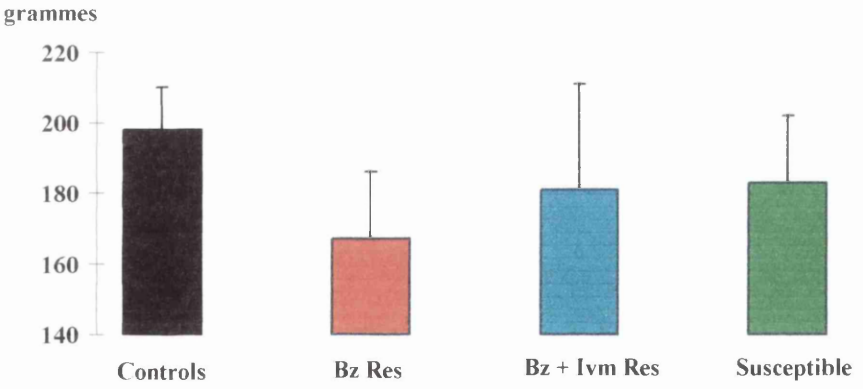

Figure 5.5 *Average daily weight gains (grammes, + SEM) of lambs infected with different isolates of T. circumcincta (4000 L_3 per day)*

5.3.3 Pepsinogen values

The average pepsinogen values of the different groups throughout the study are shown in Figure 5.6. Pepsinogen values became elevated in the infected groups from day 14, from which point the uninfected control group values remained significantly lower throughout the rest of the study. At day 14 the pepsinogen values of the SCRI (Bz + Ivm) group were significantly lower than the other infected groups ($P < 0.05$) but thereafter there were no differences between the infected groups. The pepsinogen values for lamb No. 2444 were similar to those of the other lambs in the Bz + Ivm resistant group.

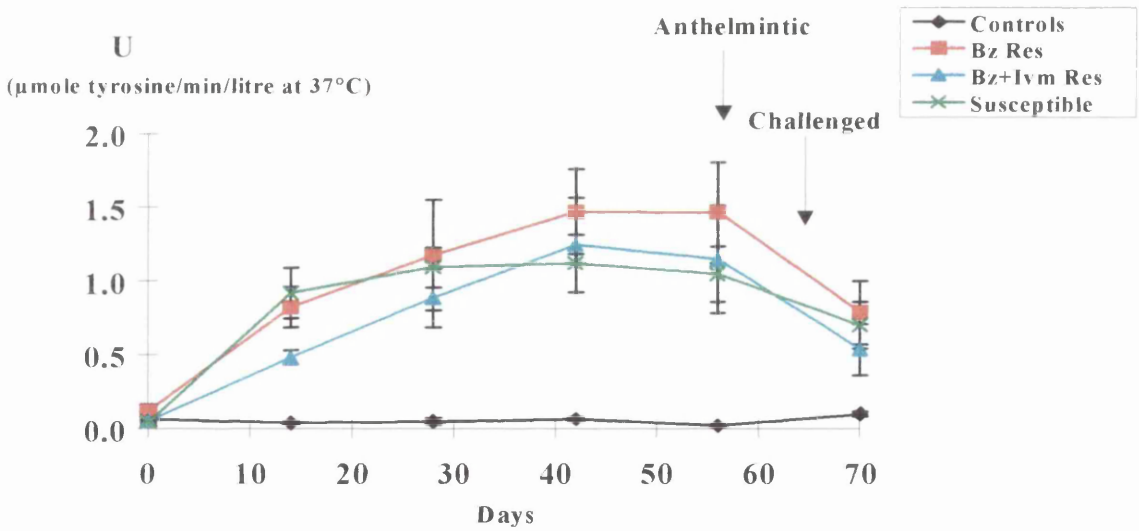

Figure 5.6 *Average pepsinogen values (±SEM) of lambs infected daily with 4000 L_3 of different isolates of T. circumcincta*

5.3.4 Abomasal worm burdens

The *T. circumcincta* burdens of the groups following challenge with 10,000 L_3 are shown graphically in Figure 5.7. The individual *T. circumcincta* burdens for each animal are shown in Table 5.2. The numbers of male and female middle and late L_4 stage larvae were approximately equal in every lamb where worms were counted. One parasite naive control lamb (No. 2349) challenged with the Bz resistant isolate had a worm burden of only 600 late L_4 stage larvae. With the exception of this animal the establishment rates in the parasite naive challenged controls were similar for both the MOSI (susceptible) and MORI (Bz resistant) isolates, being 45.5 and 39.3 % respectively. The challenge controls had significantly higher numbers of late fourth larval stage and overall worm burdens than all of the trickle infected groups ($P< 0.01$). There were no differences between the dosed groups in terms of worm burdens or development stages although the susceptible isolate (MOSI) tended to have lower numbers of worms than the other two isolates. Animal No. 2444 from the SCRI (Bz + Ivm) group did have a reasonably high worm burden of 3,950 although the majority of the established worms were at the early fourth larval stage.

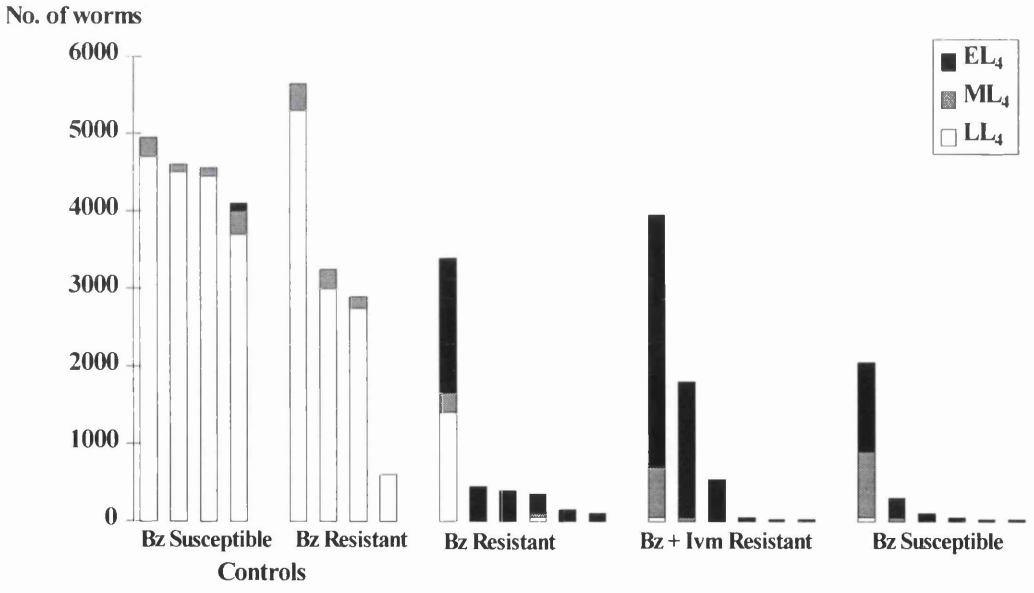

Figure 5.7 *Early, middle and late fourth larval stage worm burdens of lambs challenged with 10,000 L_3 of different isolates of T. circumcincta (10 days post infection)*

Table 5.2 *Individual worm burdens of lambs challenged with 10,000 L_3 of different isolates of T. circumcincta (10 days post infection)*

Group	Lamb No.	EL_4	ML_4 Males	ML_4 Females	LL_4 Males	LL_4 Females	Total (± SD)
	2265	100	0	300	1700	2000	4100
	2342	0	0	250	2150	2550	4950
(MOSI)	2380	0	0	100	2150	2300	4550
	2263	0	0	100	2350	2150	4600
	Mean	**25**	**0**	**188**	**2088**	**2250**	**4550 (± 349)**
Controls							
	2434	0	0	150	1300	1450	2900
	2397	0	150	200	2600	2700	5650
(MORI)	2349	0	0	0	300	300	600
	2403	0	50	200	1400	1600	3250
	Mean	**0**	**50**	**138**	**1400**	**1513**	**3100 (± 2067)**
	2269	400	0	0	0	0	400
	2271	100	0	0	0	0	100
MORI	2425	1750	250	0	650	750	3400
(Bz resistant)	2273	250	50	0	50	0	350
	2134	450	0	0	0	0	450
	2335	150	0	0	0	0	150
	Mean	**517**	**50**	**0**	**117**	**125**	**809 (± 1278)**
	2348	550	0	0	0	0	550
	2381	1750	50	0	0	0	1800
SCRI	2330	0	0	0	0	0	0
(Bz + Ivm res.)	2444	3250	350	300	50	0	3950
	2404	0	0	0	0	0	0
	2254	50	0	0	0	0	50
	Mean	**934**	**67**	**50**	**9**	**0**	**1059 (± 1577)**
	2272	1150	500	350	0	50	2050
	986	250	0	50	0	0	300
MOSI	2388	100	0	0	0	300	400
(susceptible)	2357	0	0	0	0	0	0
	1005	0	0	0	0	0	0
	2258	50	0	0	0	0	50
	Mean	**259**	**84**	**67**	**0**	**59**	**467 (± 793)**

5.3.5 Abomasal mucosal mast cells

The average number of mucosal mast cells counted from sections of an abomasal fold taken from each lamb were expressed as mast cells per 0.02 mm^2 of mucosa and are shown graphically in Figure 5.8. The individual mucosal mast cell numbers for each animal are shown in Table 5.3. There were no differences between the numbers of mucosal mast cells in the lambs from the control group challenged with either the MORI (Bz resistant) or MOSI (susceptible) isolates. No data was available for one lamb in the MOSI trickle challenged group (No. 2272) as the section taken at *post mortem* was inadvertently destroyed. Each of the trickle challenged groups had significantly higher abomasal mucosal mast cell counts than the challenged controls ($P < 0.001$). There were no differences between any of the trickle infected groups in terms of mucosal mast cell counts.

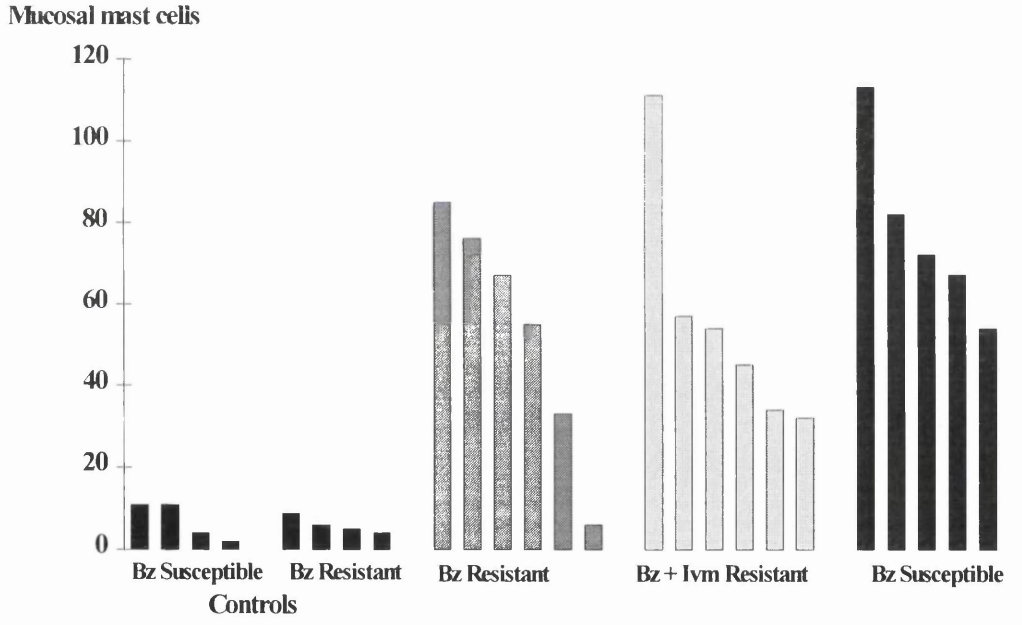

Figure 5.8 *Average abomasal mucosal mast cell numbers (per 0.02 mm^2 tissue) of lambs challenged with 10,000 L_3 of different isolates of T. circumcincta (10 days post infection)*

Table 5.3 *Individual abomasal mucosal mast cell numbers (per 0.02 mm^2 tissue) of lambs challenged with 10,000 L_3 of different isolates of T. circumcincta (10 days post infection)*

Group	Lamb No.	No. MMC ($0.02mm^2$)	Group	Lamb No.	No. MMC ($0.02mm^2$)
	2265	4		2434	9
Controls	2342	11	**Controls**	2397	5
(MOSI)	2380	11	**(MORI)**	2349	6
	2263	2		2403	4
	Mean (± SD)	**7 (4.7)**		**Mean (± SD)**	**6 (2.2)**
	2269	67		2348	34
	2271	76		2381	57
	2425	6		2330	45
MORI	2273	55	**SCRI**	2444	54
	2134	85		2404	111
	2335	33		2254	32
	Mean (± SD)	**54 (29.5)**		**Mean (± SD)**	**56 (29)**
	2272	*			
	986	113			
	2388	72			
MOSI	2357	82			
	1005	54			
	2258	67			
	Mean (± SD)	**78 (22.2)**			

* data not available.

MOSI, susceptible isolate

MORI, Bz resistant isolate

SCRI, Bz + Ivm resisatnt isolate

5.4 Discussion

No differences were apparent in the immunogenicity or pathogenicity of the two Bz-resistant isolates of *T. circumcincta* investigated in comparison to that of an unselected susceptible isolate. Faecal egg counts, plasma pepsinogen values and liveweight gains were similar for all three groups during the period of trickle challenge. There was a notable difference in the pre-patent period of the different isolates with the susceptible (MOSI) parasites producing significantly higher faecal egg counts by day 14 of the study ($P < 0.05$). Five of the six MOSI infected lambs had positive faecal egg counts (FECs) on day 14 with an averge of 54 EPG. This was compared to 2/6 and 3/6 lambs in the MORI and SCRI infected groups with average FECs of 5 and 3 EPG respectively. There were also differences in the profiles of the three isolates with peak FECs on days 31, 35 and 42 for the MOSI, MORI and SCRI infections and average counts of 78, 61 and 28 EPG respectively. The counts remained reasonably low once infections had reached patency despite continued infection, suggesting that regulation of egg output was occurring in these animals.

The MOSI has been used in several studies at Moredun with variable results in terms of peak average FEC and days to peak count, the results of which are summarised in table 5.4. Animal age (Douch & Morum, 1993), genotype (Abbot *et al*, 1984) and nutrition (Coop & Holmes, 1996) can influence faecal egg output and may account for the large variation seen in studies with this isolate. The lambs were aged 6 months at the start of the present study and on a high plane of nutrition which may help to explain why the FECs remained reasonably low. It is interesting to note that there were no marked differences in terms of weight gain between the trickle infected groups and the uninfected controls. Significant differences in terms of weight gain were noted by Sykes & Coop (1977) and Jackson & Christie (1984) using the MOSI and a similar dosing regime but the lambs in these studies were aged 4 months and there were also breed differences. The average plasma pepsinogen value for the SCRI group was lower on day 14 ($P<0.05$) but there were no marked differences between either of the infected groups throughout the remaining trickle infection. The pepsinogen values were elevated in the present study suggesting that these lambs were experiencing some degree of sub-clinical pathophysiological

damage, although this was not associated with significant reductions in lamb performance.

Following the administration of anthelmintic to remove residual worms from the trickle infection, no differences between the infected groups, in terms of worm burden or mucosal mast cell numbers, were evident as a result of challenge with 10,000 L_3. Although anthelmintic controls were not employed, previous studies have demonstrated the susceptibility of the SCRI (Jackson *et al.*, 1992a), MORI and MOSI (F. Jackson, unpublished data) to levamisole. Furthermore, the lambs were treated with anthelmintic on two consecutive days in an effort to ensure the removal of histotrophic stages.

Table 5.4 *Comparison of several studies using the Moredun susceptible isolate (MOSI) of T. circumcincta in terms of daily infective dose, average peak faecal egg counts and days to achieve peak counts*

Authors	Age (months)	Breed	Daily dose (L_3)	Peak FEC (EPG)	Days to peak
The present study	6	G x S	4000	78	42
Sykes & Coop (1977)	4	B/BL x S*	4000	480	63
Coop *et al.* (1982)	4	B/BL x S	1000	350	42
			3000	275	42
			5000	300	42
Jackson & Christie (1984)	3½-4	BL x S	400	200	42
			4000	190	42
Seaton *et al.* (1989b)	5	G x S	1000	240	48
Coop *et al.* (1995)	4 ½	G x S	2000	500	53

G - Greyface, B - Blackface, BL - Border Leicester, S - Suffolk
* Male lambs only

The degree of immunity that the infected groups acquired during the trickle challenge was similar for each of the isolates with no significant differences in terms of total worm burden, stage of worm development or mucosal mast cell numbers. Average establishment rates (± SD) in the previously infected lambs for the MORI

(Bz-resistant), SCRI (Bz + Ivm resistant) and MOSI (susceptible) challenge infections were 8.1 (± 12.8), 10.6 (± 15.8) and 4.7 (± 7.9) % respectively. The MOSI has also been used in several challenge studies at Moredun involving control and secondary infections, the results of which are summarised in table 5.5. Differences in animal age, daily larval dosage and size of challenge infection may account for the variable establishment rates seen in these studies.

Table 5.5 *Comparison of several studies using the Moredun susceptible isolate (MOSI) of T. circumcincta in terms of establishment (Est.) rates for control (C) and secondary (S) infections. Age of sheep is given at time of challenge, following period of trickle infection. Greyface x Suffolk lambs were used in each study and worm burdens taken on day 10 post challenge*

Authors	Age (months)	Daily dose (L_3)	Trickle (weeks)	Challenge (L_3)	Est. (%)
Present study	8	4,000	8	10,000	(C) 45.5 (S) 4.7
Smith *et al.* * (1983b)	11	2,000	8 - 10	50,000	(C) 29.2 (S) 11.0
Smith *et al.* (1984a)	10	2,000	9	50,000	(C) 46.2 (S) 4.8
				1,000	(C) 26.8 (S) 12.4
Smith *et al.* (1985)	4 ½	2,000	8 - 9	50,000	(C) 27.4 (S) 22.8
				50,000	(C) 63.0 (S) 30.6
Coop *et al.* (1995)	7	2,000	8	50,000	(C) 32.8 (S) 39.2

* Smith, Jackson, Jackson & Williams (1983b)

Average abomasal mucosal mast cell numbers (± SD) for the infected sheep challenged with the MORI, SCRI and MOSI isolates were 54 (± 29), 56 (± 29) and 78 (± 22) cells per 0.02 mm^2 tissue respectively. There was a very good relationship between the number of mast cells and the number of worms present in each animal.

The overall value, including worm burdens and mast cell counts of the challenge controls, resulted in an inverse correlation of 0.72, suggesting that mucosal mast cell counts are a good indicator of immunogenicity. Although the average mast cell count for the MOSI group was slightly higher, the tissue sample for the lamb with the highest worm burden in this group was missing. In view of the correlation between worm burden and mucosal mast cell count, it likely that the average number of mast cells would be lower for the MOSI group if the counts for this particular animal were available. The heterogeneity in the immune responses of the sheep in the present study has also highlighted the difficulty in making compararisons between different studies on immunogenicity.

Previous work, such as that of Kelly *et al.* (1978), has been criticized because comparisons were made between isolates with very different origins and histories and results may have reflected ecotypic differences. The same criticism applies to the present study since the isolates had very different backgrounds in regard to their exposure to anthelmintics. The MOSI and MORI may however, due to extensive movement of animals between Firth Mains and the Moredun site, have at some time shared a common ancestry. The main cause for Bz resistance in both *H. contortus*, *T.colubriformis* (Kwa *et al.*, 1994) and *T. circumcincta* (Elard *et al.*, 1996) is a single mutation from phenylalanine to tyrosine at amino acid position 200 in the β-tubulin isotype 1 gene. It seems reasonable to suggest that alleles conferring resistance may be advantageous for survival if selected for by anthelmintics, but whether such a minor change in the genetic code would modify fundamental characteristics such as larval establishment and development, and adult persistence and fecundity, is not clear. Beech, Prichard & Scott (1994) studied the genetic variability of β-tubulin genes in Bz-susceptible and Bz-resistant isolates of *H. contortus*. Examining the allelic variation at two β-tubulin loci, these workers concluded that resistance is associated with changes in allele frequency at these loci rather than novel genetic rearrangements. If anthelmintic-resistant alleles are linked to increased infectivity these parasites might indeed be more pathogenic but, conversely, more likely to kill their hosts and incidentally themselves (Le Jambre *et al.*, 1982).

Nevertheless, the purpose of the present study was to compare and assess the relative effects of infection of the three different isolates of *T. circumcincta*. The

MOSI has been used in all of the pathogenicity and production studies at Moredun since it's isolation in 1979 and had been passaged 64 times in donor lambs before this study. Although there were no statistical differences, the MORI (Bz resistant) isolate tended to show a higher degree of pathogenicity in terms of lower faeal consistencies over the first two weeks of infection, higher worm burdens and reduced liveweight gains compared to the other isolates. Average daily weight gains for the MORI (Bz resistant), SCRI (Bz + Ivm resistant) and MOSI (susceptible) isolates were 167, 181 and 183 grammes per day respectively. Similarly, the respective geometric mean burdens for each of the groups following challenge were 384, 76 and 48 worms. It seems that the Bz resistant *T. circumcincta* isolated from Firth Mains is not atypical for this species and it's degree of fitness within the host may indeed be greater than that of our unselected susceptible isolate. The results of the three year field study on production are therefore supported by this present study since neither the pathogenicity or immunogenicity of the MORI are atypical for this species. This also strengthens the suggestion made in chapter 3 that resistant parasites may well be controlled with anthelmintic treatments which are not fully effective, at least when dealing with species of a low biotic potential such as *Teladorsagia.*

CHAPTER 6

Anthelmintic resistance status of parasites on the paddocks at Firth Mains

6.1 Introduction

Following the characterization of the Bz-resistant *T. circumcincta* isolated from Firth Mains in 1983, drugs within the Bz class were withdrawn from the farm and sheep on the contaminated paddocks were treated on an annual basis with either Lev or Ivm. Results of the faecal egg count reduction tests (FECRTs) and controlled efficacy tests (CETs) conducted as part of the three year field study on production suggested that resistance at Firth Mains may have extended to the imidazothiazole (levamisole) class of anthelmintic and possibly to the Fbz + Lev combination. However, as discussed in chapter 4, the treatment efficacies seen with naturally infected animals can be influenced markedly by the degree of immunity acquired by each lamb throughout the season. This in turn can be influenced by a number of factors including genotype, nutrition and the degree and duration of exposure to infection. Therefore, in an attempt to determine the resistance status of the parasites on each paddock, a CET was performed in accordance with WAAVP guidelines (Wood *et al.*, 1995) which require artificial infection of parasite naive animals.

6.2 Materials and methods

6.2.1 Infective larvae

A naturally infected male lamb from each of the treatment group paddocks at Firth Mains was housed towards the end of the study in 1995 and its faeces collected to provide infective larvae. Methods of larval culture, recovery, storage and infection techniques were those described in chapter 2.2.5. Since it was near the end of the season, larval cultures also contained *T. vitrinus*. The relative proportions of *T. circumcincta* and *T. vitrinus* in each larval culture was determined by examining the tail characteristics of 100 exsheathed infective larvae under a compound microscope. The larvae were exsheathed by the addition of 200 µl of sodium hypochlorite (Milton sterilizing solution) per 1ml of larval suspension and left for 2 minutes to exsheath before the addition of one drop of helminthological iodine.

6.2.2 Experimental Design

Details of the experimental design are summarized in Table 6.1. Thirty two parasite naive Suffolk-cross lambs, aged six months, were allocated into four groups of 8 animals, balanced in terms of bodyweight and sex. The different isolates collected from each donor were randomly assigned to groups of lambs, each of which was challenged with 10,000 L_3 on day 0. Two lambs of similar weight from each group, one male and one female, were assigned as untreated controls. The groups remained housed in separate concrete pens, the surfaces of which were cleaned daily and replaced with fresh straw. The lambs were fed with hay and feed concentrates daily and water was provided *ad libitum*. On day 28 lambs from each group, except the controls, were treated on the basis of liveweight with their respective anthelmintic at the relevant MRD. The lambs were slaughtered 7 days post-treatment and their abomasa and small intestines removed for worm burden estimation using 2 % sub-samples as described in chapter 2.3.

Table 6.1 *Experimental design of study to determine resistance status of anthelmintics at Firth Mains*

Group (n = 8)	Anthelmintic used on paddock from which donor lamb grazed	Day 0 (infected)	Day 28* (anthelmintic)	Day 35
1	Ivm	10,000 L_3	Ivm (0.2 mg kg^{-1})	Post mortem
2	Fbz	10,000 L_3	Fbz (5 mg kg^{-1})	Post mortem
3	Lev	10,000 L_3	Lev (7.5 mg kg^{-1})	Post mortem
4	Fbz/Lev	10,000 L_3	Fbz/Lev (5 + 7.5 mg kg^{-1})	Post mortem

* Two lambs from each group remained as untreated controls

6.2.3 Parasitological parameters

Lambs were sampled *per rectum* weekly and their faecal egg counts determined using the methods described in chapter 2.2. Eggs from each group were pooled and identified by means of an image shearing technique, described in chapter 2.2.4.

The FECRT treatment efficacies for each of the anthelmintics were calculated according to WAAVP guidelines (Coles *et al.,* 1992) although day 7 counts were necessary due to the experimental design :

% Reduction = 100 (1 - Xt / Xc), where Xt and Xc are the treated and control group arithmetic mean faecal egg counts respectively on day 7.

The CET treatment efficacies were calculated according to WAAVP guidelines using both arithmetic and geometric mean worm data since either method should be equally acceptable for determining efficacy with artificially induced infections in parasite naive animals (Wood *et al.*, 1995):

% Reduction = 100 (1 - Xt / Xc), where Xt and Xc are the treated and control group arithmetic/geometric mean worm counts respectively at slaughter.

6.2.4 Statistical analyses

Differences between group faecal egg counts were determined by Log_{10} (x + 1) transformation prior to analysis of variance (Minitab, version 10.0).

6.3 Results

6.3.1 Specific composition of larval cultures

The relative proportions of *T. circumcincta* and *T. vitrinus* in each larval culture, as determined by examining the tail characteristics of 100 infective larvae, are shown in Table 6.2. The Lev group were infected with the highest proportion of *T. vitrinus* larvae (36 %) whereas the respective infective dose for the Fbz/Lev and Fbz groups was composed of 21 % and 25 % of this small-intestinal species. The larval culture used to infect lambs within the Ivm group had comparatively low numbers of *T. vitrinus* with only 2 % of the overall dose comprising of this species.

6.3.2 Faecal egg count reduction test (FECRT)

The faecal egg counts of the different groups are shown in Figure 6.1. Patent infections were apparent in some of the lambs by day 14 of the study. Faecal egg counts were similar in all of the infected groups by day 21. Lambs infected with

larvae from the Fbz and Fbz/Lev paddocks, as well as the control lambs, had significantly lower faecal egg counts ($P < 0.05$) than the Ivm group on day 28. There were no significant differences between the faecal egg counts of the Ivm and Lev groups at this point. Following anthelmintic, all of the treated groups had significantly lower faecal egg counts than the untreated controls ($P < 0.001$).

Faecal egg count reductions for each of the anthelmintic treatments, calculated according to WAAVP guidelines, are shown in Table 6.3. Pre- and post-treatment egg measurements for each group are shown in Table 6.4. According to WAAVP faecal egg count reduction test guidelines (Coles *et al.,* 1992), resistance is present if the percentage reduction is less than 95 % and the lower confidence interval is less than 90 %. If only one of these two criteria is met then resistance is suspected. Under these assumptions, the Fbz/Lev combination and Ivm treatments have remained fully effective, both approaching reductions in faecal egg count of 100 %. Results of the FECRT confirmed the presence of Fbz resistance at Firth Mains although the treatment efficacy of 77.8 % is somewhat higher than was seen in naturally infected animals. The treatment efficacy of just over 89 % seen with Lev suggests that resistance to this anthelmintic is also present, at least on the particular paddock were sheep have been treated with this drug for three seasons.

Table 6.2 *Relative proportions of T. circumcincta and T. vitrinus in larval cultures from donor lambs grazing different paddocks, as determined by the tail characteristics of 100 exsheathed infective larvae*

Paddock on which lamb was grazing.	**Anthelmintic employed on paddock from 1993 - 1995**	**% *T. circumcincta***	**% *T. vitrinus***
5	Ivm	98	2
3	Fbz	75	25
2	Lev	64	36
1	Fbz/Lev	79	21

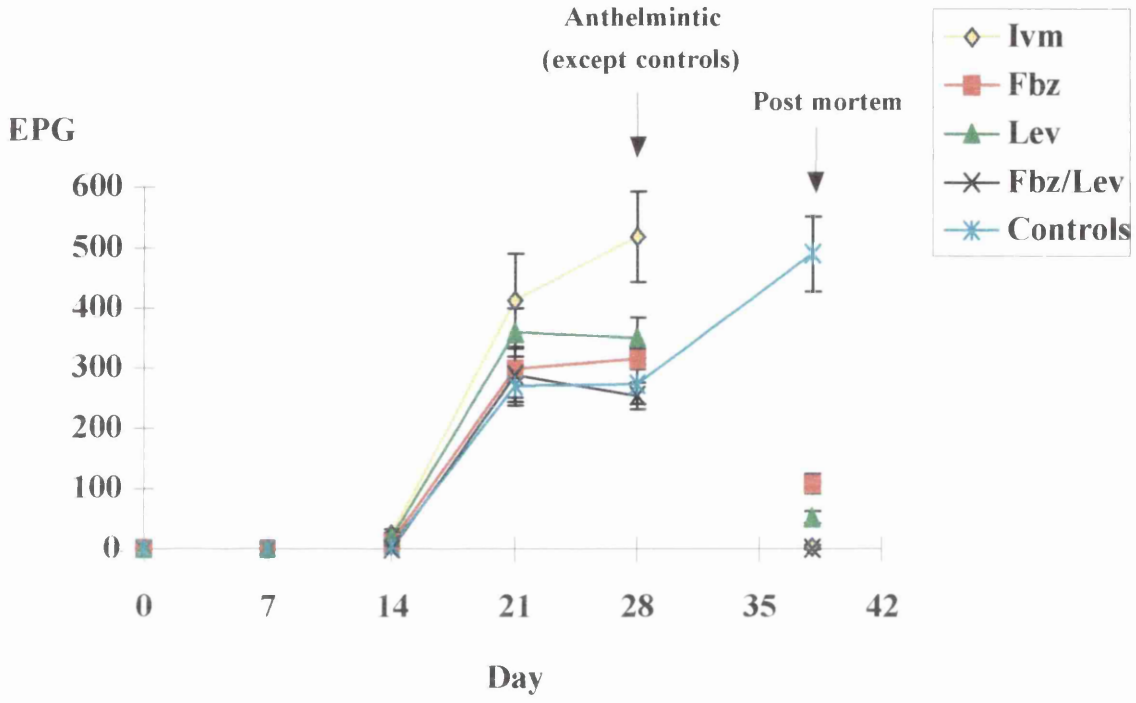

Figure 6.1 *Average faecal egg counts (±SEM) of groups of lambs (n = 8) infected with 10,000 L_3 from a donor lamb which had grazed a particular paddock, the lambs of which had been treated with either Ivm, Fbz, Lev or Fbz/Lev using a non-suppressive treatment regime for three seasons. Two lambs from each group represented the untreated controls*

Table 6.3 *Faecal egg count reductions in groups of sheep infected with 10,000 L_3 28 days previously, including 95 % confidence intervals*

Treatment (day 0)	EPG (± SD) day 0	EPG (± SD) day 10	% Reduction	95 % CIs
Untreated controls	274.5 (91.3)	490.1 (174.4)	-	-
Ivm (0.2 mg kg^{-1})	519.0 (183.3)	3.2 (7.3)	99.3	95, 100
Fbz (5 mg kg^{-1})	316.5 (45.7)	109.0 (40.4)	77.8	66, 86
Lev (7.5 mg kg^{-1})	351.0 (83.1)	53.0 (23.6)	89.2	83, 93
Fbz/Lev (5 + 7.5 mg kg^{-1})	255.0 (56.3)	0.7 (1.2)	99.9	99, 100

Table 6.4 *Identification of pre- (day 0) and post-treatment (day 10) faecal egg count measurements of sheep infected with 10,000 L_3 28 days previously*

Treatment group	*(%) T. circumcincta*		*(%) T. vitrinus*	
	day 0	day 10	day 0	day 10
Untreated controls	91	90	9	10
Ivm (0.2 mg kg^{-1})	100	*	0	*
Fbz (5 mg kg^{-1})	83.3	85.7	16.7	14.3
Lev (7.5 mg kg^{-1})	89.5	90.9	10.5	9.1
Fbz/Lev (5 + 7.5 mg kg^{-1})	91.3	*	8.7	*

* insufficient eggs

6.3.3 Controlled efficacy test (CET)

6.3.3.1 Abomasal worm burdens

The abomasal worm burdens of the groups following anthelmintic treatment are shown graphically in Figure 6.2. The individual abomasal worm burdens for each animal are shown in Table 6.5. Only adult *T. circumcincta* were present and the proportion of male and females were similar in all of the lambs where worms were counted. The average number of *T. circumcincta* present in each pair of infected controls was 2775, 3225, 2750 and 3850 for the Ivm, Fbz, Lev and Fbz/Lev larval cultures respectively. Anthelmintic efficacies, calculated using both arithmetic and

geometric mean worm burden data, are shown in Table 6.6. Both the Fbz/Lev combination and the Ivm treatments were highly effective against abomasal species using either arithmetic or geometric mean data, with worm reductions of over 99 %. Fbz treatment resulted in abomasal worm reductions of approximately 70 % using either method, confirming the presence of Bz-resistant *T. circumcincta* on the paddocks at Firth Mains. The efficacy for Lev treatment calculated using geometric data was over 99 %, whereas reduction using arithmetic worm burdens was 97.9 %.

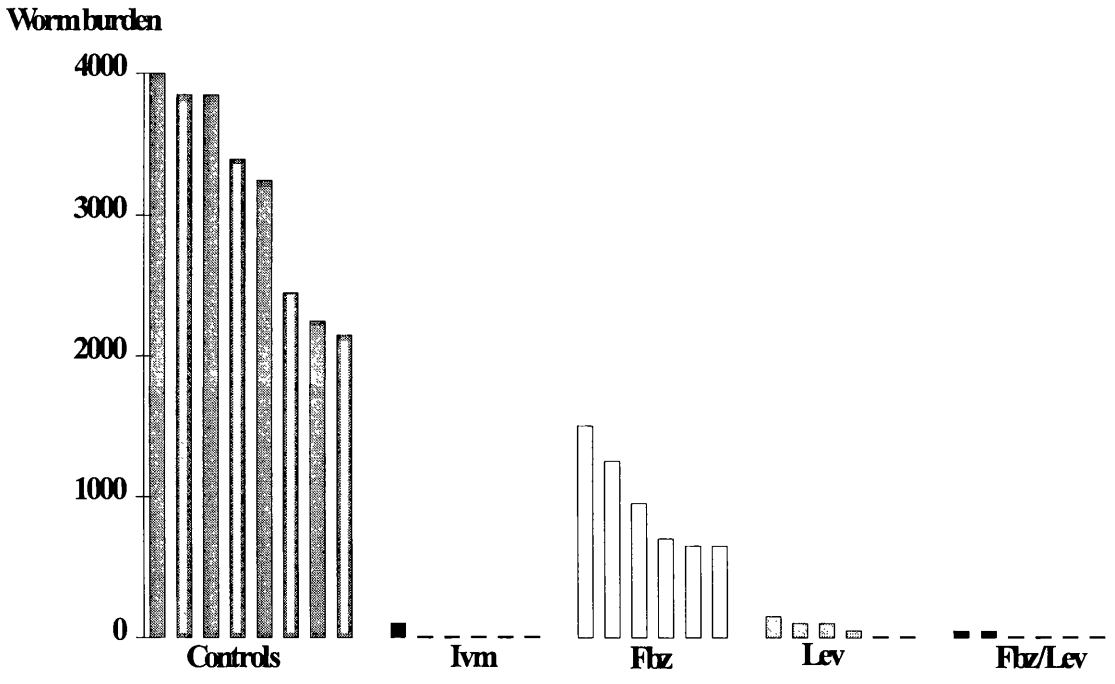

Figure 6.2 *T. circumcincta burdens of untreated controls and lambs treated with either Ivm (0.2 mg kg^{-1}), Fbz (5 mg kg^{-1}), Lev (7.5 mg kg^{-1}) or a Fbz/Lev (5 + 7.5 mg kg^{-1}) combination. Lambs were infected 28 days pre-treatment with 10,000 L_3 from a donor lamb which had grazed the particular treatment paddock with two lambs from each group remaining as controls*

Table 6.5 *Individual T. circumcincta burdens of untreated controls and lambs treated with either Ivm, Fbz, Lev or a Fbz/Lev combination. Lambs were infected 28 days pre-treatment with 10,000 L_3 from a donor lamb which had grazed the particular treatment paddock with two lambs from each group remaining as controls*

Group	Lamb No.	Males	Females	Total (± SD)
	603 (Ivm)	1600	1800	3400
	1275 (Ivm)	1050	1100	2150
	592 (Fbz)	1150	1300	2450
Controls	601 (Fbz)	1750	2250	4000
	397 (Lev)	900	1350	2250
	591 (Lev)	1400	1850	3250
	394 (Fbz/Lev)	1850	2000	3850
	678 (Fbz/Lev)	1500	2350	3850
	Arithmetic mean	**1400**	**1750**	**3150 (± 763)**
	Geometric mean	**1362**	**1695**	**3064**
	386	0	0	0
	644	0	0	0
	649	0	0	0
Ivm	670	0	100	100
	693	0	0	0
	1268	0	0	0
	Arithmetic mean	**0**	**16.7**	**16.7 (± 41)**
	Geometric mean	**0**	**1.2**	**1.2**
	370	500	150	650
	576	400	250	650
	695	900	350	1250
Fbz	696	700	800	1500
	1254	600	350	950
	1282	450	250	700
	Arithmetic mean	**591.7**	**358.3**	**950 (± 357)**
	Geometric mean	**569.2**	**311.8**	**898.7**
	391	100	0	100
	392	50	100	150
	610	0	0	0
Lev	624	0	50	50
	662	50	50	100
	1287	0	0	0
	Arithmetic mean	**33.3**	**33.3**	**66.7 (± 61)**
	Geometric mean	**7.0**	**7.0**	**19.7**
	393	0	0	0
	396	0	0	0
	588	0	50	50
Fbz/Lev	639	0	0	0
	646	50	0	50
	661	0	0	0
	Arithmetic mean	**8.3**	**8.3**	**16.7 (± 26)**
	Geometric mean	**0.9**	**0.9**	**2.7**

Table 6.6 *Abomasal worm burden reductions in groups of sheep artificially infected with 10,000 L_3 28 days pre-treatment, calculated using arithmetic and geometric mean worm burden data*

Treatment (day 0)	Arithmetic mean worm burden (± SD) day 10	% Reduction	Geometric mean worm burden day 10	% Reduction
Untreated controls	3150 (763)	-	3064	-
Ivm (0.2 mg kg^{-1})	17 (41)	99.5	2	99.9
Fbz (5 mg kg^{-1})	950 (357)	69.9	899	70.7
Lev (7.5 mg kg^{-1})	67 (61)	97.9	20	99.3
Fbz/Lev (5 + 7.5 mg kg^{-1})	17 (26)	99.5	3	99.9

6.3.3.2 Small intestinal worm burdens

The small intestinal worm burdens of the groups following anthelmintic treatment are shown graphically in Figure 6.3. Only *Trichostrongylus* spp were identified from lambs in these studies and examination of the male worms confirmed that they were *T.vitrinus*. The individual *T.vitrinus* burdens for each animal are shown in Table 6.7. The ratio of male to female worms was approximately equal in those lambs with reasonable burdens. The average number of small intestinal worms present in each pair of infected controls was 50, 2050, 3050 and 1150 for the Ivm, Fbz, Lev and Fbz/Lev larval cultures respectively. Efficacies were calculated using both arithmetic and geometric mean worm burden data, the results of which are shown in Table 6.8. The particularly low worm burden of the Ivm controls was to be expected since these lambs were infected with an isolate having the lowest proportion (2 %) of *T. vitrinus* larvae. Efficacies calculated with the omission of the Ivm control lamb worm burdens are shown in Table 6.9. Using either arithmetic or geometric mean data, and with or without the Ivm controls, both the Fbz/Lev combination and the Ivm treatments were 100 % effective at removing small intestinal species. Inclusion of the Ivm controls resulted in the lowest calculated efficacies for both Fbz and Lev treatment, with arithmetic data being least effective. Nevertheless, Fbz was still highly effective with a minimum efficacy of 98.9 %. The efficacy of Lev treatment however, calculated using arithmetic data, was moderate

with 82 % and 86.4 % reductions with and without the Ivm controls respectively. Geometric data on the otherhand resulted in reductions of 96 % and 98.9 % with and without the Ivm controls respectively.

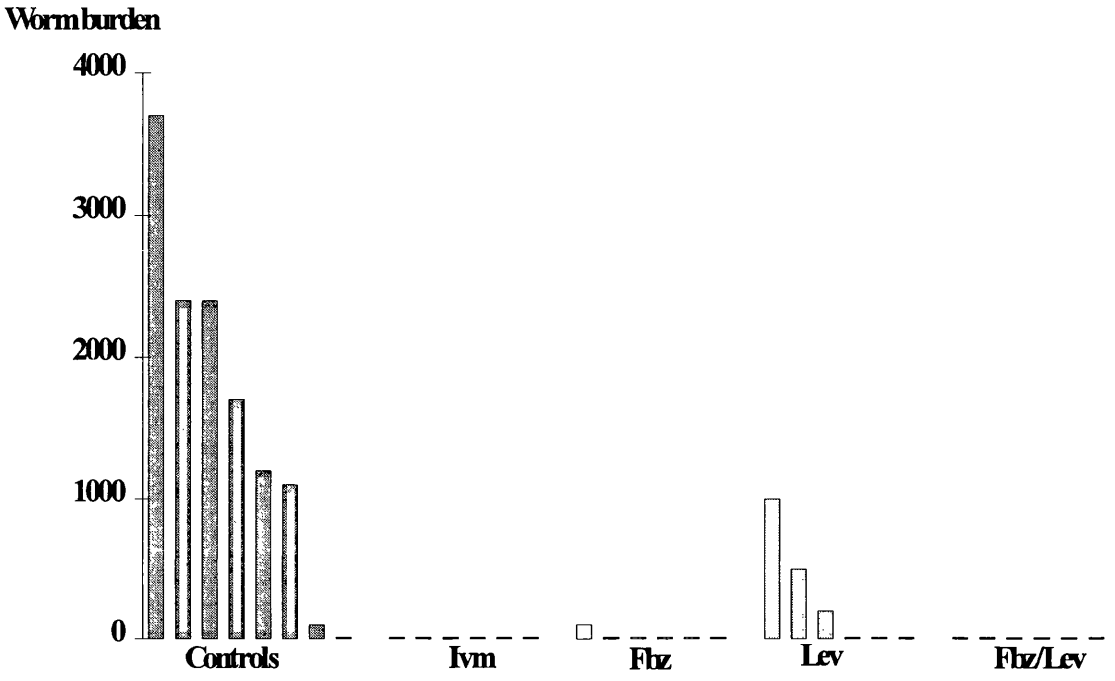

Figure 6.3 *T.vitrinus burdens of lambs treated with either Ivm (0.2 mg kg^{-1}), Fbz (5 mg kg^{-1}), Lev (7.5 mg kg^{-1}) or a Fbz/Lev (5 + 7.5 mg kg^{-1}) combination. Lambs were infected 28 days pre-treatment with 10,000 L3 from a donor lamb which had grazed the particular treatment paddock with two lambs from each group remaining as controls*

Table 6.7 *Individual T.vitrinus burdens of untreated controls and lambs treated with either Ivm, Fbz, Lev or a Fbz/Lev combination. Lambs were infected 28 days pre-treatment with 10,000 L_3 from a donor lamb which had grazed the particular treatment paddock with two lambs from each group remaining as controls*

Group	Lamb No.	Males	Females	Total (± SD)
	603 (Ivm)	100	0	100
	1275 (Ivm)	0	0	0
	592 (Fbz)	700	1000	1700
Controls	601 (Fbz)	1000	1400	2400
	397 (Lev)	1600	2100	3700
	591 (Lev)	1100	1300	2400
	394 (Fbz/Lev)	500	700	1200
	678 (Fbz/Lev)	300	800	1100
	Arithmetic mean	**662.5**	**912.5**	**1575 (± 1248)**
	Geometric mean	**255.7**	**194.7**	**512.7**
	386	0	0	0
	644	0	0	0
	649	0	0	0
Ivm	670	0	0	0
	693	0	0	0
	1268	0	0	0
	Arithmetic mean	**0**	**0**	**0**
	Geometric mean	**0**	**0**	**0**
	370	0	0	0
	576	0	0	0
	695	0	100	100
Fbz	696	0	0	0
	1254	0	0	0
	1282	0	0	0
	Arithmetic mean	**0**	**16.7**	**16.7 (± 41)**
	Geometric mean	**0**	**1.2**	**1.2**
	391	200	0	200
	392	600	400	1000
	610	0	0	0
Lev	624	300	200	500
	662	0	0	0
	1287	0	0	0
	Arithmetic mean	**183.3**	**100**	**283.3 (± 402)**
	Geometric mean	**17.2**	**5.6**	**20.6**
	393	0	0	0
	396	0	0	0
	588	0	0	0
Fbz/Lev	639	0	0	0
	646	0	0	0
	661	0	0	0
	Arithmetic mean	**0**	**0**	**0**
	Geometric mean	**0**	**0**	**0**

Table 6.8 *Small intestinal worm burden reductions in groups of sheep artificially infected with 10,000 L_3 28 days pre-treatment, calculated using arithmetic and geometric mean worm burden data*

Treatment (day 0)	Arithmetic mean worm burden (± SD) day 10	% Reduction	Geometric mean worm burden day 10	% Reduction
Untreated controls	1575 (1249)	-	513	-
Ivm (0.2 mg kg^{-1})	0 (0)	100	0	100
Fbz (5 mg kg^{-1})	17 (41)	98.9	1	99.8
Lev (7.5 mg kg^{-1})	283 (402)	82.0	21	96.0
Fbz/Lev (5 + 7.5 mg kg^{-1})	0 (0)	100	0	100

Table 6.9 *Small intestinal worm burden reductions in groups of sheep artificially infected with 10,000 L_3 28 days pre-treatment, calculated using arithmetic and geometric mean worm burden data (omitting worm burdens of the Ivm control lambs since these animals had negligible burdens of T. vitrinus)*

Treatment (day 0)	Arithmetic mean worm burden (± SD) day 10	% Reduction	Geometric mean worm burden day 10	% Reduction
Controls (not Ivm)	2083 (970)	-	1905	-
Ivm (0.2 mg kg^{-1})	0 (0)	100	0	100
Fbz (5 mg kg^{-1})	17 (41)	99.2	1	99.9
Lev (7.5 mg kg^{-1})	283 (402)	86.4	21	98.9
Fbz/Lev (5 + 7.5 mg kg^{-1})	0 (0)	100	0	100

6.4 Discussion

In an effort to reduce the number of animals required, it was decided to use pooled control data for the pairs of animals infected with each of the isolates. This was based on previous studies at Moredun which have shown that there are no significant differences between different isolates, at least with *T. circumcincta*, in terms of initial establishment in parasite naive lambs (F. Jackson, unpublished data). The establishment rates of the isolates used in chapter 5 also support this statement. Tables 6.10 and 6.11 show the respective abomasal and small intestinal calculated efficacies using only the two control lambs infected with each particular isolate compared to values using pooled worm burdens. The values compare very well indeed and support the results obtained using this experimental design.

Table 6.10 *Arithmetic (and geometric) efficacies calculated using the abomasal worm burdens of the two control animals infected with the particular isolate compared to that of values obtained using pooled controls*

Group	Pooled controls	Ivm controls	Fbz controls	Lev controls	Fbz/Lev controls
Ivm	99.5 (99.9)	99.4 (99.9)			
Fbz	69.9 (70.7)		70.5 (71.3)		
Lev	97.9 (99.3)			97.6 (99.3)	
Fbz/Lev	99.5 (99.9)				99.6 (99.9)

Table 6.11 *Arithmetic (and geometric) efficacies calculated using the small intestinal worm burdens of the two control animals infected with the particular isolate compared to that of values obtained using pooled controls*

Group	Pooled controls	Ivm controls	Fbz controls	Lev controls	Fbz/Lev controls
Ivm	100 (100)	100 (100)			
Fbz	99.2 (99.9)		99.2 (99.9)		
Lev	86.4 (98.9)			90.7 (99.3)	
Fbz/Lev	100 (100)				100 (100)

Both the Fbz/Lev combination and the Ivm treatments were highly effective against abomasal and small intestinal species with faecal egg and worm reductions of over 99 %. Results of the FECRTs corresponded very well with those of the CETs, providing further evidence that using fewer lambs than the 15 required to satisfy WAAVP guidelines (Coles *et al.* 1992) may still provide a useful guide to anthelmintic efficacy when conducting FECRTs.

As expected the study confirmed the presence of Bz resistant *T. circumcincta* on the paddocks at Firth Mains, although the treatment efficacy of Fbz was somewhat higher than was seen with the naturally infected field infections. The calculated efficacy for Fbz treatment using the FECRT was 77.8 % with similar proportions of *T. circumcincta* and *T. vitrinus* spp eggs identified in samples taken before and after treatment suggesting we may also have Bz resistant *T.vitrinus*. The egg measuring technique employed only uses 50 % ellipses of the egg size distributions in estimating the size of specific populations and therefore has some margin of error. Eggs recovered post-treatment may also have been expelled by dying worms or from autolysis of dead worms. Results of the CET, the definitive test for resistance, showed calculated efficacies for Fbz of over 99 % against small intestinal species using either arithmetic or geometric mean worm burdens suggesting that Bz resistant *T. vitrinus* is not a problem.

According to WAAVP guidelines standard parametric or nonparametric worm burden data should be equally acceptable for determining efficacy of an anthelmintic with artificially induced infections in young parasite naive animals in which infections are relatively uniform (Wood *et al.*, 1995). The calculated efficacy for Fbz treatment in the CET using arithmetic and geometric mean abomasal worm burden data were 69.9 and 70.7 % respectively. In the corresponding 1995 end of season CET conducted on lambs from this paddock, Fbz treatment efficacy was only 49.4 %, confirming that treatment efficacies are likely to be lower when using naturally infected animals. Benchaoui & McKellar (1994) used the same Bz-resistant isolate of *T. circumcincta* in parasite naive Suffolk cross sheep in their study but achieved geometric mean worm reductions of only 1 % following Fbz treatment. However,

this work is not directly comparable since these lambs were also infected with *H. contortus* and mixed infections are known to affect drug pharmacokinetics (Landuyt, Debackere, Vercruysse & McKellar, 1995). The treatment efficacy of Fbz, as determined by a CET with naturally infected Greyface x Suffolk lambs at Firth Mains in 1991, was 43.9 % (Jackson *et al.*, 1993). The results are encouraging therefore since they provide no evidence of any decline in the efficacy of Fbz despite its continued use over three successive seasons.

Results of the study also suggest that Lev resistance may be present at Firth Mains on the paddock where this drug was used in a non-suppressive treatment regime. The calculated efficacy for Lev using the FECRT was 89.2 %, with similar proportions of *T. circumcincta* and *T. vitrinus* spp eggs identified in samples taken before and after treatment. However, calculated efficacies for Lev treatment in the CET using arithmetic and geometric mean abomasal worm burden data were over 97.9 and 99.3 % respectively, suggesting that Lev resistance is not yet a problem with *T. circumcincta.* With the omission of the Ivm controls, due to the low levels of *T. vitrinus* infection in these animals, arithmetic and geometric small intestinal worm reductions for Lev compared to pooled controls were 86.4 and 98.9 % respectively. The arithmetic and geometric efficacies against *T. vitrinus* calculated using only the two control animals infected with the Lev isolate were 90.7 and 99.3 % respectively. The arithmetic data in each case clearly suggesting Lev resistance in the *T.vitrinus* population on the particular paddock were Lev was employed throughout the study.

In the corresponding 1993-1995 end of season CETs conducted on lambs from the Lev treatment paddock, efficacies against *T. circumcincta* were 82.1, 80.7 and 82% respectively. However, as previously mentioned, the accuracy of CETs conducted on naturally infected animals containing a variety of worm stages is questionable. Whether or not we have Lev resistant *T. circumcincta* species is on this particular paddock is unclear and further investigations are currently being undertaken at Moredun.

In the corresponding 1993-1995 end of season CETs conducted on lambs from the Lev treatment paddock, efficacies against *T. vitrinus* were 98.8, 97.5 and 97.5% respectively. However, the calculated efficacies in the present study using arithmetic data were 86.4 and 90.7% using the pooled and paired controls respectively.

Furthermore, since the highest proportion of *T. vitrinus* was isolated from this paddock it seems likely that we may be seeing the beginnings of Lev resistance in this species. A study is currently being undertaken at Moredun to determine whether this is the case or not. Using surgical transfer of identified adults a pure isolate of *T.vitrinus* from the Lev treatment paddock has been obtained and is currently being used in a CET.

The apparent development of resistance to Lev at Firth Mains is interesting since the animals contaminating this paddock have been exposed to only five treatments over three years. In a FECRT conducted in 1992 there was no evidence of Lev resistance at Firth Mains where the treatment efficacy of Lev in young lambs was greater than 98 % (Scott, Robbins, Jackson & Jackson, 1993/94). Following the discovery of Bz resistance at Firth Mains in 1983, drugs from within this class were suspended and animals were treated with Lev or Ivm in an annual rotation. Therefore, prior to the start of the study in 1993 there will have been some selection for Lev resistance. Nevertheless, the rapid development of resistance to Lev is not uncommon; in Australia resistance to this drug was selected for in both *Ostertagia* and *Trichostrongylus* spp within one year of its introduction (Anderson, Martin, Jarrett, Brown & Miller, 1988b). The potential for misinterpretation of the FECRT when assessing Lev resistance is well known (Grimshaw *et al.*, 1996). However, in this present study only adult parasites were present and thus any reduction in the calculated efficacies cannot be due to stage specific differences in susceptibility.

Geometric mean worm burdens of the untreated control lambs in the 1995 CET agree reasonably well with those seen in the larval cultures used in this study. For instance, the proportion of *T. vitrinus* in the larval cultures obtained from the Ivm, Fbz, Lev and Fbz/Lev treatment paddocks were 2, 25, 36 and 25 % respectively. The % of the total geometric mean worm burdens which were *T. vitrinus* in the 1995 CET were 1, 14, 22 and 22 % for the untreated control animals grazing the Ivm, Fbz, Lev and Fbz/Lev treatment paddocks respectively. The donor lambs taken from each paddock appear to be representative of their group and support the results of this study on the anthelmintic resistance status of the parasites on each of the paddocks at Firth Mains.

CHAPTER 7

The effects of feed withdrawal, divided dosing and co-administration of piperonyl butoxide upon the pharmacokinetics of fenbendazole

7.1 Introduction

The increasing prevalence of anthelmintic resistance has focused attention on the need to maximize the useful life span of the present and any future anthelmintic compounds. Consequently, recent research has investigated means of increasing drug bioavailability in order to maximize treatment efficacy against heterozygous and homozygous resistant individuals. Since the mode of action of Bz anthelmintics requires sustained presentation for activity rather than high initial concentrations (Lacey, 1988), altering drug administration offers a means of increasing the duration of availability and efficacy against resistant parasites.

It is known that reductions in feed result in reduced flow rates of digesta through the gastrointestinal tract (Coombe & Kay, 1965). Taylor *et al.* (1992) investigated the importance of feed intake upon the pharmacokinetics of Fbz, as well as Ivm, in sheep and cattle fed different diets. Using a chromium-EDTA marker these workers demonstrated that the rate of passage of digesta through the gut was more rapid in lambs grazing fresh pasture than that of housed animals fed a hay and concentrate diet. It was suggested that the reduced availability of the drugs in the former animals, as estimated by area under the plasma concentration-time curve (AUC), was a consequence of reduced time for absorption of the anthelmintics. Ali & Chick (1992) confirmed these observations when describing the availability of oxfendazole in plasma of sheep fed fresh pasture compared with housed sheep fed a dry ration.

The concept that a faster gastric flow rate in sheep on high feed intake decreases the time for drug absorption and recycling, contributing to a shorter duration of drug availability, has been established by the work of Ali & Hennessy (1993 and 1995a and 1995b). These workers found that halving the feed intake of sheep slowed the passage of digesta through the gastrointestinal tract and increased the period for drug release from particulate material and absorption into the bloodstream. By implementing such a regimen, 36 hours before and after drug treatment, the activity of oxfendazole was significantly increased against Bz-resistant isolates of *H. contortus* and *T. colubriformis*. Increased efficacy appears to be influenced by drug-digesta particle association in the rumen which, by regulating the rate and duration of metabolite availability, is a major determinant of the

pharmacokinetic disposition of oxfendazole in ruminants (Hennessy *et al.*, 1994). Similar results against Bz-resistant isolates of *H. contortus* and *T. colubriformis* have been demonstrated with albendazole despite intrinsic differences in pharmacokinetic behaviour compared to oxfendazole (Hennessy *et al.*, 1995).

Extending the period of drug administration by dividing the dose has likewise been shown to increase the efficacy of Bz anthelmintics against resistant parasites. Bogan *et al.* (1987), working with goats, demonstrated that the repetition of three administrations of oxfendazole at 24 hour intervals produced significant increases in the AUC compared to a single equivalent dose. Using a similar regime, Sangster *et al.* (1991b) demonstrated increased drug bioavailability and increased efficacies against Bz resistant isolates of *H. contortus*, *Ostertagia* spp and *Trichostrongylus* spp. In addition, greater efficacy was recorded by Sangster *et al.* (1991b) when the oxfendazole doses were separated by 12 hours compared to a 24 hour inter-dosing interval.

Modification of drug metabolism is another method which has been shown to enhance treatment efficacy against resistant parasites. Benzimidazole sulphide anthelmintics are extensively metabolised into their sulphoxide which in turn are oxidized into the more polar and less anthelmintically active sulphone metabolites (Prichard *et al.*, 1985). Benchaoui & McKellar (1994) investigated the effects of piperonyl butoxide, a novel Bz synergist, upon Fbz metabolism in sheep. Piperonyl butoxide (PB) is a synthetic methylenedioxyphenyl derivative widely used as an insecticide synergist (Hodgson, Ryu, Adams & Levi, 1995) and has been shown to inhibit mixed function oxidase activity in insects as well as mammals (Haley, 1978). Inhibition of cytochrome P450 activity which is involved in the mixed-function oxidization of xenobiotic compounds in order to facilitate their excretion, has been observed in liver microsomal preparations from goats treated with PB (Burley & Bray, 1983). In sheep infected with a Bz-resistant isolate of *T. circumcincta*, Benchaoui & McKellar (1994) demonstrated an increase in worm reduction of over 80 % compared to conventional Fbz treatment by co-administering PB. The enhanced treatment efficacy was attributed to a decreased rate of Fbz metabolism, resulting in an increased bioavailability of Fbz and its active sulphoxide metabolite, oxfendazole (Ofz).

Feed withdrawal, divided dosing and co-administration of PB and their effects upon the treatment efficacy of Fbz were described in chapter 4. This chapter will focus on these methods in more detail and determine their effects upon the pharmacokinetics of Fbz in sheep naturally infected with a Bz-resistant isolate of *T.circumcincta.*

7.2 Materials and methods

7.2.1 Divided dosing and feed withdrawal

The effects of divided dosing and feed withdrawal upon the treatment efficacy and pharmacokinetics of Fbz were investigated in the mid-seasonal FECRT in 1994. The naturally infected 5 month old ewe and wether Suffolk-cross lambs were grazing the Fbz treatment paddock, known to be contaminated predominantly with a Bz-resistant isolate of *T. circumcincta* (Coop *et al.*, 1993). Lambs were faecal sampled and weighed two days before treatment and allocated into balanced groups such that each group had members with similar faecal egg count and bodyweight. Group treatments (n = 6) were assigned randomly and the group which were used in the feed reduction study housed 24 hours prior to drug administration. This group were allowed water *ad libitum* but denied access to feed whilst the other groups remained at pasture.

All animals were faecal sampled again on day 0 and treated on the basis of liveweight with Fbz (2.5 % w/v Panacur, Hoechst UK Ltd.) at the relevant MRD for sheep. Blood samples were taken as previously described in chapter 2.4.2 at 0, 4, 8, 12, 20, 24, 36, 48, 72, 120 and 168 hours following treatment and plasma stored at –20 °C for subsequent analysis. Group 1 lambs were orally administered with Fbz at the MRD for sheep (5 mg kg^{-1}). Group 2 were also given Fbz at 5 mg kg^{-1} but the dose was divided with half the MRD given at time 0 and half after a 12 hour interval (2.5 + 2.5 mg kg^{-1}). Those of group 3 were denied access to food for 24 hours before being treated with Fbz at the standard dose rate (5 mg kg^{-1}). Following treatment all animals were returned to pasture.

7.2.2 Co-administration of piperonyl butoxide

Lambs from the same paddock, now aged 7 months, were used to investigate the effects of co-administering piperonyl butoxide (90 % w/v, Aldrich Chemicals Co., UK) upon the treatment efficacy and pharmacokinetics of Fbz in the 1994 end of season CET. A dose rate of 63 mg kg^{-1} for PB was selected as the optimal concentration following titration studies (Benchaoui & McKellar, 1994). Eight lambs were drafted in from the control group, two of which were assigned to each of the groups in order to provide sufficient animals for statistical comparisons. All animals were faecal sampled and weighed two days before treatment and allocated into balanced groups such that each group had members with similar faecal egg count and bodyweight. Group treatments (n = 6) were assigned randomly and faecal samples re-taken on day 0 before treating animals on the basis of liveweight at the relevant dose rate.

Blood samples were taken as previously described in chapter 2.4.2 at 0, 1, 4, 8, 12, 24, 32, 48, 72, 96, 120 and 168 hours following treatment and plasma stored at –20 °C for subsequent analysis. Group 1 lambs were treated with Fbz at the MRD for sheep (5 mg kg^{-1}) whereas those of group 2 remained as the untreated controls. Group 3 were orally administered PB (63 mg kg^{-1}) and acted as controls for this compound. Group 4 were co-administered both Fbz and PB (5 + 63 mg kg^{-1}). The animals remained housed until necropsy on day 14 post-treatment.

7.2.3 Parasitological parameters

Treatment efficacies for the FECRT and CET were calculated according to WAAVP guidelines as described previously in chapter 4.2.

7.2.4 HPLC analyses

Fbz and its sulphoxide (Ofz) and sulphone ($FbzSO_2$) metabolites were extracted from plasma and quantified by HPLC analysis. The preparation of standards and samples, extraction procedures and HPLC run conditions are described in chapter 2.7. All HPLC analyses were carried out under good laboratory practice

(GLP) in the department of veterinary pharmacology laboratory at Glasgow University Veterinary School.

7.2.5 Pharmacokinetic and statistical analyses

The maximum plasma concentration (Cmax), time to maximum concentration (Tmax), the area under the plasma concentration-time curve computed to the last observation (AUC_{last}) and the area under the first-moment curve computed to the last observation ($AUMC_{last}$) were derived from fitted profiles of individual animals using the pharmacokinetic modelling program PCNONLIN, version 4.0 (Statistical Consultants Inc., Lexington, USA). The mean residence time computed to the last observation (MRT_{last}) was calculated as the ratio $AUMC_{last}/AUC_{last}$. The half-life ($T_{1/2}$) was calculated by multiplying the MRT by the natural log of 2 (0.693). Differences in the pharmacokinetic parameters between groups were determined by analysis of variance (Minitab, version 10.0), with a value of $P < 0.05$ being considered statistically significant.

7.3 Results

7.3.1 Divided dosing and feed withdrawal

Plasma concentration profiles of Fbz, and its sulphoxide (Ofz) and sulphone ($FbzSO_2$) metabolites following feed withdrawal, divided dosing and conventional treatment are shown in Figures 7.1, 7.2 and 7.3 respectively. The respective mean pharmacokinetic parameters for these metabolites are shown in Tables 7.1, 7.2 and 7.3. The Cmax and AUC values for Fbz, Ofz and $FbzSO_2$ following feed withdrawal were consistently higher than the values obtained by conventional treatment and divided dosing. The maximum concentration of 0.17 $\mu g\ ml^{-1}$ achieved for the active Ofz metabolite following feed withdrawal was an increase of over 40 % ($P<0.05$) compared to the other treatment methods. Although the highest concentrations were achieved following feed withdrawal, the longest residence times were achieved following a divided dose. Using this approach the Tmax was increased by over 14 hours ($P<0.001$) and the MRT by nearly 6 hours ($P<0.05$) compared to conventional

treatment. The Tmax value for Fbz using a divided dose was also 8 hours longer than was achieved using feed withdrawal ($P<0.05$).

The FECRT calculated efficacies for the different methods of drug presentation are shown in Table 7.4. Pre- and post-treatment egg measurements identified 100 % *Teladorsagia* confirming that the paddock was contaminated predominantly with this species at this point in the season. Dividing the dose with a 12 hour interval resulted in an increased efficacy of 28.8 % compared to conventional treatment although this was not statistically significant. Administering the MRD following a 24 hour period of food withdrawal on the otherhand resulted in a significant increase compared to conventional treatment of 39.7 % ($P < 0.05$).

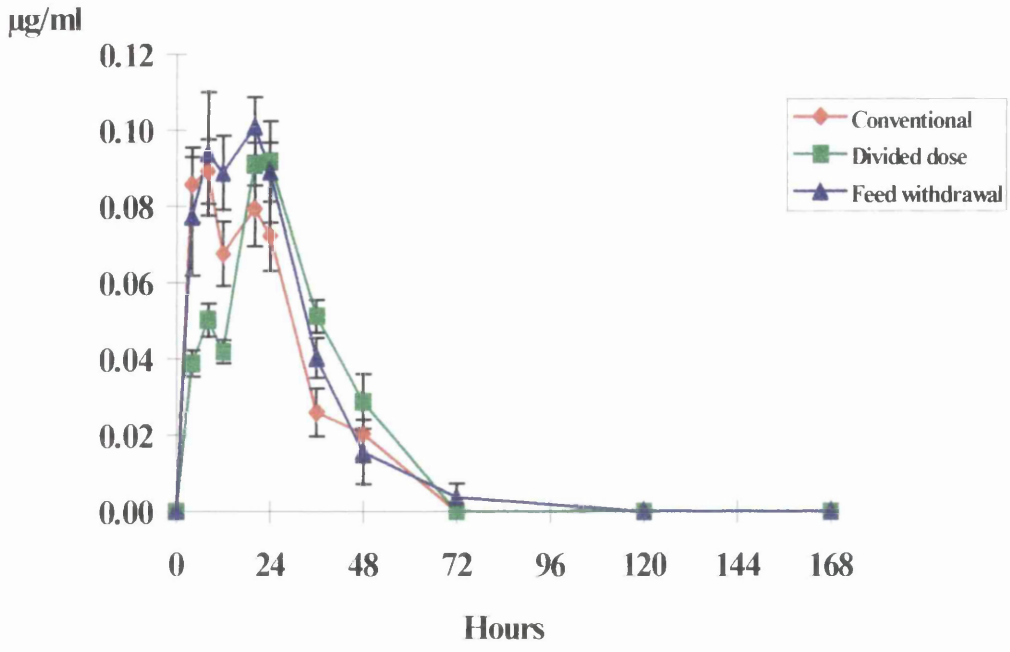

Figure 7.1 *Mean plasma concentrations (± SEM) of fenbendazole (Fbz) in groups of sheep (n = 6) treated with Fbz either conventionally (5 mg kg $^{-1}$), as a divided dose (2.5 + 2.5 mg kg $^{-1}$) with a 12 hour treatment interval or following 24 hours of feed withdrawal (5 mg kg $^{-1}$)*

Table 7.1 *Pharmacokinetic parameters (mean ± SD) of fenbendazole (Fbz) in groups of sheep (n = 6) treated with Fbz either conventionally (5 mg kg $^{-1}$), as a divided dose (2.5 + 2.5 mg kg $^{-1}$) with a 12 hour treatment interval or following 24 hours of feed withdrawal (5 mg kg $^{-1}$)*

Parameter	Conventional(a) (5 mg kg^{-1})	Divided dose(b) (2.5 + 2.5mg kg^{-1}) 12 hour interval	Feed withdrawal(c) (24 hours) followed by 5 mg kg^{-1}
Cmax (µg ml^{-1})	0.09 (0.02)	0.10 (0.01)	0.12 (0.02)
Tmax (h)	8.0 (6.2)	22.7 (2.1) a*** c*	14.0 (8.3)
$T_{1/2}$ (h)	14.3 (2.5)	18.3 (2.3) a*	15.3 (4.5)
AUClast (µg.h ml^{-1})	2.84 (1.04)	3.03 (0.52)	3.43 (1.07)
AUMClast (µg.h^2 ml^{-1})	61.24 (29.63)	80.96 (21.51)	80.24 (50.14)
MRTlast (h)	20.59 (3.60)	26.40 (3.28) a*	22.10 (6.55)

* significantly different from suffix group $P < 0.05$, ***$P < 0.001$

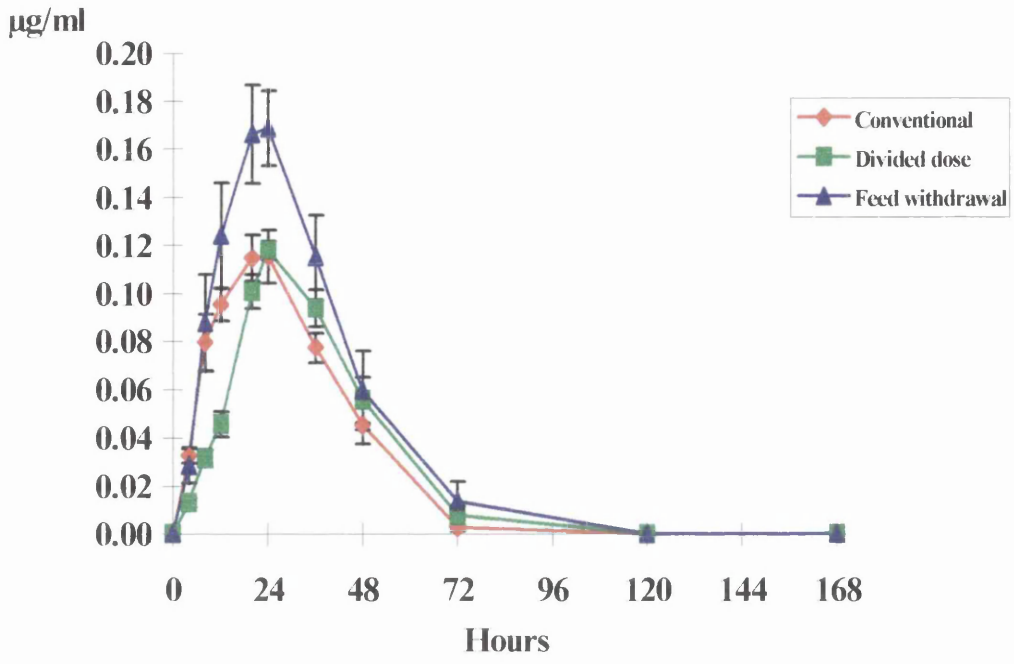

Figure 7.2 *Mean plasma concentrations (±SEM) of fenbendazole sulphoxide (oxfendazole, Ofz) in groups of sheep (n = 6) treated with Fbz either conventionally (5 mg kg $^{-1}$), as a divided dose (2.5 + 2.5 mg kg $^{-1}$) with a 12 hour treatment interval or following 24 hours of feed withdrawal (5 mg kg $^{-1}$)*

Table 7.2 *Pharmacokinetic parameters (mean ±SD) of fenbendazole sulphoxide (Ofz) in groups of sheep (n = 6) treated with Fbz either conventionally (5 mg kg $^{-1}$), as a divided dose (2.5 + 2.5 mg kg $^{-1}$) with a 12 hour treatment interval or following 24 hours of feed withdrawal (5 mg kg $^{-1}$)*

Parameter	Conventional(a) (5 mg kg^{-1})	Divided dose(b) (2.5 + 2.5mg kg^{-1}) 12 hour interval	Feed withdrawal(c) (24 hours) followed by 5 mg kg^{-1}
Cmax (µg ml^{-1})	0.12 (0.03)	0.12 (0.01)	0.17 (0.04) a* b*
Tmax (h)	22.7 (2.1)	24.0 (0.0)	22.7 (2.1)
$T_{1/2}$ (h)	19.3 (1.6)	22.8 (3.1) a*	20.6 (3.9)
AUClast (µg.h ml^{-1})	4.47 (1.10)	4.41 (0.99)	6.50 (2.58)
AUMClast (µg.h^2 ml^{-1})	125.1 (37.1)	147.8 (50.6)	201.1 (110.5)
MRTlast (h)	27.83 (2.28)	32.96 (4.53) a*	29.75 (5.61)

* significantly different from suffix group $P < 0.05$

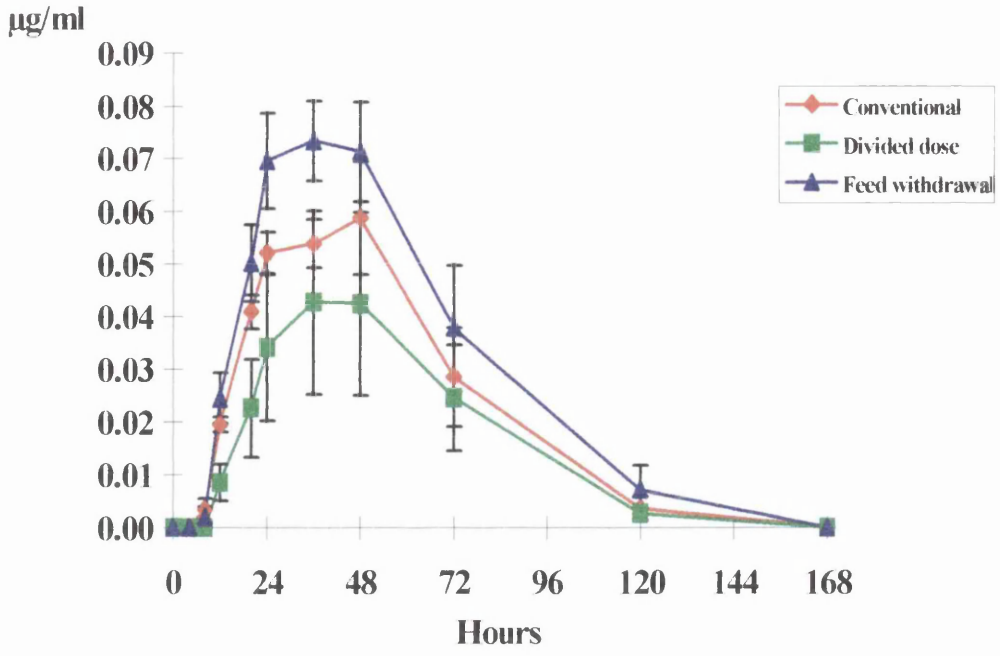

Figure 7.3 *Mean plasma concentrations (±SEM) of fenbendazole sulphone (Fbz SO_2) in groups of sheep (n = 6) treated with Fbz either conventionally (5mg kg^{-1}), as a divided dose (2.5 + 2.5 mg kg^{-1}) with a 12 hour treatment interval or following 24 hours of feed withdrawal (5 mg kg^{-1})*

Table 7.3 *Pharmacokinetic parameters (mean ±SD) of fenbendazole sulphone (FbzSO_2) in groups of sheep (n = 6) treated with Fbz either conventionally (5mgkg^{-1}), as a divided dose (2.5 + 2.5 mg kg^{-1}) with a 12 hour treatment interval or following 24 hours of feed withdrawal (5 mg kg^{-1})*

Parameter	Conventional(a) (5 mg kg^{-1})	Divided dose(b) (2.5 + 2.5mg kg^{-1}) 12 hour interval	Feed withdrawal(c) (24 hours) followed by 5 mg kg^{-1}
Cmax (µg ml^{-1})	0.06 (0.02)	0.05 (0.01)	0.08 (0.02) b**
Tmax (h)	36.0 (10.7)	42.0 (6.6)	42.0 (10.0)
$T_{1/2}$ (h)	33.7 (3.8)	34.8 (5.5)	33.8 (7.3)
AUClast (µg.h ml^{-1})	3.69 (1.46)	2.75 (1.0)	4.88 (2.18)
AUMClast (µg.h^2 ml^{-1})	185.1 (96.1)	144.1 (81.8)	253.7 (160.2)
MRTlast (h)	48.59 (5.53)	50.18 (7.92)	48.75 (10.55)

** significantly different from suffix group $P < 0.01$

Table 7.4 *Faecal egg count reductions in groups of sheep (n = 6) naturally infected with Bz-resistant T. circumcincta, treated with Fbz either conventionally (5mg kg $^{-1}$), as a divided dose (2.5 + 2.5 mg kg $^{-1}$) with a 12 hour treatment interval or following 24 hours of feed withdrawal (5 mg kg $^{-1}$)*

Group (n=6)	Treatment	EPG (± SD) day -2	EPG (± SD) day 0	EPG (± SD) day 10	% Reduction	95 % CIs	P value
1	Conventional (5 mg kg^{-1})	218 (198)	171 (126)	86.7 (77)	49.3	0, 81	-
2	Divided dose (2.5 + 2.5 mg kg^{-1}) (12 hour interval)	211 (243)	155 (115)	34 (45)	78.1	21, 94	0.13
3	24 hour food withdrawal followed by 5 mg kg^{-1}	223 (235)	318 (196)	35 (50)	89.0	59, 97	0.03*

* Significantly different compared to conventional treatment $P< 0.05$

7.3.2 Co-administration of piperonyl butoxide

Plasma concentration profiles of Fbz, and its sulphoxide (Ofz) and sulphone ($FbzSO_2$) metabolites following conventional treatment with Fbz or with its co-administration with piperonyl butoxide (PB) are shown in Figures 7.4, 7.5 and 7.6 respectively. The respective mean pharmacokinetic parameters for these metabolites are shown in Tables 7.5, 7.6 and 7.7. The mean maximum concentrations (Cmax) achieved following conventional treatment for Fbz and its Ofz and $FbzSO_2$ metabolites were 0.05, 0.09 and 0.04 μg ml^{-1} respectively. Increases of over 200 % ($P<0.001$) for each of these values was achieved with the co-administration of PB. Similar increases in AUC's ($P<0.01$) for each of the metabolites were seen as a result of co-administration but there were no statistical differences in the Tmax, $T_{1/2}$ or MRTs compared to conventional treatment.

CET calculated efficacies for the different methods of drug presentation are shown in Table 7.8. Only reductions in abomasal worms are shown since Fbz was fully effective against small intestinal species. Due to the wide range of burdens seen in the untreated and PB control animals these groups were pooled in order to calculate treatment efficacies. The co-administration of PB resulted in an increase in worm reduction of over 27 % compared to conventional treatment, although this figure was not statistically significant.

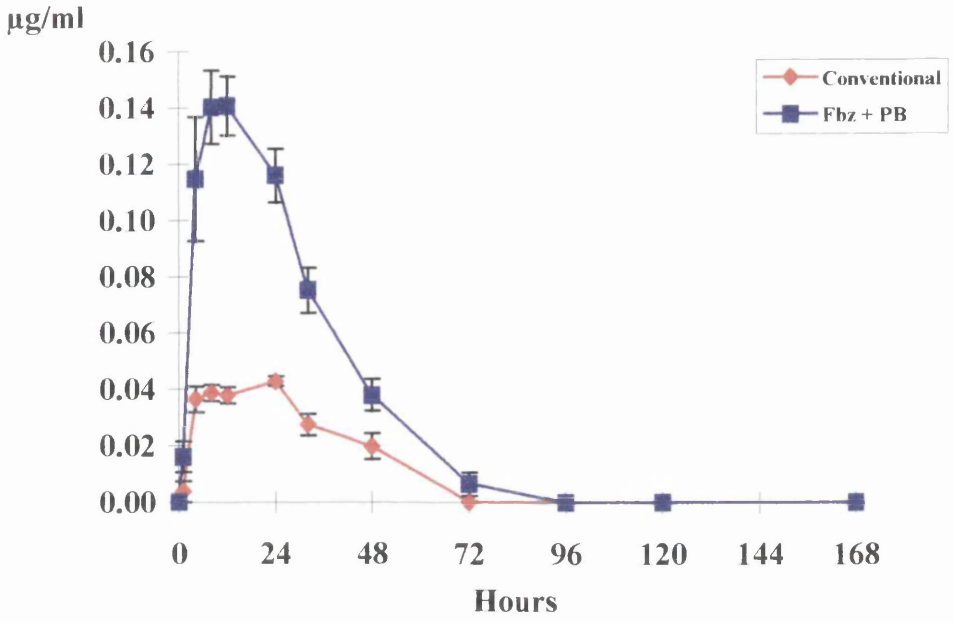

Figure 7.4 *Mean plasma concentrations (±SEM) of fenbendazole (Fbz) in groups of sheep (n = 6) treated with Fbz either conventionally (5 mg kg $^{-1}$) or in combination with piperonyl butoxide (5 + 63 mg kg $^{-1}$)*

Table 7.5 *Pharmacokinetic parameters (mean ±SD) of fenbendazole (Fbz) in groups of sheep (n = 6) treated with Fbz either conventionally (5 mg kg $^{-1}$) or in combination with piperonyl butoxide (5 + 63 mg kg $^{-1}$)*

Parameter	Conventional (5 mg kg^{-1})	Fbz + PB (5 + 63 mg kg^{-1})
Cmax (µg ml^{-1})	0.05 (0.01)	0.15 (0.02) ***
Tmax (h)	18.0 (9.4)	10.7 (7.0)
$T_{1/2}$ (h)	17.62 (2.59)	16.60 (2.01)
AUClast (µg.h ml^{-1})	1.74 (0.37)	5.09 (1.11) ***
AUMClast (µg.h^2 ml^{-1})	45.09 (13.80)	123.90 (41.38) **
MRTlast (h)	25.43 (3.74)	23.95 (2.90)

** significantly different from conventional treatment $P < 0.01$, ***$P < 0.001$

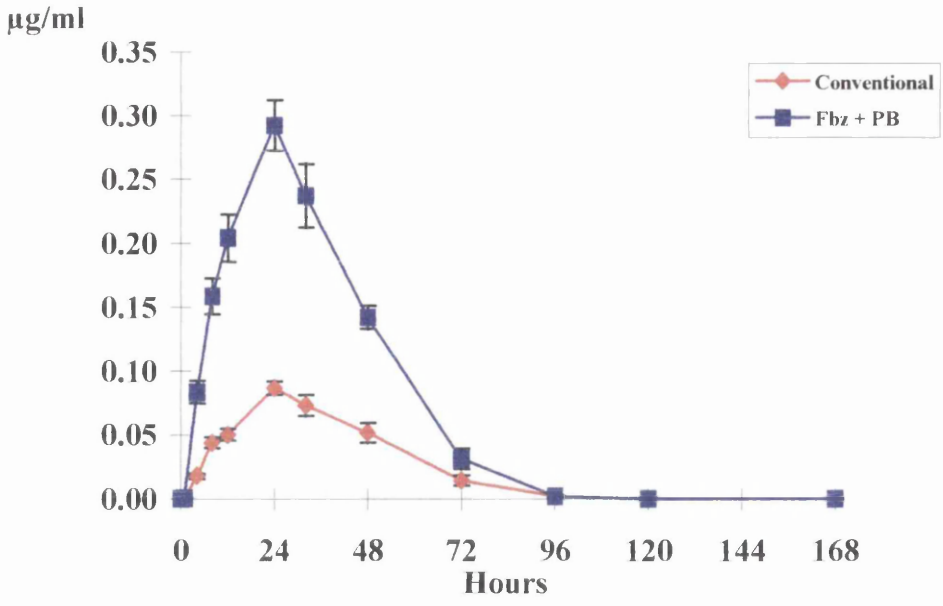

Figure 7.5 *Mean plasma concentrations (±SEM) of fenbendazole sulphoxide (oxfendazole, Ofz) in groups of sheep (n = 6) treated with Fbz either conventionally (5 mg kg $^{-1}$) or in combination with piperonyl butoxide (5 + 63 mg kg $^{-1}$)*

Table 7.6 *Pharmacokinetic parameters (mean ±SD) of fenbendazole sulphoxide (Ofz) in groups of sheep (n = 6) treated with Fbz either conventionally (5 mg kg $^{-1}$) or in combination with piperonyl butoxide (5 + 63 mg kg $^{-1}$)*

Parameter	Conventional (5 mg kg^{-1})	Fbz + PB (5 + 63 mg kg^{-1})
Cmax (µg ml^{-1})	0.09 (0.02)	0.29 (0.05) ***
Tmax (h)	25.3 (3.3)	24.0 (0.0)
$T_{1/2}$ (h)	23.98 (2.98)	22.06 (1.78)
AUClast (µg.h ml^{-1})	3.81 (0.96)	11.98 (2.34) ***
AUMClast (µg.h^2 ml^{-1})	134.5 (49.2)	382.6 (87.1) ***
MRTlast (h)	34.60 (4.30)	31.83 (2.57)

*** significantly different from conventional treatment $P < 0.001$

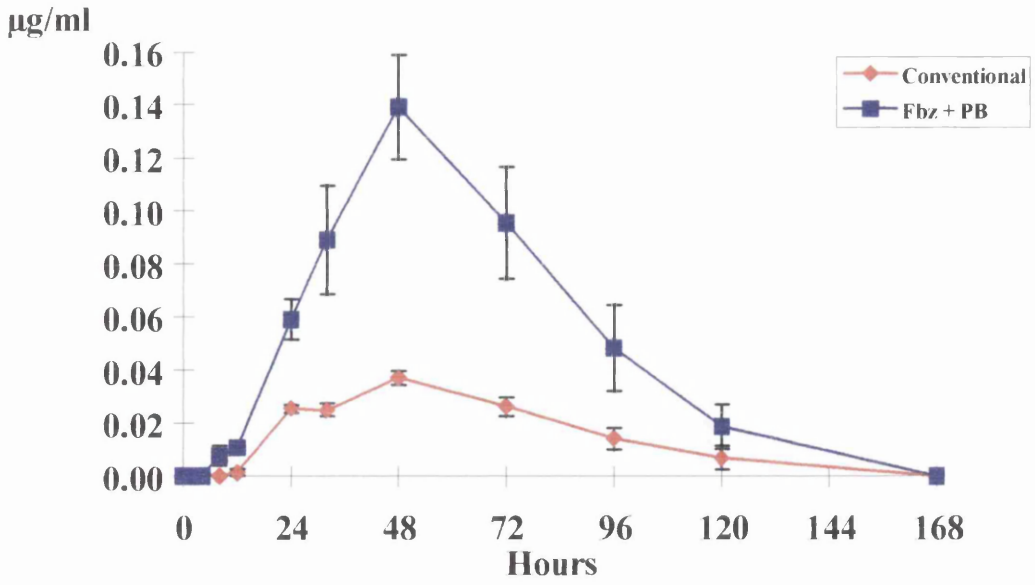

Figure 7.6 *Mean plasma concentrations (±SEM) of fenbendazole sulphone ($FbzSO_2$) in groups of sheep (n = 6) treated with Fbz either conventionally (5mgkg^{-1}) or in combination with piperonyl butoxide (5 + 63 mg kg^{-1})*

Table 7.7 *Pharmacokinetic parameters (mean ± SD) of fenbendazole sulphone ($FbzSO_2$) in groups of sheep (n = 6) treated with Fbz either conventionally (5mgkg^{-1}) or in combination with piperonyl butoxide (5 + 63 mg kg^{-1})*

Parameter	Conventional (5 mg kg^{-1})	Fbz + PB (5 + 63 mg kg^{-1})
Cmax (µg ml^{-1})	0.04 (0.01)	0.14 (0.05) ***
Tmax (h)	48.0 (0.0)	48.0 (0.0)
$T_{1/2}$ (h)	41.55 (6.74)	42.00 (4.22)
AUClast (µg.h ml^{-1})	2.52 (0.99)	8.68 (4.19) **
AUMClast (µg.h^2 ml^{-1})	158.59 (89.83)	539.98 (313.03) *
MRTlast (h)	59.95 (9.72)	60.60 (6.09)

* significantly different from conventional treatment $P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Table 7.8 *Abomasal worm reductions in groups of sheep (n = 6) treated with Fbz either conventionally (5 mg kg $^{-1}$) or in combination with piperonyl butoxide (5 + 63 mg kg $^{-1}$)*

Group (n=6)	Treatment (day 0)	Geometric mean worm burden (± SD) day 14	% Reduction*
1	Conventional Fbz (5 mg kg^{-1})	4861 (3633)	25.7
2	Untreated controls	4623 (7640)	-
3	PB controls (63 mg kg^{-1})	9267 (4684)	-
4	Fbz + PB (5 + 63mg kg^{-1})	3071 (1789)	53.1

*using pooled geometric mean worm burdens of untreated and PB controls

7.4 Discussion

The 'effective' concentrations of a drug to which a parasite population must be exposed have never been specifically defined, nor are they likely to be, given the multitude of variables which are associated with host and parasite (Hennessy, 1994). In his review, Hennessy (1994) proposed that the metabolite concentration in the respective compartment which is midway between zero and the maximum could be regarded as the minimum 'effective' level (Figure 7.7). Assuming these proposals, the effective concentrations for the active Fbz and Ofz metabolites were extended for each of the enhancement methods, particularly co-administration of PB.

As well as sulphoxidation, Fbz and Ofz may undergo hydroxylation, the metabolites of which are also thought to contribute to anthelmintic activity (Short *et al.*, 1988). These hydroxylated metabolites are then conjugated to increase their solubility before the majority are secreted in bile, of which about 40 % are reabsorbed and enterohepatically recycled (Hennessy, Steel & Prichard, 1993b). The extensive metabolic sequences which are necessary for the clearance of Fbz are further complicated by the involvement of gut microflora which may reduce Ofz back to its parent molecule within the ruminal environment. Similarly, biliary metabolites in the large intestine may be deconjugated by bacteria to further promote

absorption (Hennessy, 1993). Assessment of the pharmacokinetics of Fbz and its metabolites in plasma, therefore, is likely to be only an approximation of the situation at the pharmacodynamic level. Nevertheless, any such alterations would be expected to increase the duration of the plasma-tissue recycling process, leading to increased concentrations of active drug traversing the gastrointestinal mucosa.

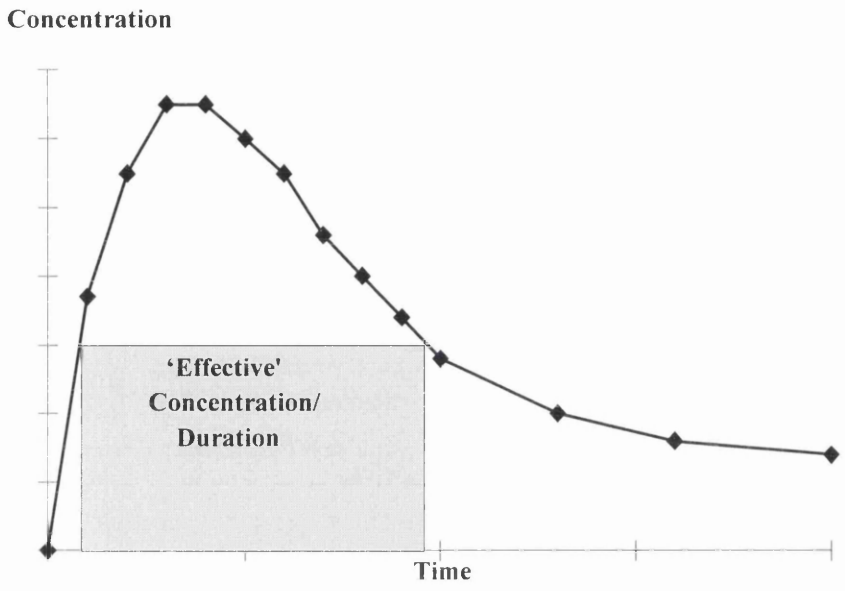

Figure 7.7 *Typical concentration-with-time profile of a drug metabolite in plasma demonstrating first-order absorption and elimination processes. Adapted from Hennessy (1994)*

The increases seen in both drug availability and treatment efficacy as a result of divided dosing and feed withdrawal serve to confirm the merits of these techniques in the combat against resistant parasites. The mean half life of the parent compound of just over 14 hours following conventional treatment serves to illustrate the necessity of a 12 hour treatment interval when employing a divided dose. Considering that the clearance of a single dose follows first order principles, a 24 hour treatment interval would expose worms to two discrete intervals of drug which is not as effective as the sustained exposure conferred by a 12 hour interval.

The increases in drug availability that resulted from the co-administration of PB, although more clear-cut, were not mirrored with increases in treatment efficacy of the same magnitude. This may be explained by the inclusion of naturally infected control animals. As discussed in chapter 4, treatment efficacies calculated using untreated control animals with varying degrees of acquired immunity will underestimate reductions that would be expected when employing parasite naive animals. Since their seasonal treatments were not fully effective the majority of lambs grazing the Fbz paddock will have had constant infections and the potential to mount solid immune responses. Although the lambs were allocated into balanced groups on the basis of faecal egg counts, this is often a poor indicator of worm burden, especially with older animals (McKenna, 1981). These remarks are supported by the work of Benchaoui & McKellar (1994) who demonstrated an increase in worm reduction of over 80 % with the co-administration of PB compared to conventional Fbz treatment. The parasite naive lambs used by these workers were also Suffolk-cross, but more importantly the Bz-resistant *T. circumcincta* used for artificial infection was isolated from the same pastures at Firth Mains.

The co-administration of PB produced increases in Cmax and AUC of approximately 200 % for each of the Fbz metabolites compared to conventional treatment. However, no differences were apparent between either treatment regime in terms of residence parameters. PB has been shown to produce a distinct biphasic effect on several hepatic enzyme activities in mice (Kinsler, Levi & Hodgson, 1990). Inhibition and subsequent induction of these enzymes may explain why the residence times were not extended to the same degree as the initial metabolite concentrations.

Another interesting observation from this work was the difference between the pharmacokinetic parameters of the conventionally treated animals in either study. The Cmax and AUC were consistently lower for each of the metabolites in the conventionally treated lambs of the PB study. Retention parameters (Tmax, $T_{1/2}$ and MRT), on the otherhand, were extended in the same animals. These observations may be explained by the fact that the lambs used in the second study were treated at the end of the season when infections with *T. vitrinus* were also apparent. Parasitism with small intestinal species is associated with increased digesta transit time at this site (Gregory, Wenham, Poppi, Coop, MacRae & Miller, 1985) as well as villous

atrophy (Coop *et al.*, 1979), both of which may affect the pharmacokinetics of anthelmintic drugs. Similar results, reduced Cmax and extended Tmax values, have recently been reported in lambs infected with *T. colubriformis* (Landuyt *et al.*, 1995). Lambs used in the divided dosing/reduced feed study returned to pasture following treatment whereas those in the second study remained housed and were fed hay and concentrates. The differences between these diets may have also affected the pharmacokinetics of the conventionally treated lambs in either study (Taylor *et al.*, 1992).

This present study on the co-administration of PB has provided valuable data as to whether or not such combinations may be effective in the control of resistant parasites. Other compounds such as the anti-thyroid drug methimazole and the anti-steroid drug metyrapone have been reported to inhibit the microsomal oxidation of different xenobiotics (Tynes & Hodgson, 1983). The influence of these compounds upon the pharmacokinetics of the pro-benzimidazole anthelmintic netobimin in sheep was investigated by Lanusse & Prichard (1991). More recently, Lanusse, Gascon & Prichard (1995) investigated the effect of methimazole upon the pharmacokinetics of Fbz and Ofz in sheep. Improved pharmacokinetic profiles were obtained in both studies and, since both compounds are inexpensive and relatively safe, further research is required to evaluate their potential.

Parbendazole, a Bz of only moderate anthelmintic potency, has also been shown to enhance the anthelmintic efficacy of Ofz against resistant isolates (Hennessy, Lacey, Prichard & Steel, 1985; Hennessy, Steel, Prichard & Lacey, 1992). The latter workers concluded that the presence of parbendazole temporarily slowed the hepatic metabolism and biliary secretion of Ofz and its metabolites. This depressed liver activity also affected the relative metabolite proportions with increased concentrations of anthelmintically active hydroxy-Fbz (OH.Fbz) compared to conventional treatment. Although parbendazole is a strong inhibitor of mammalian microtubule assembly (Lacey, Brady, Prichard & Watson, 1987) the study of Hennessy *et al.* (1992) demonstrated that these effects were reversible and that the use of such a combination may be sufficiently safe for commercial application.

Although the use of slow-release devices would similarly prolong the duration for absorption of Bz compounds from the gastrointestinal tract, the use of feed reduction offers a means of achieving this goal. Using existing preparations, the manipulation of feed intake requires only that suitable penning is available to enable animals to be held around the time of treatment. Withholding feed offers a simple and practical approach to maximizing the bioavailability and efficacy of Bz drugs and may prolong their useful life-span against resistant parasites. Increases in efficacy of the magnitude reported here may still be valuable in strategic and prophylactic regimens aimed at controlling ostertagiosis, where the principal aim is to reduce parasite impact to levels which do not compromise animal performance and welfare.

CHAPTER 8

Investigation into the use of arbitrarily primed polymerase chain reaction (AP-PCR) in the detection of drug resistant nematode parasites

8.1 Introduction

The polymerase chain reaction (PCR) technique promotes the amplification of a segment of DNA that is flanked by two regions of known sequence. Double-stranded DNA is first denatured by increasing the temperature to provide two single-stranded DNA templates. By lowering the temperature, oligonucleotide primers, with complementary sequences to flanking regions, are allowed to anneal to each strand. This provides a starting point for the synthesis of a new strand of DNA, catalyzed by the enzyme DNA polymerase. The discovery of a heat stable DNA polymerase (Taq) from the thermophilic bacterium, *Thermus aquaticus*, allows repeated cycles of denaturation, annealing and DNA extension. As a result, the production of an exponential number of copies of the central DNA segment is possible without inactivating the enzyme (Saiki, Scharf, Faloona, Mullis, Horn, Erlich & Arnheim, 1985).

Arbitrarily primed PCR (AP-PCR) is a recently developed technique (Welsh & McClelland, 1990; Williams, Kubelik, Livak, Rafalski & Tingey, 1990) which employs a single primer of arbitrary nucleotide sequence which simultaneously amplifies a number of loci distributed throughout the genome. Each primer gives a different pattern of products, revealed by electrophoresis, each with the potential of detecting polymorphisms between strains. It is a relatively simple process that does not require nucleotide sequence information or experience of molecular techniques such as the cloning of probes or hybridization methods. The pattern of products resulting from AP-PCR are referred to by some workers as random amplified polymorphic DNA markers (RAPD) and their use here will be synonymous.

Resistance to the Bz anthelmintics appears to be due to a change in the β-tubulin isotype pattern, resulting in the loss of high affinity receptor binding sites. Using an allele-specific PCR, Kwa *et al.* (1994) demonstrated that the main cause for Bz resistance in both *H. contortus* and *T. colubriformis* is a single mutation from phenylalanine to tyrosine at amino acid position 200 in the β-tubulin isotype 1 gene. The same mechanism for resistance to Bz drugs has also been shown to exist in *T.circumcincta* (Elard *et al.*, 1996). A prerequisite of allele-specific PCR however, is that the gene(s) associated with resistance to a particular anthelmintic is/are already defined. Preliminary findings at Moredun (D. Knox & R. C. Moore, unpublished

data) have suggested that AP-PCR may be able to differentiate between Bz-resistant and susceptible populations of *T. circumcincta* and *H. contortus* without the need for specific probes. The aim of this study was to confirm these findings and investigate variation at the single parasite level. Furthermore, since knowledge of the site of action and the molecular mechanisms of levamisole/morantel and ivermectin resistance is limited, it was hoped that such a technique may also be applicable in the detection of parasites resistant to either of these anthelmintics.

8.2 Materials and methods

8.2.1 Parasite material

All of the parasites used in this study were isolated from field material and maintained at Moredun in worm-free donor lambs. Adult worms were collected at necropsy as described in chapter 2.3. Methods of larval culture, recovery and storage are described in chapter 2.2.5. Details of the nematode isolates used in the study are described in chapter 2.2.6. The two *H. contortus* isolates used in the preliminary investigation were the Moredun Bz-susceptible strain (HcS) and a multiple resistant (Bz + Ivm) white river strain from South Africa (HcR). The two *T. circumcincta* isolates used were the Moredun Bz-susceptible strain (MOSI) and a multiple resistant (Bz + Ivm) caprine strain (SCRI).

8.2.2 Genomic DNA

Methods of extracting genomic DNA from adult populations and individual larvae are described in chapter 2.6.1. DNA extracted from adult populations was diluted using sterile distilled water to a concentration of approximately 8 ng μl^{-1} before storing aliquots at –20 °C. The concentration of DNA extracted from individual larvae was not quantified.

8.2.3 Primers

The oligonucleotide primers available for the study were purchased from Oswell DNA Service (Edinburgh, UK) and are listed in Table 8.1. Primer stock

solutions were diluted to a concentration of 10 μM using sterile distilled water (Appendix A).

Table 8.1 *Primers tested for arbitrarily primed PCR of genomic DNA from adult populations of H. contortus and T. circumcincta*

Primer	Sequence*
391 R	5'-ACC.GCA.TGC.AAC.ACG.AGC.ACC.-3'
392 R	5'-GCG.GGT.CCG.CAT.TTC.AAT.CCT.-3'
393 R	5'-GTT.GGT.GAT.CTT.GGA.AAT.-3'
394 R	5'-ATT.TCC.AAG.ATC.ACC.AAG.-3'
395 R	5'-TTT.TCC.GAG.ATC.ATC.-3'
508 G	5'-ACA.GAA.TTC.CAG.GGI.CAG.TGC.GGI.TCI.TGC.TGC.TGG.-3'
509 G	5'-ACA.AAG.CTT.GTA.ICC.ICC.GTT.GCA.ICC.CTC.-3'

*A = Adenine, C = Cytosine, G = Guanine, T = Thymine, I = Inosine

8.2.4 AP-PCR amplification

8.2.4.1 Adult populations

Optimal amplification conditions were investigated using genomic DNA extracted from the Bz-susceptible isolate of *H. contortus* (HcS) diluted to a concentration of 8 ng μl^{-1}. Amplifications were performed in 25 μl reaction volumes, the protocol of which is described in chapter 2.6.2. Primers 391R and 509G were chosen, as was an annealing temperature of 45 °C and a final $MgCl_2$ concentration of 4 mM, to proceed the investigation with resistant and susceptible isolates.

8.2.4.2 Individual larvae

In order to achieve amplification from individual larvae the AP-PCR protocol had to be redeveloped such that there were lower concentrations of primer and $MgCl_2$ but increased amounts of polymerase enzyme. Primer 509G was chosen to investigate the optimal amplification conditions for genomic DNA from individual larvae. Extracted DNA was re-suspended in 20 μl of sterile distilled water the day

before AP-PCR. Amplifications were performed in 50 µl reaction volumes with the following amendments to the protocol: 5 µl PCR incubation buffer (Appendix A), 2.0 µl Primer, 2.0 µl $MgCl_2$ (25 mM, final concentration of 1 mM) and 9.0 µl of sterile water. After boiling the tubes for 5 minutes, 10 µl of stock dNTPs and 2.0 µl of Taq DNA polymerase (1.0 Units μl^{-1}, Boehringer, UK) was added before overlaying with 50 µl of mineral oil. Amplification conditions were the same as those described in chapter 2.6.2.

8.2.5 Analysis of PCR products

All AP-PCR reactions were routinely analysed using 1.4 % agarose gel electrophoresis, described in chapter 2.6.3. If greater resolution was required samples were analysed using 7.5 % polyacrylamide gel electrophoresis, described in chapter 2.6.4. A large (300 ml) 1.4% agarose gel (run at 25V overnight) and two 10% polyacrylamide gels (20mA for 16 hours) were used to compare individual larvae from MOSI and SCRI, using sample volumes of 20 µl.

8.3 Results

8.3.1 Adult populations

Amplification products for the different primers using genomic DNA from HcS with an annealing temperature of 45 °C and a final $MgCl_2$ concentration of 4 mM are shown in Plate 8.1. Controls containing all the components except the template DNA were always negative. Primers 391R and 509G produced a more clearly defined range of banding patterns and were chosen to proceed the investigation with the resistant and susceptible isolates. The amplification products using genomic DNA from the susceptible and resistant isolates of *H. contortus* and *T. circumcincta*, as revealed by agarose and polyacrylamide gel electrophoresis, are shown in Plates 8.2 and 8.3 respectively. There were distinct differences in the banding profiles of the susceptible and resistant isolates using either primer. These differences were enhanced by polyacrylamide gel electrophoresis which allowed the resolution of more detailed banding.

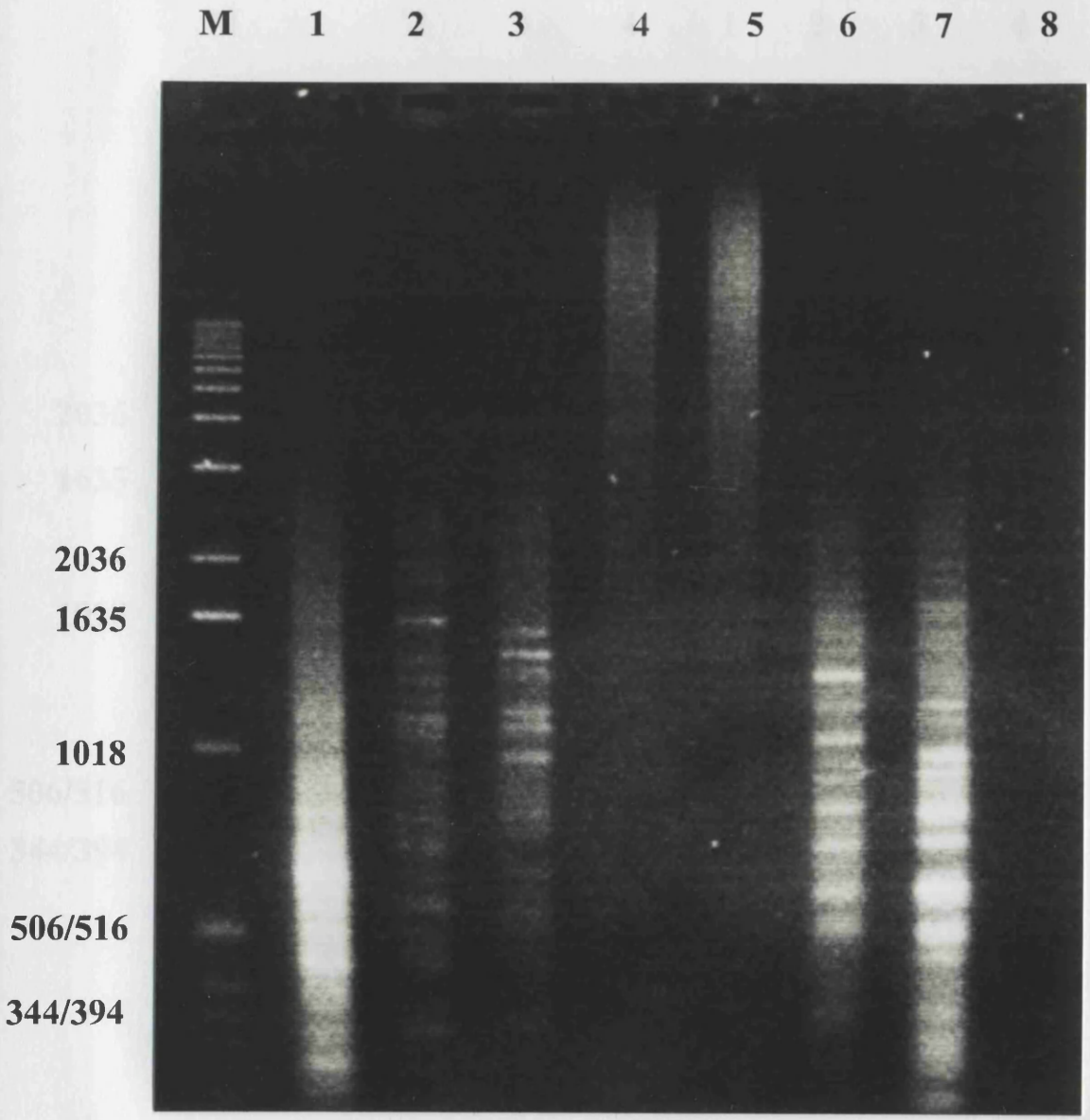

Plate 8.1 *Amplification products for different primers using genomic DNA from a Bz-susceptible isolate of H. contortus (HcS), an annealing temperature of 45 °C and a final $MgCl_2$ concentration of 4 mM. M = Molecular weight markers, Primers: 1 = 391R, 2 = 392R, 3 = 393R, 4 = 394R, 5 = 395R, 6 = 508G, 7 = 509G, 8 = –ve Control. Visualized using 1.4 % agarose gel electrophoresis*

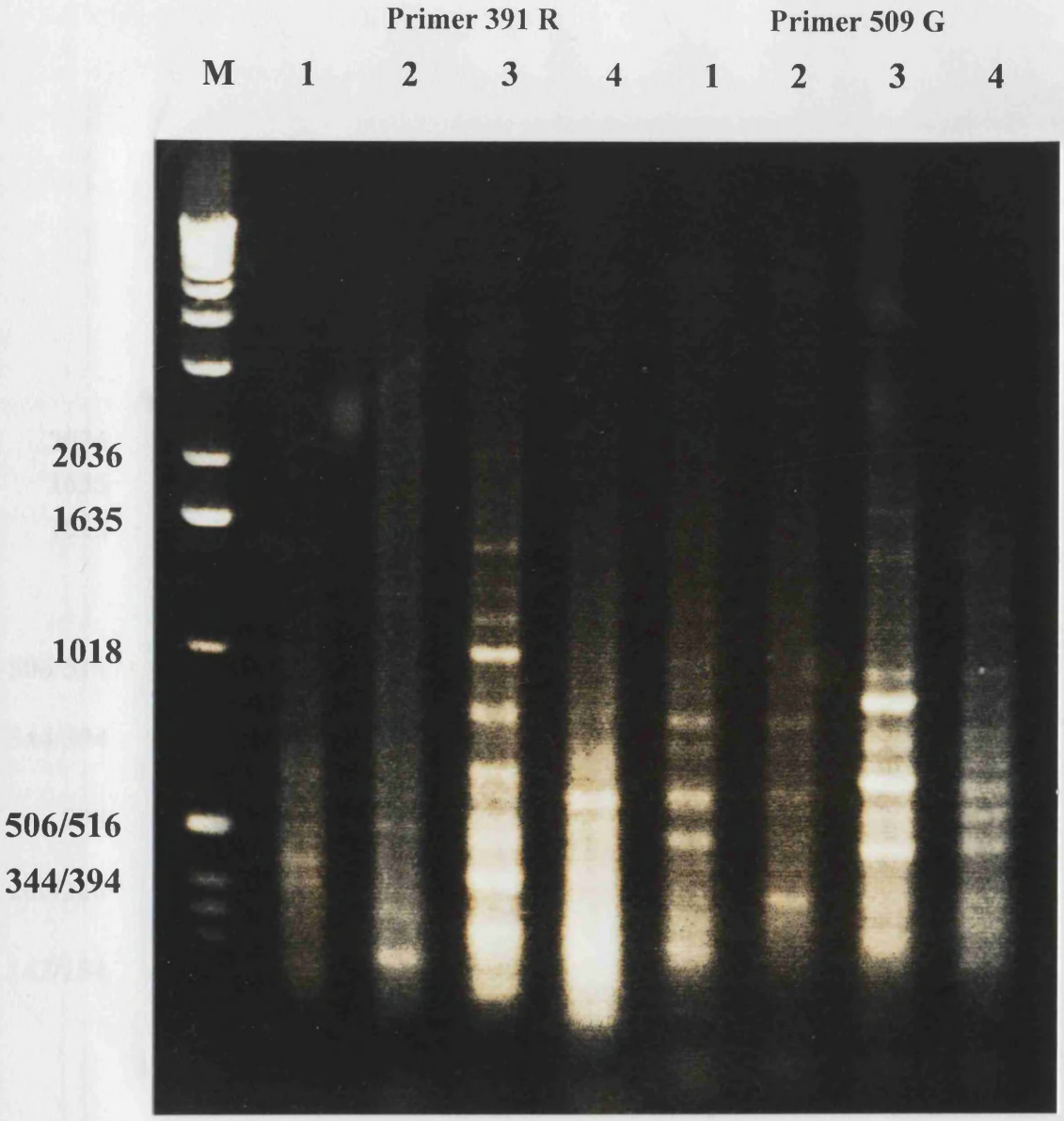

Plate 8.2 *Amplification products using primers 391R or 509G and genomic DNA from a Bz-susceptible isolate of H. contortus (HcS), a multiple resistant (Bz + Ivm) isolate of H. contortus (HcR), a Bz-susceptible isolate of T. circumcincta (MOSI) and a multiple resistant (Bz + Ivm) isolate of T. circumcincta (SCRI). M = Molecular weight markers, 1 = HcS, 2 = HcR, 3 = MOSI, 4 = SCRI. Visualized using 1.4 % agarose gel electrophoresis*

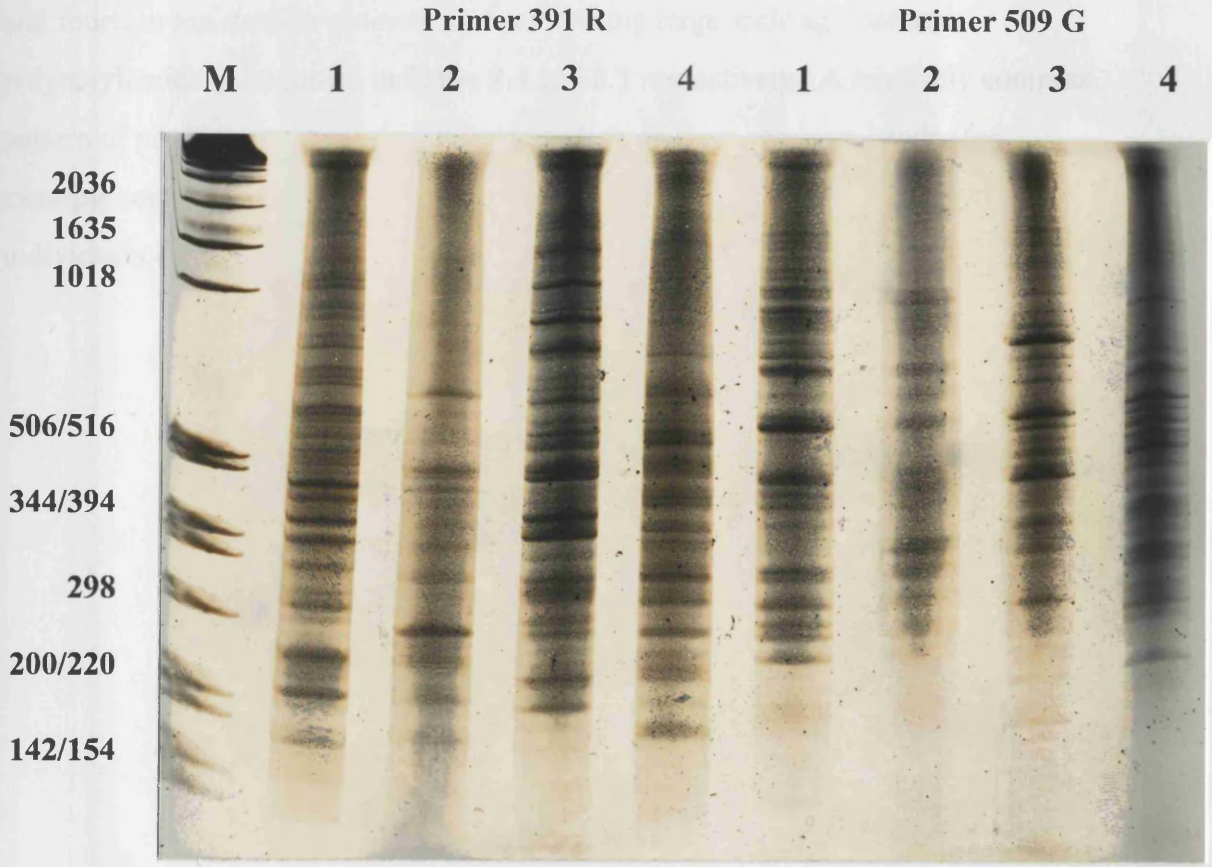

Plate 8.3 *Amplification products using primers 391R or 509G and genomic DNA from a Bz-susceptible isolate of H. contortus (HcS), a multiple resistant (Bz + Ivm) isolate of H. contortus (HcR), a Bz-susceptible isolate of T. circumcincta (MOSI) and a multiple resistant (Bz + Ivm) isolate of T. circumcincta (SCRI). M = Molecular weight markers, 1 = HcS, 2 = HcR, 3 = MOSI, 4 = SCRI. Visualized using 7.5 % polyacrylamide gel electrophoresis*

8.3.2 Individual larvae

Differences in the banding intensity were observed between and within different AP-PCR runs. Controls containing all the components except the template DNA were always negative. Non-discrete size ranges of amplification products, appearing as a 'smear' as visualized by electrophoresis were also a problem between and within runs. Nevertheless, successful amplifications from fourteen susceptible and fourteen resistant larvae were compared using large scale agarose and polyacrylamide gels, shown in Plates 8.4 and 8.5 respectively. A relatively complex pattern of products was revealed and although there were common bands (for example see arrow in Plate 8.5) there was also considerable variation between individuals of each particular isolate.

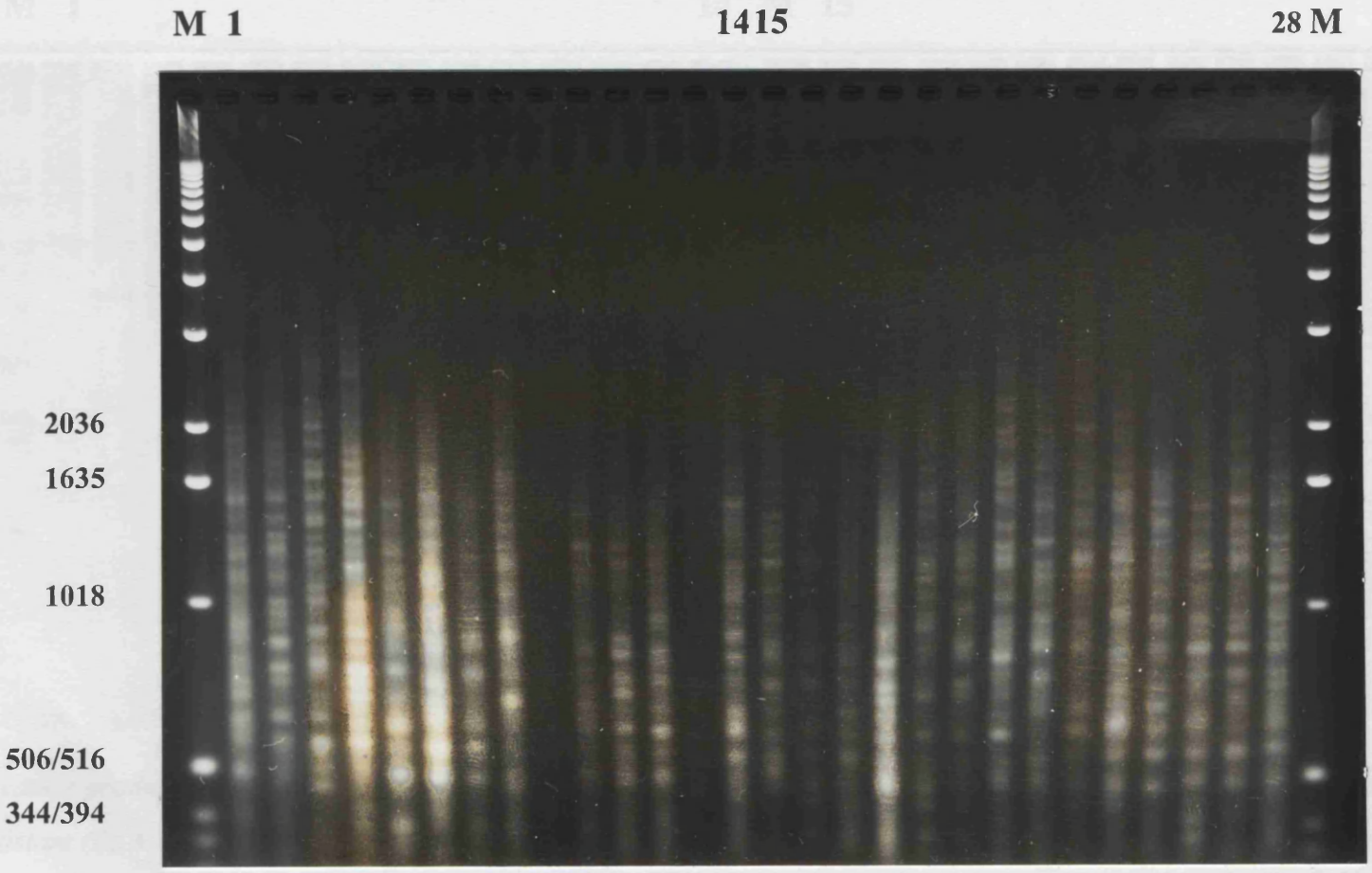

Plate 8.4 *Amplification products using primer 509G and genomic DNA from individuals of a Bz-susceptible isolate of T. circumcincta (MOSI) and a multiple resistant (Bz + Ivm) isolate of T. circumcincta (SCRI). M = Molecular weight markers, 1 to 14 = MOSI, 15 to 28 = SCRI. Visualized using 1.4 % agarose gel electrophoresis*

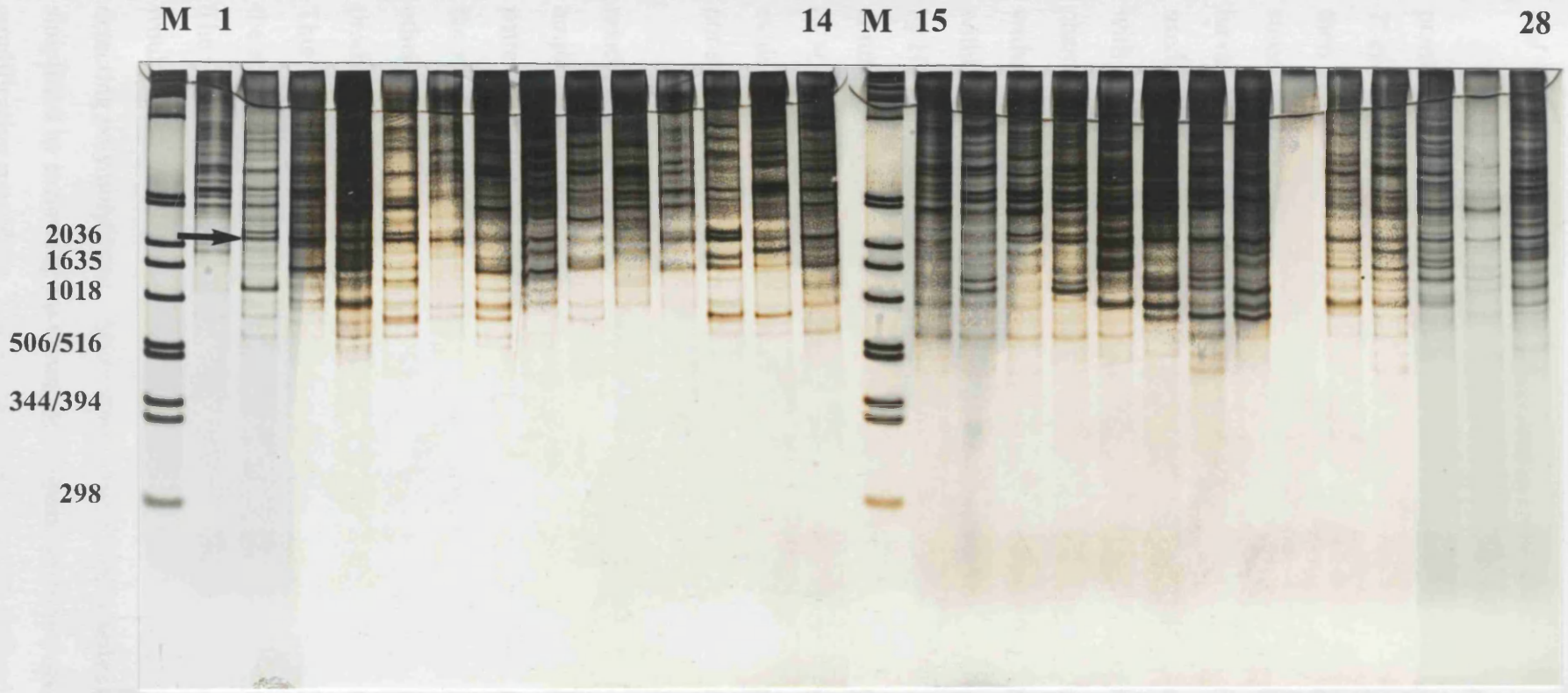

Plate 8.5 *Amplification products using primer 509G and genomic DNA from individuals of a Bz-susceptible isolate of T. circumcincta (MOSI) and a multiple resistant (Bz + Ivm) isolate of T. circumcincta (SCRI). M = Molecular weight markers, 1 to 14 = MOSI, 15 to 28 = SCRI. Visualized using 7.5 % polyacrylamide gel electrophoresis*

8.4 Discussion

Results of AP-PCR using DNA from adult populations suggested that it may be possible to differentiate between susceptible and resistant isolates of *H. contortus* or *T. circumcincta*. If a particular band was repeatedly amplified in either population then it is possible that it could be used as a diagnostic marker for resistance or susceptibility. However, there was also variation in the banding patterns of single larvae from within a population. It is unlikely therefore, that this approach could be used routinely to differentiate between susceptible and resistant parasites (at least with the primer employed in this study). Grant & Whitington (1994), whilst characterizing a library of DNA probes, demonstrated extensive genetic variability within and between two isolates of *T. colubriformis*. The intraspecific variability within species was also very large for most of the trichostrongylid nematodes studied by Humbert & Cabaret (1995). Blouin, Yowell, Courtney & Dame (1995) also commented on the unusually high within-population diversity of mitochondrial DNA of *H. contortus* and *T. circumcincta*. The results of the present study provide further evidence of the considerable DNA polymorphism within helminth parasites and it is not surprising therefore, that such variability in banding patterns were achieved.

The method developed for the extraction of DNA from individuals was relatively simple and did not require exsheathment of larvae. Although separate amplifications from the same individual were not attempted the reproducible banding patterns achieved whilst developing the technique (not shown) support the validity of the variation seen at the individual level, a conclusion which has also been drawn by other workers (Humbert & Cabaret, 1995). However, the quality of amplification products did vary between runs and the methodology requires further fine tuning. This was probably a reflection of the quality of the template DNA and demonstrates the sensitivity of the technique to any changes in PCR components (Black, 1993). The use of different primers also merits further study. Shorter oligonucleotides would be expected to produce more detailed patterns with a greater potential of detecting polymorphisms. Alternatively, the complex series of bands could be simplified by increasing the annealing temperature to improve the stringency of the amplification procedure. The use of primers towards defined elements within a

species (Gasser, Nansen & Bøgh, 1995) could also increase the precision of this approach.

In the case of Bz resistance the AP-PCR products could be Southern blotted and a β-tubulin probe employed to determine whether or not one or more of the bands is mutually exclusive for this genotype. This approach would specifically visualize PCR products encompassing all, or part, of the β-tubulin gene and has the potential of differentiating between resistant and susceptible banding profiles. However, until the molecular mechanisms of levamisole/morantel or ivermectin resistance are determined, it is unlikely that such an approach could be adopted for these drug classes. AP-PCR clearly has its limitations that need to be considered when assessing its potential. Since electrophoresis separates bands on the basis of molecular weight, it is possible that apparently homologous fragments do not correspond to the same portion of genomic DNA (Dias Neto, Pereira de Souza, Rollinson, Katz, Pena & Simpson, 1993). Furthermore, as AP-PCR is not quantitative, it is unknown whether individuals whose DNA yields a specific fragment are heterozygous or homozygous for an amplifiable allele (Williams *et al.*, 1990). Therefore the usefulness of such a technique in detecting heterozygous resistant individuals is questionable. Ideally a genetically based method is required that can identify these genotypes so that resistance can be diagnosed earlier and reversion given a chance to succeed. Nevertheless, AP-PCR does have the potential to monitor resistance and provide a more accurate diagnosis of the problem. Its application as an epidemiological tool in the assessment of different approaches to the prevention and reversion of anthelmintic resistance also merits further study.

CHAPTER 9

General discussion

9.1 Discussion

The results from this series of studies have provided useful data on the epidemiology, diagnosis and control of anthelmintic resistant populations of *Teladorsagia* and the effects of these populations upon lamb performance. Production losses that result from nematode parasitism can be very difficult to assess or quantify, particularly in the field (Sykes, 1994). Most of our knowledge on the impact of infection comes from pathophysiological and nutritional studies with artificial infections. Suckling lambs at pasture with their mothers will be exposed to small numbers of larvae from a very early age which may have important consequences on the development of immunity. Another important aspect concerning artificial infection is the age of the infective larvae, since storing larvae at 4 °C for differing periods of time can result in different infectivity levels (Kerboeuf, Hubert & Mallett, 1989) and may explain discrepancies seen in trials of similar design. The naturally infected lambs in this field study however provided a valuable model for studying grazing performance in set stocked animals on permanent pasture. There was little evidence of marked differences in performance in the Fbz treated lambs over the three year study despite the presence of a Bz resistant isolate of *Teladorsagia* on the paddocks at Firth Mains. The maximum difference in weight gain between the Fbz and best performing group at the end of the 1993, 1994 and 1995 seasons were 3.1, 3.6 and 2.6 kgs, reductions of 12.6, 13.5 and 9.5 % respectively. In this present study, as in previous studies (Rowlands & Probert, 1972; Coop *et al.* 1985), the two principal gastrointestinal nematodes, *N.battus* and *T.circumcincta* both compromised performance in the absence of correctly timed control measures. Although the results are encouraging with regard to the use of resistance selected drugs (RSDs) in the control of less pathogenic species with a relatively low biotic potential such as *Teladorsagia*, it should be noted that only a modest stocking rate was employed and that there was no evidence of clinical disease. The effects of using less effective drugs upon lamb performance may well be more serious in situations where challenge from pasture is greater or the development of immunity compromised by nutritional stress or trace element deficiencies.

The importance of acquired immunity in the regulation and limitation of pasture contamination was also highlighted in this study. By mid-season of each year there was evidence of regulation of egg output despite increasing pasture contamination. It should be noted however, that the particularly dry spells in 1994 and 1995 may have produced atypical epidemiological patterns. The population dynamics of gastrointestinal nematodes can be influenced by a number of seasonal, nutritional and managemental factors which are depicted in Figure 9.1. The potential for complex interactions between the various elements make it very difficult to compare lamb performance between seasons. Nevertheless, *Teladorsagia* populations tend to be remarkably stable which is thought to be a consequence of stereotypical and density dependent effects upon faecal egg output seen with this species (Barger, 1987). Climatic conditions especially favourable for hatching and survival of eggs and larvae, or a succession of unfavourable seasons, tend therefore not to lead to escalating or declining *Teladorsagia* populations.

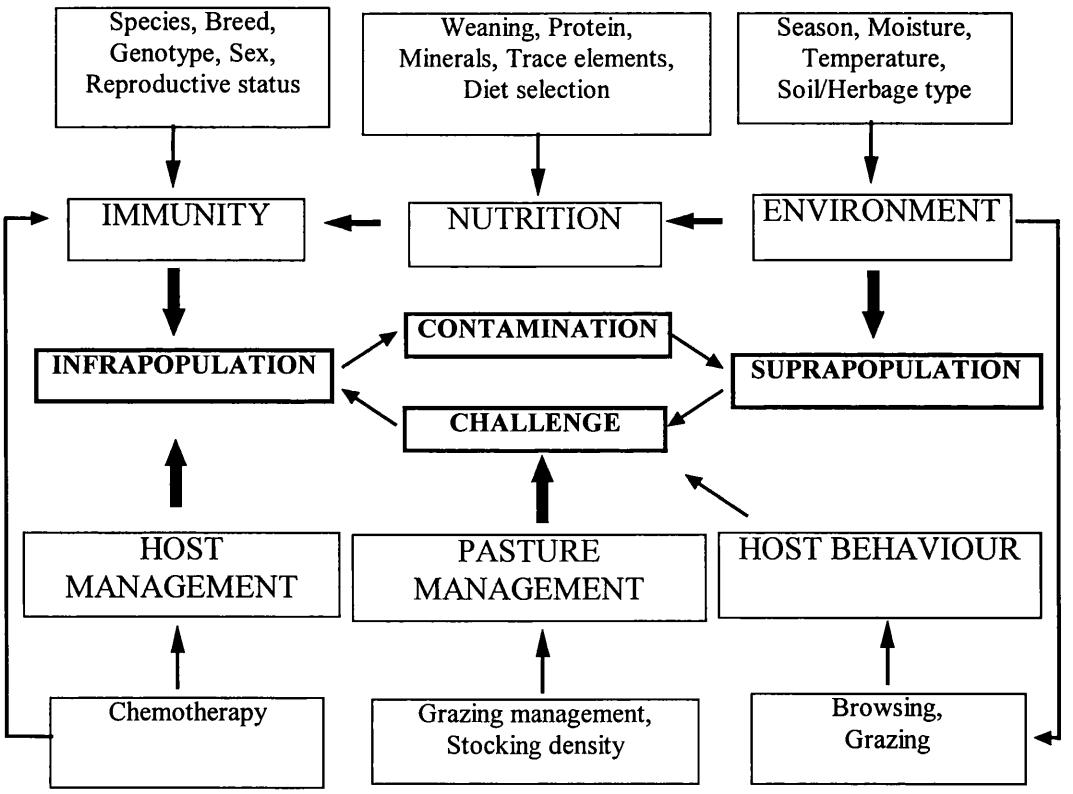

Figure 9.1 *Interactive elements that influence parasite population dynamics*

One interesting aspect of this study was the continued highly efficacious use of the Fbz/Lev combination both in terms of lamb performance and treatment efficacy. The principal theory behind the use of combination drenches is largely based on the proposition that dual resistance is likely to be comparatively rare and that worms surviving one of these drugs will be removed by the other (McKenna *et al.*, 1996). Modelling studies have also suggested that the use of Bz-Lev combinations may well be a more effective means of delaying resistance than administering the same drugs in an annual alternation (Smith, 1990; Barnes *et al.*, 1995). Although advocated to slow down the development of anthelmintic resistance, the short term benefits of using such combinations when resistance is present are clear. The significance of this statement is further emphasized by the suspected development of Lev resistance on the paddock at Firth Mains where Lev was used. These findings provide some support therefore for the view expressed by McKenna (1990b) that the selection for multiple resistance is unlikely to be greater than that which would otherwise develop from the use of each component separately. Furthermore, evidence from Australia (Anderson *et al.*, 1991; Overend *et al.*, 1994) and New Zealand (McKenna *et al.*, 1996) have shown that the use of Bz-Lev combinations may also be effective on some properties where resistance is present against both anthelmintics. Combination drenches consisting of mixtures of Bz and Lev drugs have been released onto the anthelmintic market in Australia and New Zealand in recent years but at present are not licensed for use in the UK. Whether such combinations ever become available in the UK will depend not only on their perceived commercial benefits but also upon the attitude of the licensing authorities. Evidence from this study certainly indicates that manufacturer's should consider the introduction of combinations as a means of prolonging the useful life of both Bz and Lev anthelmintics.

The FECRT remains the method of choice for the routine monitoring of resistance, being capable of evaluating any anthelmintic and the advantage of simplicity. However, it has been estimated that it is only when the proportion of resistant parasites is above 25% that this *in vivo* test can detect anthelmintic resistance (Martin *et al.*, 1989). It is also important that the species of nematode eggs

pre- and post-treatment are differentiated to avoid interpretive errors of the FECRT (McKenna, 1996) since the identification of species surviving treatment is meaningless without a corresponding measure of their initial abundance. Seasonal changes in the relative numbers of the various nematode parasites of sheep (Boag & Thomas, 1977) emphasizes the necessity of pre-treatment evaluation. Egg differentiation techniques (Christie & Jackson, 1982) were employed to identify genera in these studies, but coproculture and larval identification could also have been used.

This study has provided further evidence to suggest that the interval before post-treatment sampling in the FECRT may need to be reconsidered, at least for the levamisole class of anthelmintics (Grimshaw *et al.*, 1996). These workers concluded that it may be necessary to take faecal samples less than 7 days post-treatment to avoid misinterpretation associated with the subsequent development of immature stages. This is especially relevant to *Teladorsagia* spp., the immature stages of which are thought to be particularly refractory to levamisole). Similarly, the WAAVP guideline of 10-14 days for post-treatment sampling may have to be reconsidered for both the host and nematode species under investigation. Discrepancies between the results of FECRTs in sheep and goats were noted by Jackson (1993) and it was suggested that longer resampling periods may be required when investigating Ivm resistance in the caprine host. Furthermore, the fecundity of parasites capable of surviving anthelmintic treatment appears to be variable, which reduces the value of the test as a quantitative assay. For example, McKellar *et al.* (1988) noted that the numbers of eggs *in utero* of *Cooperia* were markedly reduced following treatment with Ivm. On the otherhand Scott *et al.* (1991) noted that the numbers of eggs *in utero* increased in adult multiple resistant *Haemonchus* within 7 days post-treatment with Ivm but were lower following the administration of oxfendazole. The likelihood of false positive results for *Nematodirus* spp. when using an interval of 14 days was also demonstrated in this study. It appears that the FECRT may need to be tailored to suit the host, drug and nematode species under investigation.

Several other points need to be considered before results of the FECRT can be regarded with confidence. Firstly, the FECs of the untreated control lambs in this

study increased markedly upon housing. Increases in faecal egg count following the cessation of larval dosing have been noted in previous experimental infections with *Teladorsagia* species (Coop *et al.*, 1982 and 1985). These authors suggested that the antigenic stimulus from incoming larvae may have elicited responses that depressed the egg laying capacity of existing adult worm populations and/or retarded the development of larvae through to the adult stage. The removal of this stimulus was thought to be the primary factor for the abrupt changes in egg count. However, the possibility of dietary changes influencing faecal egg concentration should also be considered. In the present study faecal consistencies did not change and there was no evidence of inappetence upon housing and it was concluded that the increase in FECs was caused by a relaxation in immunity resulting from the removal of larval antigenic stimulus. Although housing animals offers the substantial benefit of convenience for resistance screening, the results from our studies suggest that faecal egg output should be closely monitored. It may be necessary to allow some time for the worm populations to stabilize and to obtain pre-treatment FEC data to enable the selection of comparable groups for the FECRT. Another convenient time to conduct FECRTs in the case of ewes is during the PPRI. However, this also poses some problems since the degree and extent of relaxation appears to very variable. Recent studies in New Zealand, in which the faecal egg output of *Teladorsagia* was suppressed by the inclusion of a non-rumen degradable protein, have shown that the effects of the PPRI may also be markedly influenced by nutrition (M. F. J. van Houtert, personal communication).

The definitive test for determining drug susceptibility/resistance to all stages of development is the CET. However, results from this present study have also identified weaknesses in this test when applied to naturally infected animals. The heterogeneity of host immunoresponsiveness, reflected in the worm burdens of untreated control animals, affects the calculated efficacy of RSDs. The influence of acquired immunity upon CET findings was exemplified in untreated control animals at the end of each season, some of which had very low worm burdens. This also raises the question as to whether low burdens in treated animals were the result of anthelmintic treatment, acquired immunity or the additive effects of the two. The obvious response would be to test lambs before they have acquired immunity but

there is a drawback to this apparently simple solution. It is well established that there is a succession in prevalence of nematode species throughout the season (Boag & Thomas, 1977). In naturally infected animals CETs against species such as *T.vitrinus* for example, which become more numerous in lambs later in the season, would be compromised by age based restrictions.

Between season differences in climate, herbage availability and parasite contamination will also affect the development of immunity (Figure 9.2). These uncontrolled seasonally varying factors make it very difficult therefore, to compare changes in population susceptibility/resistance over time. The presence of inhibited larvae having different rates of metabolism/susceptibility may also be expected to affect treatment efficacy for certain drugs. A fuller understanding of the sources of trial variability is required to increase the sensitivity of the CET in field infections.

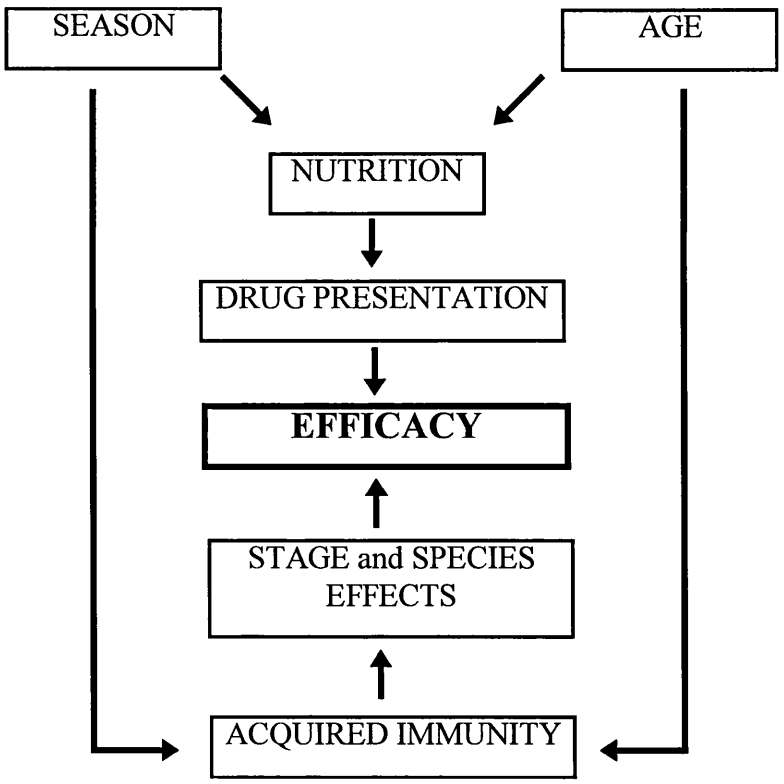

Figure 9.2 *Interactive elements which may affect treatment efficacy*

The use of highly effective drugs may also interfere with the development and maintenance of immunity. There was clear evidence in the study of Barger (1988) that untreated lambs, but not lambs given one or more treatments, had acquired immunity against *H. contortus* by the age of 4 months. Recent studies have suggested that anthelmintics may exert direct effects upon the immune system of sheep and possibly hormone levels. Cabaj, Stankiewicz, Jonas & Moore (1994 and 1995) studied the effects of Fbz and Lev in uninfected sheep which were injected with soluble (ovalbumin) and particulate (human erythrocytes) antigens following drenching on days 0 and 28. In both studies there were variable humoral immune responses to the different antigens and decreased serum complement activity was noted 3 days after the second antigen injection. Interestingly, however, at the end of each experiment the drenched lambs were significantly heavier than the non-drenched control lambs. In the case of Fbz these differences were not attributed to increases in levels of growth promoting hormones whereas in the Lev study there was a small decrease in insulin-like growth factor-1 (IGF-1) hormone levels 4 days after the first drench. In each case the authors concluded that the growth enhancing effects of these drugs requires further observation. The same group of workers (Stankiewicz, Cabaj, Jonas, Moore, Millar & Ng Chie, 1995) studied the effects of Ivm upon the immune responses of lambs using an identical experimental design. Similar antibody responses were seen for the drenched and control lambs although the secondary response to soluble antigen was significantly depressed in the Ivm-drenched lambs. The ability of lymphocytes to divide *in vitro* was also decreased but no effects were seen on the complement system between Ivm-treated and non-drenched control lambs. Ivermectin had variable effects upon the growth hormones studied but no differences were observed in terms of body weight gain. The authors concluded that the immunosuppressive property of Ivm may be important in parasite removal without concomitant inflammation. It is apparent that anthelmintics do exert a variety of direct/indirect effects upon the immune system of sheep, but the interactions are very complex and clearly require further study.

In non-suppressive treatment regimes, any effects upon acquired immunity resulting from anthelmintic treatment should be relatively small. However, in suppressive regimes or those incorporating persistent anthelmintics or

sustained/pulse release delivery devices this may not be the case. At present the use of an albendazole bolus is restricted to sheep greater than 35 kg bodyweight, by which time they should have acquired some degree of immunity. Moxidectin is a more recently introduced anthelmintic for sheep having an extended activity in comparison with Ivm (Taylor, Edgar & Kenny, 1993). Other anthelmintics in the avermectin class such as doramectin also have persistent activity (Weatherley, Hong, Harris, Smith & Hammet, 1993). Although currently only available for cattle it is possible that doramectin may be introduced for sheep in the near future. Whether or not these long acting drugs will compromise developing immunity is a matter of some debate and requires further investigation.

The question as to whether geometric or arithmetic mean worm burden data should be used in the calculation of CET treatment efficacies was also considered in this study. Reductions calculated using arithmetic means generally provided a more conservative estimate of anthelmintic efficacy whereas geometric data tended to bias in favour of the anthelmintic. However, as discussed in chapter 4, a common sense approach is required when evaluating worm burden data. Both calculations should be quoted so that any significant discrepancies can be examined more carefully. This would prevent not only false impressions of anthelmintic success but also misguided conceptions of resistance.

It is well established that biochemical or genetic assays are required in order to make a more direct measurement of resistance. The main cause of Bz resistance in both *H. contortus* and *T. colubriformis* (Kwa *et al.*, 1994) and *T. circumcincta* (Elard, *et al.*, 1996) is a single mutation from phenylalanine to tyrosine at amino acid position 200 in the β-tubulin isotype 1 gene. Although genetic probes are available for Bz's, these tests are relatively expensive and currently applicable as research tools only. With an improved understanding of both the mode of action of drugs and the mechanism(s) of resistance, routine diagnostic tests may become available. This would not only allow the detection of resistance at the earliest possible stage but could provide accurate data on any changes that occur in the frequency of resistance genes with the continued use of RSDs.

The findings from the studies on increasing treatment efficacy against resistant parasites are similar to others using a variety of Bz-resistant isolates in Australia (Ali

& Hennessy 1993; Hennessy *et al.*, 1994 and 1995). Not only are the findings on food withdrawal prior to treatment and divided-dosing relevant for the management of resistance in the UK, but it is also clearly important for farms where resistance has not yet developed. Modelling studies, incorporating varying degrees of resistant alleles, suggest that drug efficacy will decline at a slower rate when using methods such as modified feed management (Barnes *et al.*, 1995). Furthermore, it has been predicted that the rate at which resistance develops is slowest when anthelmintics are capable of killing the largest fraction of parasites heterozygous for resistance (Smith & Grenfell, 1994). Using approaches that maximize drug efficacy may therefore delay resistance and prolong the useful life of anthelmintic classes. If suitable penning is available, withholding feed offers a simple and practical approach to maximizing the bioavailability and efficacy of Bz drugs against resistant parasites. Obviously this approach would not be suitable for pregnant ewes since it may increase the risk of toxaemia. Increases in efficacy of the magnitude reported here may still be valuable in strategic and prophylactic regimens aimed at controlling parasitic gastroenteritis in which *Teladorsagia* is the principal species, where the aim is to reduce parasite impact to levels which do not severely compromise animal performance and welfare.

The influence of diet on the pharmacokinetics of Bz anthelmintics is known to be important in cattle as well as sheep (Taylor *et al.*, 1992; Sanyal, Knox, Singh, Hennessy & Steel, 1995). The increased period for drug release from particulate material and extended absorption/recycling that results from slowing the passage of digesta may also be important in goats (Barrett, Jackson, Patterson, Jackson & McKellar, in press). Despite differences in metabolism compared to Bz's, Taylor *et al.* (1992) noted that plasma concentrations of Ivm were also lower in lambs grazing fresh pasture. Recent work by Ali & Hennessy (1996) has confirmed the importance of diet upon the pharmacokinetic disposition and efficacy of Ivm. By reducing the level of feed intake these workers noted extended residence times and greater availability of Ivm compared to sheep on a high feed intake. This resulted in a 97% reduction of Ivm-resistant *H. contortus* burdens compared to only 53% in sheep maintained on a high feed intake. The increase in Lev treatment efficacy following feed withdrawal seen in the present study suggests that diet may also have some

impact on the pharmacokinetics of this drug class. Differences in gastrointestinal transit times and hepatic metabolism of Bz anthelmintics are thought to account for the large variation in pharmacokinetics seen in ruminant species (McKellar & Benchaoui, 1994). It is important therefore, that enhancement methods for anthelmintic classes are assessed for each host and that dosage rates are not extrapolated between species.

The requirement of a 12 hour treatment interval to achieve maximum efficacy when dividing Bz anthelmintic treatments was demonstrated in this study. Although divided dosing is less practical, it provides an alternative to the provision of penning or modified feed management. It is important however, to establish a balance between the minimal number of anthelmintic treatments required and the maximum benefit gained. In addition to the problem of tissue residues, it is possible that sustained release or extended pulse release devices may have detrimental effects on Bz anthelmintics. Gleizes, Eeckhoutte, Pineau, Alvinerie & Galtier (1991), using hepatic microsomes isolated from rabbits, demonstrated that successive oxfendazole treatments produced an induction of specific isoenzymes of the cytochrome P450 system. The induction of sulphonating enzymes would be expected to increase the metabolism of Bz anthelmintics. The production of anthelmintically inactive sulphone moieties would not only reduce treatment efficacy but may well select for resistant parasites. It is possible that the development of a short term erodible bolus might ensure/maintain the success of this approach. A formulation that could mimic two or three 12 hour pulses would be ideal for Bz's and may well be suitable for the other anthelmintic classes. The sustained presentation of avermectin or levamisole drug classes are unlikely to be affected by any induction of hepatic enzymes since these drugs are not metabolized to the same degree as Bz's and tend to be excreted as parent molecules.

The pharmacokinetics of different anthelmintics may also increase the selection pressure for resistant species. The depot effect of lipophilic drugs such as Ivm, doramectin and moxidectin could increase the selection pressure in two ways. Firstly, any persistence within the host should effectively screen a larger population of parasites thereby selecting for hetero- and homozygous resistant individuals. Secondly, the elimination of drugs results in a 'tail' effect during which the

anthelmintic concentration decreases enabling heterozygous individuals to survive. Whether or not this 'tail' is prolonged with persistent anthelmintics is an important consideration since a slowly declining efficacy may result in subtherapeutic levels which may enable heterozygous resistant individuals to survive (Taylor *et al.*, 1993).

During the course of these studies there was no evidence of any resistance against Ivm and with the exception of goat studies (Jackson *et al.*, 1992) there appears to be little avermectin/milbemycin resistance in the UK. The relatively slow rate of development of resistance in the UK appears to be due, at least in part, to the ability of the suprapopulation to survive for prolonged periods under UK climatic conditions on pasture (Waller & Thomas, 1978; Rose & Small, 1984) and in soil (Al Saqur, Bairden, Armour & Gettinby, 1982). The extended survival on pasture of our common gastrointestinal parasites thereby provides a reservoir of susceptibility which ensures a slower rate of accumulation of resistance genes in the population as a whole.

Drugs within the avermectin/milbemycin class are extremely potent having helminthocidal effects at very low concentrations for some species (Conder & Campbell, 1995). One possible explanation for the decline in the *T. vitrinus* population on the Ivm treatment paddock over the course of the study may lie in the sensitivity of this species to the drug. It is conceivable that for highly sensitive species the prolonged tail effect may well extend its period of activity beyond that seen with less sensitive species. For example, Shoop, Mrozik & Fisher (1995) noted that a compound may kill one species at 200 $\mu g\ kg^{-1}$ whilst it may kill other species at varying dosages between 2 and 200 $\mu g\ kg^{-1}$. Clearly the prophylactic potential of longer acting drugs and delivery systems has important implications as regards the development of chemical and managemental strategies for the control of ovine nematodes in the UK.

The co-administration of Fbz and PB has provided valuable data as to whether or not such combinations may be effective in the control of resistant parasites. Improved pharmacokinetic profiles of the pro-benzimidazole anthelmintic netobimin was demonstrated by Lanusse & Prichard (1991) using either the anti-thyroid drug methimazole or the anti-steroid drug metyrapone. Enhanced pharmacokinetic profiles of Fbz and Ofz in combinations with methimazole were also observed in the

study of Lanusse *et al.* (1995). Parbendazole, a Bz of only moderate anthelmintic potency, has been shown to slow down the metabolism of Ofz with consequent increases in efficacy against resistant isolates (Hennessy *et al.*, 1985 and 1992). Altering the metabolism and elimination of anthelmintics to increase systemic availability and efficacy against hetero- and homozygous resistant parasites offers a cost effective alternative to developing new drug classes and the use of such combinations merits further research.

Considerable research and development is still required before nematode vaccines become a commercial reality. Furthermore, multi-species vaccines may be required since monovalent vaccines may simply allow non-targeted species to pose a threat to livestock. The ease with which helminths have countered all classes of anthelmintics and the apparent high levels of polymorphism in helminth populations suggests that hidden antigen vaccines may also prove to be vulnerable to the adaptive capabilities of the parasite (Jackson, 1993; Grant, 1994). Breeding for immunoresponsiveness is inevitably a lengthy process and is unlikely therefore to have any immediate impact. Embryo manipulation and transfer does offer an opportunity for acceleration, however, the possibility of genetic restriction makes this a hazardous approach. Schwaiger, Gostomski, Stear, Duncan, McKellar, Epplen & Buitkamp (1995) for example concluded that the selection of particular alleles may hinder the protective immune response to other pathogens. Selecting 'resistant' lines of sheep on the basis of low faecal egg counts may inadvertently select hypersensitive sheep that suffer significantly more diarrhoea (Larsen, Vizard & Anderson, 1995), thereby affecting animal performance. It seems therefore, that an immunological approach is unlikely to offer a sole means of control but may simply complement existing and future methods.

Although naturally acquired immunity plays an important role in parasite population regulation its acquisition takes some time to develop in naive animals and it is likely to be compromised at points throughout an animals life. Conditions may sometimes permit large numbers of larvae on pasture leading to occasional outbreaks of clinical disease. Besides growing lambs, acquired immunity is liable to fail in ewes undergoing PPRI and in animals under stress, such as poor nutrition. For these reasons the immediacy offered by chemotherapy will ensure that there will always be

a need for anthelmintics and they will remain an important means of achieving control (Gutteridge, 1989). Recent advances in drug targeting and new delivery systems will hopefully maintain the use of anthelmintics. For example, the use of zeolite, a synthetic crystal matrix, was shown to prolong the delivery of tetramisole in rats, producing improved efficacy against *N. brasiliensis* (Shaker, Dyer & Storey, 1992). More recently the release of levamisole from biodegradable poly-lactide-co-glycolide microspheres was shown to be extended in the study of Fitzgerald & Corrigan (1996). The encapsulation of albendazole into liposomes has also provided promising results (D. R. Hennessy, personal communication). The protection afforded by the liposomes resulted in better drug delivery with consequent increases in drug bioavailability and increased efficacies against abomasal and small intestinal species.

In Australia a series of regional, epidemiologically based strategic programmes such as 'Wormkill' and 'Drenchplan' have been developed and successfully adopted by farmers (Waller, Dash, Barger, Le Jambre & Plant, 1995). Given the shortage of research funds for applied parasitological research it is hardly surprising that similar programmes have not been developed and promoted in the UK or indeed Europe. If such schemes are ever to be developed then it is clear that they must not only include managemental and immunological approaches but also an improved understanding of parasite epidemiology. Information from current research on anthelmintics and their pharmacokinetics needs to be included in any such programmes. The establishment of sheep nematode control schemes also requires improved communication between scientists, veterinarians, livestock owners and pharmaceutical companies. The recognition of deficiencies in chemical control and the development of improved strategies are also in the best interests of the pharmaceutical industry (Herd, 1993). It is only with the disclosure of reliable information however, that control strategies will have the chance to succeed. The importance of regular monitoring of faecal egg counts should be emphasized. Similarly, farmers should understand that anthelmintics are valuable but extremely limited resources that should not be squandered by excessive and unnecessary use (Barger, 1993b).

Alternative methods of control such as the utilization of supplementary protein (Coop *et al.*, 1995), nematophagous fungi (Charles *et al.*, 1996) or the use of

different herbage species (Scales *et al.*, 1995) need to be evaluated in the field. Simply removing faeces has been shown to reduce the level of contamination for horses with consequent increases in the amount of grazable pasture available (Herd, 1990). Currently, and for the foreseeable future, the most important means of control will be chemotherapy. This study has shown that the development of resistance need not necessarily signal the end of an anthelmintic class. Under careful management it may be possible to re-introduce RSDs into slow chemoprophylactic rotations, at least when resistance involves less pathogenic species with a low biotic potential such as *Teladorsagia*. Progress in vaccine development and the implementation of breeding programmes for immunoresponsive individuals are unlikely to replace the need for chemotherapy. Indeed, the role of anthelmintics in the future of parasite control may well be to support the use of such alternatives.

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APPENDIX

General

Helminthological Iodine

250 g Potassium Iodide (Fisons, UK)

50 g resublimed Iodine (BDH chemicals Ltd., UK)

Dissolve in 500 ml of distilled water

2.5.1 Plasma Pepsinogen Assay

1% Bovine serum albumin (BSA)

0.25 g BSA dissolved in 25 ml distilled water

Glycine buffer (0.1M, pH 2)

7.5 g glycine

5.85 g NaCl

Dissolve in 800 ml of distilled water, stirring vigorously with a magnetic stirrer.

Adjust the pH to 2 with HCl. Make up to 1 litre using distilled water.

1N Sodium hydroxide (NaOH)

40 g NaOH pellets dissolved in 1 litre of distilled water.

10% Trichloroacetic acid (TCA)

100 g TCA crystals dissolved in 1 litre of distilled water.

Folins Working Reagent

5 ml Folins and Ciocalteau's reagent mixed with 10 ml distilled water.

Stock tyrosine solution

0.0362 g tyrosine dissolved in 100 ml of 0.1N HCl. (Note: shelf life 1 month at 4 °C)

Working standard is a 1 in 10 dilution of stock with distilled water.

2.6.1 Extraction of genomic DNA

Lysis Buffer

500 µl PCR Buffer 10x (without $MgCl_2$)

100 µl 1M DTT

250 µl 10 % SDS

50 µl 10% Triton X-100

4050 µl sterile distilled water

Made without Protease K and stored at –20 °C in 495 µl aliquots

Before use add 5 µl of fresh Protease K (10 mg ml^{-1}, Sigma, UK) per 495 µl aliquot.

Final concentrations (20 mM DTT, 0.5 % SDS, 0.1 % Triton X-100, 0.1 mg ml^{-1} Protease K).

1 M DTT

0.309g DTT dissolved in 2 ml of 0.01 M sodium acetate pH 5.2

Sterile filtered (0.45 µm) and stored at –20 °C.

2.6.2 Arbitrarily Primed Polymerase Chain Reaction (AP-PCR)

PCR Incubation Buffer 10x (without $MgCl_2$)

0.1 M Tris-HCl (0.157g)

0.5 M KCl (0.373g)

1 mg ml^{-1} gelatine (0.01g)

Dissolve in 8 ml sterile distilled water, adjust pH to 8.3 using NaOH, make up to 10 ml with water, sub-aliquoted and stored at –20 °C.

1 Kb DNA markers

20 µl Stock 1 Kb DNA ladder (1000 µg $µl^{-1}$, BRL, Paisley, Scotland, UK)

60 µl Bloo Joos (see below)

340 µl sterile distilled water

Stored at –20 °C

TE buffer (1x)

2 ml of 1 M Tris buffer

0.4 ml of 0.5 M EDTA (pH 8) - see below

make up to 200 ml with sterile distilled water

Deoxyribonucleoside triphosphates (dNTPs), stock solution

2 µl of each dATP, dCTP, dGTP and dTTP (Boehringer, UK)

92 µl sterile distilled water

Stored at –20 °C

Primer (10x)

The concentration of the oligonucleotide primer (Oswell DNA Service, Edinburgh, UK) was determined by dividing its OD_{260nm} by its extinction coefficient (ε).

(dATP ε = 15.4, dTTP ε = 8.8, dGTP ε = 11.7, dCTP ε = 7.3)

For example, primer 509G: OD_{260nm} ml^{-1}= 13, ε = 305.3 ∴ c = 42.58 µM

Therefore a 1 in 4.258 dilution is required for a 10 µM (10x) solution.

i.e. 100 µl stock + 326 µl sterile water

Mix, pipette into 20 µl aliquots and store at –20 °C.

2.6.3 Agarose Gel Electrophoresis.

0.5 M EDTA (pH 8)

186.1 g EDTA

Dissolve in 800ml of distilled water, stirring vigorously with a magnetic stirrer.

Adjust the pH to 8 with NaOH (approx. 20 g of NaOH pellets).

Note: EDTA does not go into solution until the pH is adjusted to approx. pH 8.

Tris-acetate buffer (TAE)

50x Concentrated stock solution (per litre)

242 g Tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (pH 8)

Dissolve the Tris in 700 ml of distilled water before adding the glacial acetic acid and the EDTA. Make up to 1 litre using distilled water.

Bloo Joos (0.2 % Bromophenol blue)

(Gel-loading buffer)

25 % Sucrose (w/v) in water

Dissolve 5 g of sucrose in 15 ml of distilled water. Add 40µl of bromophenol blue and make up to 20 ml with distilled water. Store at 4 °C.

Ethidium Bromide (EtBr) 10mg/ml.

Add 1 g of ethidium bromide to 100 ml of distilled water. Stir on a magnetic stirrer for several hours to ensure the dye has dissolved.

Store at 4 °C in a dark bottle.

Caution: EtBr is a powerful mutagen and is moderately toxic. Always wear gloves when working with solutions that contain the dye, and a mask should be worn when weighing it.

2.6.4 Polyacrylamide Gel Electrophoresis (PAGE)

Loening E buffer

10x Concentrated stock solution (per litre)

43.59 g Tris base

41.39 g sodium dihydrogen monophosphate

3.72 g EDTA

Dissolve in 800 ml of distilled water. Adjust the pH to 8.3 with NaOH. Make up to 1 litre using distilled water.

10 % Ammonium Persulphate (APS)

0.1 g of ammonium persulphate, make up to 1 ml with distilled water.

Note : Prepare fresh daily.

30 % Acrylamide

Dissolve 29.2 g of acrylamide and 0.8 g of N,N'-methylene-stock solution
bisacrylamide in 80 ml of distilled water. Make up to 100 ml with distilled water.
Filter and store at 4°C in a dark bottle.

Caution : Acrylamide is a potent neurotoxin and is absorbed through the skin. Weigh in a fume cupboard wearing gloves and a mask. Always wear gloves when handling solutions containing these chemicals. Although polyacrylamide is considered to be non-toxic, it should be handled with care because of the possibility that it might contain small quantities of unpolymerized acrylamide.

2.6.5 Silver-staining of polyacrylamide gels

FIX

10 ml ethanol.

0.5 ml acetic acid.

Make up to 100 ml with distilled water.

STAIN

0.19 g silver nitrate.

Dissolve in 80 ml of distilled water.

Make up to 100 ml with distilled water.

DEVELOPER

3 g sodium hydroxide.

0.75 ml formaldehyde.

Dissolve the sodium hydroxide in 80 ml of distilled water then add the formaldehyde.

Make up to 100 ml with distilled water.

STOP

1.9 g sodium carbonate.

Dissolve in 200 ml of distilled water. Make up to 250 ml with distilled water.

2.7 High Performance Liquid Chromatography (HPLC)

Individual stock solutions (100 μg/ml)

Stock solutions (100 μg/ml) for each compound (Albendazole (Abz), Fenbendazole (FBz), Fenbendazole sulphoxide (FBzSO), Fenbendazole sulphone ($FBzSO_2$), and Fenbendazole hydroxide (FBzOH) were prepared by weighing out accurately to four decimal places 0.0100 g of compound which was dissolved and made up to 100 ml in a volumetric flask using methanol. Standards of Abz and Fbz and its metabolites were supplied by Hoechst Ltd., Gebaude, Germany.

Fenbendazole Mixtures (FBz + FBzSO + $FBzSO_2$ + FBzOH)

10 μg/ml Mix : 20 ml of each stock (100 μg/ml) solution, made up to 200 ml with methanol.

5 μg/ml Mix : 10 ml of each stock (100 μg/ml) solution, made up to 200 ml with methanol.

2.5 μg/ml Mix : 5 ml of each stock (100 μg/ml) solution, made up to 200 ml with methanol.

1.0 μg/ml Mix : 10 ml of 10 μg/ml Mix, made up to 100 ml with methanol.

0.5 μg/ml Mix : 10 ml of 5 μg/ml Mix, made up to 100 ml with methanol.

Publications arising from this thesis

Barrett M., Jackson F., Patterson D. M., Jackson E. & McKellar Q. A. (in press). Comparative field evaluation of divided-dosing and reduced feed intake upon treatment efficacy against resistant isolates of *Teladorsagia circumcincta* in sheep and goats. *Research in Veterinary Science.*